A Mixture of Diethylhexyl, Diisononyl and Dibutyl Phthalate Decreased Anogenital Distance, Postnatal Testosterone Levels, and Changed Social Behavior in Wistar Rats

Martina MOROVÁ[1], Tomáš SENKO[1], Lucia OLEXOVÁ[1], Zuzana DZIRBÍKOVÁ[1], Lucia KRŠKOVÁ[1]

[1]Department of Animal Physiology and Ethology, Faculty of Natural Sciences, Comenius University in Bratislava, Bratislava, Slovak Republic

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Summary

Phthalates are chemicals interfering with the function of testosterone and are suspected to play a role in the emergence of neurodevelopmental diseases. This could be due to interference with brain development for which optimal testosterone levels are essential. We investigated the effect of prenatal and early postnatal exposure to a phthalate mixture on the anogenital distance (AGD), plasma testosterone levels and social behavior in rats. Pregnant rats were exposed to a mixture of diethylhexyl, diisononyl and dibutyl phthalate, each at a dose of 4.5 mg/kg/day, from gestational day 15 to postnatal day 4. A social interaction test was performed to assess sociability in the three ontogenetic stages (weaning, puberty, adulthood). AGD was measured in adulthood to assess changes in prenatal testosterone levels. Plasma testosterone levels were measured in adults by a radioimmunoassay. The total frequency and time of socio-cohesive interactions were decreased in phthalate exposed females in weaning, puberty and adulthood. Phthalate exposed males showed a decrease in the frequency of social interactions in weaning only. Shorter anogenital distance was observed in adult males exposed to phthalates. Decreased testosterone levels were observed in the exposed group in both sexes. Our results suggest that early developmental phthalate exposure may play an important role in the hormonal and behavioral changes associated with several neurodevelopmental diseases.

Key words

Phthalate mixture • Testosterone • Anogenital distance • Social behavior • Laboratory rat

Corresponding author

M. Morová, Department of Animal Physiology and Ethology, Faculty of Natural Sciences, Comenius University in Bratislava, Mlynská dolina, Ilkovičova 6, 842 15 Bratislava 4, Slovak Republic. E-mail: morova4@uniba.sk

Introduction

Phthalates are a large family of ubiquitous environmental pollutants that act as endocrine disruptors – exogenous agents that interfere with the function of natural hormones responsible for the maintenance of homeostasis and the regulation of developmental processes (Kavlock *et al*. 1996). They are produced in large quantities and widely used to provide flexibility and durability to plastic materials. They also lubricate and act as solvents. They can be found in a wide range of products used in daily life and since there are no covalent bonds between phthalates and the plastics in which they are mixed, they can leach from these products into the environment and enter the body through food consumption, inhalation, dermal contact or intravenous injection (Schettler 2006).

Exposure to endocrine disruptors such as phthalates during fetal and early life is a public health concern because these periods are characterized by high cellular plasticity when the brain is vulnerable to external stimuli. When administered during this crucial time, these chemicals can influence physiology (Kolatorovova *et al.* 2018), behavior and lead to the development of diseases (Miodovnik *et al*. 2011). Phthalates can cross the placenta

Fax +420 241 062 164, e-mail: physres@fgu.cas.cz, www.biomed.cas.cz/physiolres

(Xu *et al*. 2008) and have been found in human amniotic fluid (Silva *et al*. 2004), breast milk (Main *et al*. 2006) and in young children (Brock *et al*. 2002).

In rodent models as well as in humans, phthalates downregulate fetal testosterone production and are, therefore, considered anti-androgenic (Barakat *et al.* 2019, Pallotti *et al*. 2020) resulting in alterations in reproductive tract development (Repouskou *et al.* 2019). Prenatal exposure to these chemicals is associated with a decreased anogenital distance (AGD) (Martino-Andrade *et al*. 2016, Curi *et al.* 2019) which is related to androgen exposure during fetal development. It was also associated with the neurodevelopmental disability (Whyatt *et al.* 2012, Kim *et al.* 2018) and with the modifications of various types of behavior, e.g. sociability impairment (Miodovnik *et al*. 2011, Lee *et al*. 2016, Kougias *et al.* 2018), depression-like behavior (Xu *et al*. 2015), anxiety-like behavior (Carbone *et al*. 2013, Philippat *et al*. 2017) and connection has also been made between early developmental phthalate exposure and autism-like behavior in children (Oulhote *et al.* 2020).

Prenatal testosterone plays a key role in the development of many aspects of mammalian behavior, including anxiety- and depression-like behavior, stress response, and social behavior (Pivina *et al*. 2007, Hines *et al*. 2015, Xu *et al*. 2015). Changes in the levels of testosterone during prenatal development may lead to changes in the programming of the hypothalamic-pituitary-adrenal axis (Kapoor and Matthews 2011), that in turn can result in changes in coping strategies in social interaction situation. Individuals prenatally exposed to higher levels of testosterone tend toward a proactive (active) coping strategy, characterized by higher levels of aggression and dominance. In contrast, individuals prenatally exposed to lower levels of testosterone employ reactive strategy (passive), characterized by less aggressive behavior and social avoidance (Koolhaas *et al*. 1999, Steinman *et al*. 2017).

In this study, we investigated the effect of prenatal and early postnatal exposure to a mixture of the three most prevalent phthalates (dibutyl phthalate – DBP, diethylhexyl phthalate – DEHP and diisononyl phthalate – DINP) in the environment (Luo *et al.* 2020) on testosterone levels in adult rats, AGD (a marker of fetal androgen exposure) and the social behavior in weaning, puberty and adulthood. Based on the above information, we hypothesized that there would be decreased serum levels of testosterone in adult offspring, shortened AGD and a decrease in the number and duration of social contacts. This decrease in social behavior would indicate a shift to reactive coping strategies in social interaction situation and would be connected to the decreased level of testosterone during prenatal development.

Methods

Animal experiments were conducted in accordance with the Principles of Laboratory Animal Care issued by the Ethical Committee of Comenius University in Bratislava, Slovak Republic. The experimental design was approved by the State Veterinary and Food Administration of the Slovak Republic (protocol number: Ro-3726/16-221) and the EU Directive 2010/63/EU for animal experiments was followed.

Animals

Wistar rats were obtained from the Institute of Experimental Pharmacology and Toxicology, Dobrá Voda, Slovak Republic. The parental generation consisted of 13 females and seven males. Animals were housed in groups of three or four animals in standard light conditions (12:12 h light-dark cycle; light on at 07:00), at an average temperature of 21±1 °C and 55±10 % relative humidity. Water and food (standard laboratory chow, MP-OŠ-06 – Peter Miško, Snina, Slovak Republic) were available *ad libitum*. After an acclimatization period (7 days), parental animals were tested in an open field test to assess the level of their excitability. Ovulatory cycle phase of female rats was identified according to Gleich and Frohberg (1977). In the evening of the proestrus day, females were housed overnight with males. The presence of spermatozoa in the vaginal smears on the next morning was referred to as gestational day 0 (GD 0).

Pregnant females were divided into two, in terms of excitability balanced groups: control (Ctrl: n=7) and phthalate (Pht: n=6) and were housed in groups of two or three per cage until GD 20. One day before delivery, Ctrl and Pht females were housed individually and allowed to raise their offspring until weaning at postnatal day (PND) 21. From PND 1 to PND 4, the litters were culled to 8 animals per litter (4 males, 4 females). After weaning, rats of either sex were housed separately in groups of 4 animals per cage.

Treatment

DEHP, DINP and DBP, 100 mg liquid each were mixed into one solution of 10 ml with peanut oil as

the vehicle (10 mg/ml). Solutions were stored at 4 °C during the experiment. Each morning, from gestational day 15 (GD 15) to PND 4, dams were weighed and the appropriate amount of phthalate, diluted in peanut oil (vehicle), was prepared daily and delivered to each female on a sponge biscuit. Dams from the exposed group received 500 µl of the vehicle with an appropriate amount of solution containing DEHP, DBP, and DINP (4.5 mg/kg of each substance). Dams from the Ctrl group received 500 µl of the vehicle on a sponge biscuit (adapted from Degroote *et al*. 2014).

The dose of phthalates used in this study was the same as that reported by Degroote *et al.* (2014) and was based on their previous work and NOAEL (No Observable Adverse Effect Level) in rats to be well below toxic doses. This dose is still higher than the estimated human exposure (Katsikantami *et al*. 2016), but since phthalates can act in a dose additive manner (Howdeshell *et al*. 2015), even smaller doses in combination can cause abnormalities during development.

Social interaction test

The social interaction test was adapted from File and Hyde (1978). At weaning (PND 21-23), puberty (PND 42-44) and adulthood (PND 77-79), offspring (weaning and puberty – Ctrl: male n=12, female n=12; Pht: male n=12, female n=12; adulthood – Ctrl: male n=12, female n=10; Pht: male n=10, female n=12) were tested for their social behavior with an unknown test partner of the same sex, treatment and approximate weight. Pairs were tested between 16:00 and 19:00. The dimensions of the test box were 72×34×38 cm (l×w×h).

Animals were acclimatized to test conditions before the start of testing. Pairs of animals were placed diagonally in the opposite corners of the box. Their behavior was measured for 5 min and all sessions were recorded using a digital camera (Logitech, Lausanne, SUI). The apparatus was cleaned with water after each test to remove odors of the previous animal.

The total duration and frequency of social interactions (socio-cohesive: following, mutual sniffing, genital investigation, climbing over, crawling under, allogrooming contact, initiation of social play; socio-aversive: attack, aggressive unrest, mounting, tail biting, escape, vigilant posture, defense activity) (adapted from Senko *et al*. 2017) were recorded from the video (for each animal separately) by a trained observer who was blind to the experimental groups.

Anogenital distance

AGD was measured on male (Ctrl: n=23; Pht: n=23) and female (Ctrl: n=28; Pht: n=22) offspring from the center of the anus to the posterior edge of the genital papilla using a digital caliper (Hedue, Mönchengladbach, GER). AGD was measured at PND 82. To increase precision, each animal's AGD was measured three times by the same person; the resulting AGD value is an average of these three measures (adapted from Gallavan *et al*. 1999).

Testosterone levels

Blood was collected into heparinized tubes on PND 116-119 (following the decapitation of animals under isoflurane anesthesia). Plasma was separated by centrifugation at 2000× g for 10 min, and stored at -76 °C until hormone analyses.

Plasma testosterone concentrations were measured by direct radioimmunoassay using [1,2,6,7-^{3}H]-testosterone tracer (specific activity 95.5 Ci/mmol; PerkinElmer, USA) and a specific antibody generated in rabbits against a testosterone-3-(carboxy-methyl)oxime bovine serum albumin conjugate, following a previously published protocol (Zeman *et al*. 2016).

We used 5 and 80 µl aliquots of plasma for males and females, respectively. Male (Ctrl: n=15, Pht: n=18) and female (Ctrl: n=26, Pht: n=22) samples were run in separate assays, with a mean intra-assay variation coefficient of 7 % and an inter-assay variation coefficient of 8.3 %. The assay sensitivity was 1.5 pg of testosterone per tube.

Statistical analysis

Our data were analysed using SigmaPlot version 11.0 software (SigmaPlot, Systat Software, Inc., Erkrath, GER).

All data were tested for a normal distribution using a Kolmogorov-Smirnov test.

The frequency and time spent in socio-cohesive interactions, AGDs and plasma testosterone concentrations have natural differences between sexes, therefore, the effect of sex was excluded from the analysis. The frequency and time spent in socio-cohesive interactions, AGD and the plasma testosterone concentration were then examined using unpaired Student's *t*-tests (for parametric distributions) or Mann-Whitney tests (for non-parametric distributions).

Results

Social interaction test

Socio-aversive interactions were rarely observed in weaning and puberty, but we did not find any significant differences between groups (data not shown).

The total frequency of socio-cohesive interactions was decreased in all ontogenetic stages, mostly in females (Fig. 1). In weaning, there was found a significant decrease in Pht males ($t=-2.251$; $p<0.05$) compared to Ctrl males and also significant decrease in Pht females ($t=-2.647$; $p<0.05$) compared to Ctrl females. In puberty, there was a significant decrease only in Pht females ($t=-2.564$; $p<0.05$) compared to Ctrl females. In adulthood, Pht females ($t=-5.228$; $p<0.001$) had a significant decrease compared to Ctrl females.

Total time of socio-cohesive interactions was significantly decreased in Pht females compared to Ctrl females (Fig. 2B) in weaning ($t=-2.691$; $p<0.05$), puberty ($T=108$; $p<0.05$) and adulthood ($t<-2.385$; $p<0.001$). Pht males did not show any significant differences compared to Ctrl males (Fig. 2A).

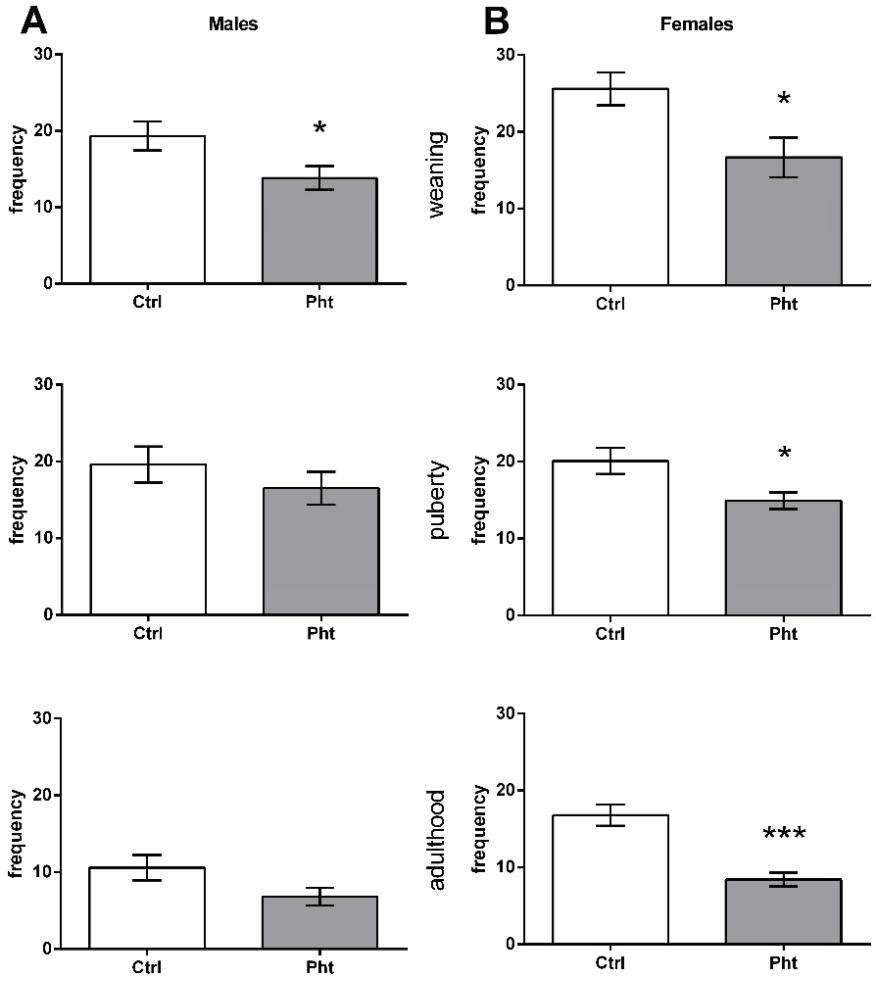

Fig. 1. The total frequency of socio-cohesive interactions in control (Ctrl) and phthalate (Pht) males (**A**) and females (**B**) in weaning (Pht: male n=12, female n=12; Ctrl: male n=12, female n=12), puberty (Ctrl: male n=12, female n=12; Pht: male n=12, female n=12) and adulthood (Ctrl: male n=12, female n=10; Pht: male n=10, female n=12). Data are expressed as mean ± SEM. Asterisks indicate significant differences (* $p<0.05$; *** $p<0.001$).

Anogenital distance

There was a significant decrease in the AGD of males treated with phthalates compared to Ctrl males ($t=2.047$; $p<0.05$) (Fig. 3A). No difference was found between the Ctrl and Pht females ($T=542$; $p=0.718$) (Fig. 3B).

Plasma testosterone levels

Prenatal and early postnatal exposure to phthalates resulted in significantly lower plasma testosterone concentration in males ($t=2.48$; $p<0.05$) (Fig. 4A) and females ($t=2.36$; $p<0.05$) (Fig. 4B).

Discussion

There has been growing evidence supporting the role of phthalates in the etiology of neurodevelopmental diseases (Jeddi *et al*. 2016). The exact mechanisms are

still not elucidated, but there seems to be a connection with changes in the prenatal levels of testosterone, a hormone important not only in brain development (Negri-Cesi *et al*. 2004) but also in the development of social behavior (Knickmeyer *et al*. 2005) and affected by phthalate exposure (Barakat *et al.* 2019, Pallotti *et al*. 2020). Therefore, in our study we examined the effect of prenatal and early postnatal exposure to a mixture of most widely used phthalates on the social behavior of laboratory rats and the anti-androgenic effect of these substances, evaluated here as the AGD and postnatal plasma testosterone levels.

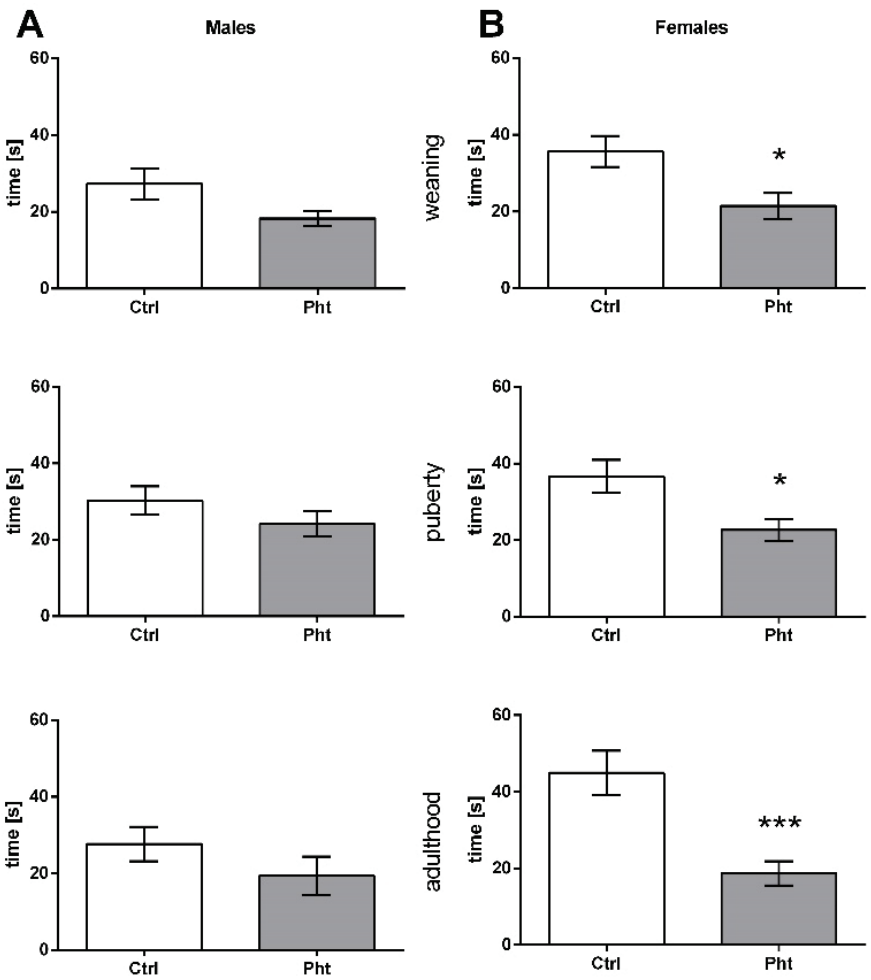

Fig. 2. The total time spent in socio-cohesive interactions in control (Ctrl) and phthalate (Pht) males (**A**) and females (**B**) in weaning (Pht: male n=12, female n=12; Ctrl: male n=12, female n=12), puberty (Ctrl: male n=12, female n=12; Pht: male n=12, female n=12) and adulthood (Ctrl: male n=12, female n=10; Pht: male n=10, female n=12). Data are expressed as mean ± SEM. Asterisks indicate significant differences (* $p<0.05$; *** $p<0.001$).

We observed, that Pht females had less socio-cohesive interactions in weaning, puberty and adulthood, both in terms of frequency and time compared to Ctrl females. Pht males had a lower frequency of socio-cohesive interactions in weaning compared to control males. AGD was affected only in males, with Pht males having shortened AGD. Plasma testosterone levels were lower in both males and females exposed to a phthalate mixture of DEHP, DBP and DINP.

In a study by Degroote *et al*. (2014), rats (PND 40) that were prenatally exposed to a mixture of endocrine disruptors, including phthalates, showed impaired social behavior. Males spent less time in social interactions, and in both sexes, escaping behavior was observed during the social interaction test. The authors speculated that this behavior could indicate higher social avoidance. Lee *et al*. (2016) found that maternal DEHP exposure induced a deficit in social interactions of mice offspring (PND 56) and in a study by Quinnies *et al*. (2017) socially investigative behaviors were reduced in mice (PND 28-32) prenatally exposed to DEHP.

In women, metabolites of phthalates during gestation were associated with greater social deficits in their children (aged 7-9) (Miodovnik *et al*. 2011). Whyatt *et al*. (2012) and Philippat *et al*. (2017) found positive associations between prenatal phthalate metabolites and the internalizing behavior of boys (aged 3). In a study by Won *et al*. (2016), in which the neurobehavioral

development of children (aged 6-18) was assessed, there was a significant association between phthalates measured in children's urine and social problems in younger children (aged 6-11), who showed greater vulnerability to phthalate exposure.

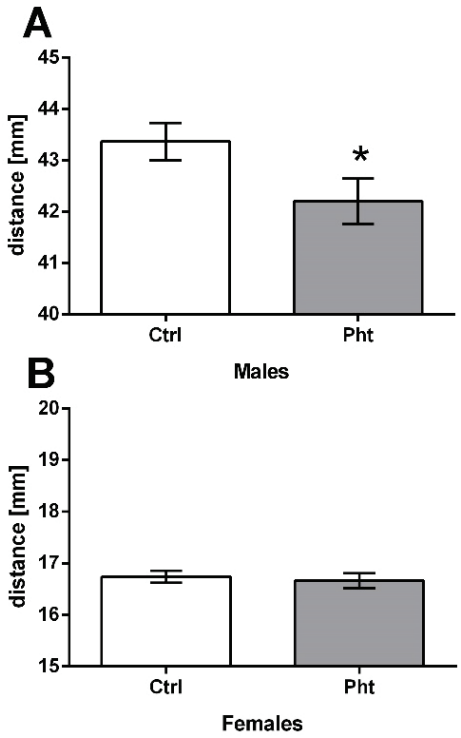

Fig. 3. Anogenital distance in control (Ctrl: n=23) and phthalate (Pht: n=23) males (**A**) and anogenital distance in control (Ctrl: n=28) and phthalate (Pht: n=22) females (**B**). Data are expressed as mean ± SEM. Asterisk indicates significant differences between groups (* p<0.05).

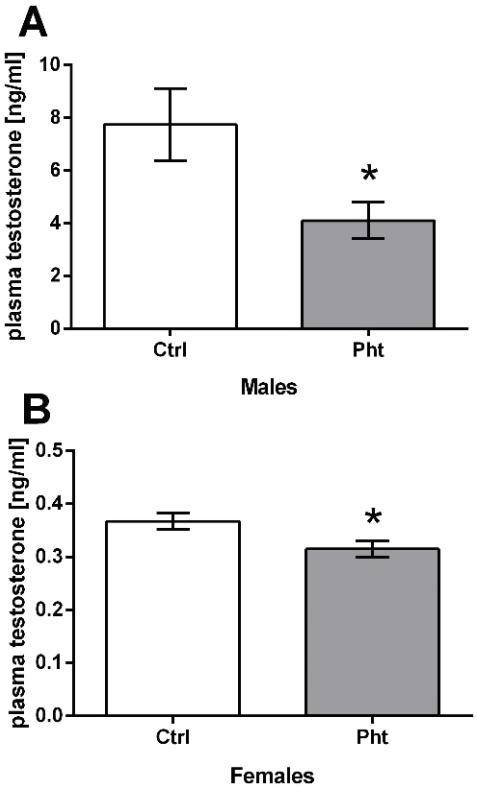

Fig. 4. The plasma testosterone concentration in control (Ctrl: n=15) and phthalate (Pht: n=18) males (**A**) and in control (Ctrl: n=26) and phthalate (Pht: n=22) females (**B**). Data are expressed as mean ± SEM. Asterisk indicates significant differences between groups (* p<0.05).

In the most studies mentioned above, social behavior was investigated in the earlier stages of postnatal development. In our study, we examined this behavior from weaning to adulthood and our findings, together with those of other authors, could support a possible role of phthalate exposure in the development of behavioral problems which last from early stages of postnatal development to adulthood.

Changes in behavior are believed to be the consequence of disruption in neurodevelopment, as the brain is the organ controlling behavior and is highly vulnerable to disruption due to exogenous agents during the critical phases of its development (Schug *et al*. 2015). In their studies, Miodovnik *et al*. (2011) and Whyatt *et al*. (2012) discuss the possible role of phthalates in influencing prenatal levels of thyroid and sex hormones, whose importance for brain development have been well described (Bernal 2005, Schwarz and McCarthy 2008).

In regard to this, we focus our research on the relationship between phthalates, testosterone production and changes in behavior due to changes in the brain. The importance of the testosterone-brain-behavior relationship was confirmed by our previous study (Dzirbíková *et al*. 2018) and considering these and previous results we propose yet another possible explanation for observed changes in social behavior.

If we apply Koolhaas's model (Koolhaas *et al*. 1999) of coping style on our data, we see that animals from Pht group seem to be more reactive (passive) in social interaction with an unknown rat. Reactive coping is associated with lower testosterone activity. We found that phthalate-affected individuals have lower testosterone levels, and this, together with our behavioral data, suggest a shift to reactive coping. The fact that prenatal testosterone plays an important role in the programming of postnatal strategies is also confirmed by our previous study. We found that testosterone application during the last third of pregnancy had an impact on social coping in social interaction test and caused a shift to proactivity (more time spent in social interaction) (Dzirbíková *et al*. 2018).

In our study, a decrease in social behavior in the Pht group was noticeable in both sexes but was more pronounced in females. This could mean that males and females react differently under the influence of exposure to prenatal phthalates, which was also suggested by other authors (Whyatt *et al*. 2012, Degroote *et al*. 2014, Won *et al*. 2016). It seems that further studies will be required to thoroughly evaluate the effect of phthalates on behavior in both sexes.

There is no doubt that phthalates are endocrine disruptors with anti-androgenic properties (Barakat *et al.* 2019, Pallotti *et al*. 2020). In our study, we detected a decrease in plasma testosterone levels in male and female adult rats after phthalate exposure. This long-term decrease in testosterone levels could indicate a decrease in its production. Many studies have described the damage to the reproductive system after prenatal phthalate exposure, including a decrease in testosterone production (Barakat *et al.* 2019, Repouskou *et al.* 2019).

AGD is determined prenatally by the testosterone metabolite, dihydrotestosterone, therefore, can be used as a marker for a decrease in the prenatal levels of testosterone (Swan *et al*. 2005). We observed shortened AGDs, which is consistent with reduced testosterone levels. In our study, only males had a reduced AGD. This result is in accordance with previous studies conducted on laboratory rodents (Parks *et al*. 2000, Wang *et al*. 2016) and could be related to the fact that reproductive tract masculinization is hormone-dependent, while development of the female reproductive tract proceeds without any specific interventions (Sharpe 2006).

There have been previous studies of laboratory animals prenatally exposed to lower doses of phthalates, which have also observed a negative effect on testosterone production and social behavior (Degroote *et al*. 2014, Sekaran and Jagadeesan 2015). Nonetheless, this is (to the best of our knowledge) the first study in which exposure to a mixture of phthalates at a relatively low dose during prenatal and early postnatal development had an observed effect on behavior and testosterone production in adulthood.

Conclusions

Our results regarding decreased social behavior are consistent with studies in human population indicating significant impacts of early life exposure to phthalates on behavioral outcomes. Changes in behavioral settings may be related to the fact that phthalates alter the levels of testosterone. Testosterone determines brain development and contributes to the formation of a coping strategy in social interactions. A decrease in its levels could therefore initiate a shift to the reactive coping strategy and lead to the decrease of social behavior. This could suggest a role of prenatal phthalate exposure in behavioral programming and a role in the emergence of neurodevelopmental diseases.

Conflict of Interest

There is no conflict of interest.

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