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Phthalate metabolite concentrations in urine samples from Swedish pet dogs

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Förekomst av ftalatmetaboliter i urinprover från svenska sällskapshundar

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SUMMARY

Phthalates are chemicals used as plasticizers and can be found in a number of different consumer products. Phthalates may leak from these products into the environment, and hereby humans and animals may be exposed to phthalates through oral intake, dermal exposure and inhalation. Experimental studies on primarily rodents have shown that phthalate exposure causes toxic effects on the reproductive system, such as effects on the levels of testosterone, progesterone and estradiol, the weight and descent of testes, the onset of puberty and maintenance of pregnancy. The purpose of this study was to quantify the concentrations of phthalate metabolites in urine samples from healthy pet dogs and to investigate relationships between the concentrations and the home environment of the dogs using a questionnaire filled out by the dogs' owners. Sixteen dogs were included in the study, with matched groups of which eight dogs lived in homes with plastic flooring and eight dogs lived in homes without plastic flooring. Urine samples were collected and analysed for ten different phthalate metabolites originating from the phthalates diethyl phthalate (DEP), dibutyl phthalate (DBP), butylbenzyl phthalate (BBzP), di(2-ethylhexyl) phthalate (DEHP) and diisononyl phthalate (DiNP). The results showed that all dogs participating in the study had identifiable levels of each of the different metabolites in their urine. The dogs living in homes with plastic flooring had higher levels of phthalate metabolites in their urine compared to the dogs living in homes without plastic flooring. This difference was statistically significant for MBzP, the metabolite of BBzP, and for MEHP, MHEHP, MOEHP and MCEPP, metabolites of DEHP. Plastic toys were also identified as a possible risk factor for higher phthalate metabolite concentrations in urine. The results of this study show that dogs are exposed to and absorb phthalates from the environment, and that dogs living in homes with plastic flooring appear to be exposed to and absorb a greater amount of phthalates than dogs living in homes with non-plastic flooring.

SAMMANFATTNING

Ftalater är en grupp kemikalier som används som mjukgörare av plaster. Ftalater förekommer i ett stort antal konsumentprodukter från vilka de kan läcka ut i miljön. Människor och djur riskerar därmed att exponeras för ftalater genom oralt intag, genom huden samt via inhalation. Ftalater har i studier på framför allt gnagare visats orsaka toxiska effekter på reproduktionssystemet, till exempel kan de påverka nivåerna av könshormoner, testiklarnas utveckling och nedvandring, tidpunkten för könsmognad och förekomsten av aborter. Syftet med den här studien var att kvantifiera halterna av ftalatmetaboliter i urinen hos friska sällskapshundar, samt att undersöka om det finns ett samband mellan halterna och hundarnas hemmiljö med hjälp av en enkät ifylld av djurägarna. Sexton hundar deltog i studien, med matchade grupper bestående av åtta hundar som bodde i hem med plastgolv och åtta hundar i hem som saknade plastgolv. Urinprover samlades in och analyserades för tio olika ftalatmetaboliter till ftalaterna dietylftalat (DEP), dibutylftalat (DBP), benzylbutylftalat (BBzP), di(2-etylhexyl)ftalat (DEHP) och diisononylftalat (DiNP). Resultaten visade att samtliga hundar i studien hade mätbara nivåer av samtliga metaboliter i sin urin. De hundar som bodde i hem med plastgolv hade högre nivåer av ftalatmetaboliter jämfört med de hundar som bodde i hem utan plastgolv. Skillnaden mellan grupperna var statistiskt signifikant för MBzP, metabolit till BBzP, och för MEHP, MHEHP, MOEHP och MCEPP, metaboliter till DEHP. Plastleksaker identifierades också som en möjlig riskfaktor för att ha högre koncentrationer av ftalatmetaboliter. Resultatet av den här studien visar att hundar exponeras för och absorberar ftalater från miljön. Dessutom indikerar det att hundar som bor i en miljö med plastgolv exponeras för och absorberar en större mängd ftalater jämfört med hundar som bor i en miljö som saknar plastgolv.

CONTENT

INTRODUCTION

Phthalates are defined as a group of chemicals with a similar chemical structure (Committee on the Health Risks of Phthalates, 2008). They are mainly used as plasticizers to give flexibility to materials such as polyvinyl chloride and other polymers (Schettler, 2006). As a consequence, they can be found in a large number of consumer products.

Phthalates, when used as plasticizers, are not chemically bound to their polymer structure, which means that they leak out into the environment (KemI, 2014; Heudorf *et al.*, 2007). This means that they can, for example, be found in indoor air, household dust and in contaminated products such as food products (Heudorf *et al.*, 2007). Some phthalates are considered to be a part of the cocktail of endocrine-disrupting chemicals in our environment (Diamanti-Kandarakis *et al.*, 2009; Wooten & Smith, 2013), and the phthalate chemicals have in many studies been associated with toxic effects on the development of the reproductive system in laboratory animals, primarily in rodents (Committee on the Health Risks of Phthalates, 2008; KemI, 2014).

Since phthalates may affect fertility and reproductive development it is of interest to investigate to what degree dogs are exposed to these chemicals. Both to identify if there is a risk for pet morbidity, but also to evaluate if data from dogs can be used as an indicator for human health risks, seeing as dogs share the same home environment as their human owners.

The purpose of this study is to quantify the concentrations of phthalate metabolites in urine from healthy pet dogs and to investigate relationships between the concentrations and the home environment of the dogs. This study compares a group of dogs living in homes with plastic flooring to a group of dogs living in homes without plastic flooring. The hypothesis is that dogs living in homes with plastic flooring are exposed to a higher amount of phthalates, and should therefore have a higher amount of phthalate metabolites in their urine. This would indicate that dogs, in similarity to humans, are exposed to and absorb phthalates from the environment.

LITERATURE REVIEW

Phthalates

Phthalates are a group of chemicals with a similar chemical structure (Committee on the Health Risks of Phthalates, 2008). The base structure of phthalates is built up of diesters of benzenedicarbxylic acid. The length and structure of the ester side chains varies, thereby giving each phthalate its own characteristics (Committee on the Health Risks of Phthalates, 2008). In Sweden and Europe the most commonly used phthalates are DIDP (diisodecyl phthalate), DPHP (di(2-propyl heptyl) phthalate), DEHP (di(2-ethylhexyl) phthalate) and DiNP (diisononyl phthalate)(KemI, 2014).

Phthalates are predominately used as plasticizers in order to give flexibility to materials such as polyvinyl chloride and other polymers (Schettler, 2006). Phthalates are used in a large number of consumer products, for example pharmaceuticals and medical devices, insecticides,

cosmetics, food packaging, personal-care products, toys, building and cleaning materials and household furnishings (Schettler, 2006). A survey conducted by the Swedish Chemicals Agency found that on the Swedish market, phthalates are primarily found in PVC (polyvinyl chloride) products (KemI, 2014). Other areas of use are rubber products and chemical products such as glue, paint and glazing agents (KemI, 2014).

Exposure

Phthalates are not chemically bound to their polymer structure (KemI, 2014; Heudorf *et al.*, 2007). This allows them to leach, migrate or evaporate into the environment, causing phthalates to be found in indoor air and atmosphere, foodstuff and other materials (Heudorf *et al.*, 2007). Human exposure is mainly through direct contact and use of products containing phthalates, or by phthalates leaking into other products or contaminating the environment (Schettler, 2006). Humans are exposed to phthalates by inhalation, ingestion and dermal exposure throughout their whole lifetime, even during intrauterine development (Heudorf *et al.*, 2007). A study of German children from 3-14 years of age showed that phthalate urine metabolite concentrations decreased with increasing age, and that all children had three to five times higher concentrations compared to levels found in adults (Becker *et al.*, 2009). The relatively higher exposure for young children could be due to behaviour such as mouthing of toys or other PVC products, or through contact with dust when playing on the floor (Becker *et al.*, 2004; Wittassek *et al.*, 2007; Sathyanarayana, 2008).

As companion animals, dogs share the same living environment as their owners. This means that they most likely are exposed to phthalates from the environment in a similar way to humans, and that they too may risk long-term exposure to phthalates (Pathirana *et al.*, 2011). Since dogs spend a lot of time on the floor, it is possible that the exposure for dogs may be even greater than for their adult owners, following that both the floor itself as well as the indoor dust may contain phthalates.

Phthalates such as DiNP and DEHP have been found in children's toys made out of PVC plastics (Stringer *et al.*, 2000). The phthalates DEP (diethyl phthalate) and BBzP (butylbenzyl phthalate) could also be found in children's toys but in smaller amounts. Since phthalates are present in children's toys, it is possible that they also exist in toys or training equipment used for dogs. Wooten & Smith (2013) confirmed that canine toys do contain and leak phthalates (DEHP, BBzP, DEP, DBP) into saliva. The study showed that behaviour such as chewing (through a simulation) increases the amount of phthalates leaking out. Phthalates (DEHP, DiBP) have also been identified in dog toys available on the Swedish market (Nohrborg, 2015). Conclusively, this means that dog toys are a possible source of phthalate exposure for dogs.

Legislation

Because of the toxic properties of phthalates, their use is regulated. Since February 2015, the phthalates DBP (dibutyl phthalate), DEHP, BBzP and DiBP (diisobutyl phthalate) are included on the Authorisation List in Annex XIV, in Regulation (EC) No 1907/2006 of the

European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC[1]. Following this, the use of these four phthalates is inhibited, unless a special permission has been received. There are further specific restrictions for the use of DEHP, DBP and BBzP, DiNP, DIDP (diisodecyl phthalate) and DNOP (di-n-octyl phthalate) in children's toys and childcare products described in Regulation (EC) No 1907/2006, Annex XVII.

However, there are exceptions from the Authorisation List for use in packaging of medical products and the legislation does not include products imported into the European Union. This means that products containing these phthalates will still exist inside the European Union, and that exposure to phthalates will continue to be a relevant issue.

Metabolism

To this date there are few available studies carried out on the metabolism and pharmacokinetics of phthalates in dogs. However, Kao *et al.* (2012) have studied the metabolism and pharmacokinetics of DEP and its metabolite MEP in juvenile dogs. They found that following either intravenous or oral administration of MEP, 91 % of the administered dose was excreted in urine over 24 hours, and 92 % over 72 hours. Similar results were found after intravenous administration of DEP where 96 % of the dose was excreted in urine within 72 hours, and 90 % of the dose was excreted in urine following oral administration. Only 3 % of the dose of both MEP and DEP was excreted in faeces. In other words, according to this study, DEP seems to mainly be excreted in urine in dogs.

The absorption and metabolism of the phthalates was rapid in the study by Kao *et al.* (2012). The elimination half-life was estimated to 1 hour. 24 hours after intravenous administration, the concentrations of MEP and DEP in plasma were undetectable. The study also showed that DEP was rapidly and almost completely metabolised to MEP. 5 minutes after intravenous administration of DEP, MEP was the predominant metabolite in plasma. The authors discuss that this rapid pace suggests that the metabolism of DEP to MEP in dogs is mostly taking place in the blood and liver (Kao *et al.*, 2012).

In an older study by Ikeda *et al.* (1980), beagle dogs were treated with radioactive DEHP through oral administration. 67 % of the dose was excreted in urine and faeces during the first 24 hours, and excretion was completed in 4 days. In this study faecal excretion was predominant in dogs. The elimination half-life was rapid also in this study, and was estimated to 1.4 hours.

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[1] OJ L 396, 30.12.2006, p. 1–849, Celex 32006R1907

Taken together, these studies indicate that the elimination half-life and excretion of the phthalates DEP and DEHP are rapid. However the studies vary by showing that DEP is mainly excreted in urine (Kao *et al.*, 2012) while for DEHP faecal excretion was predominant (Ikeda *et al.*, 1980).

Toxic effects

Phthalates have been shown to exert toxic effects on the reproductive system through their anti-androgenic properties (Gray *et al.*, 2000; Parks *et al.*, 2000). The reproductive toxicity of phthalates is most pronounced during the early development of the reproductive system (Akingbemi *et al.*, 2001). The phthalates DEHP, DBP, BBzP and DiNP all alter sexual differentiation in male rats (Gray *et al.*, 2000; Parks *et al.*, 2000). Since phthalates have different structures and properties, the degree of toxicity varies. For example, the phthalate DiNP is described as 10-20 times less potent than BBzP or DEHP, however still displaying anti-androgenic activity (Gray *et al.*, 2000). In addition to their anti-androgenic activity, phthalates also target the ovary, affecting the processes of folliculogenesis and steroidogenesis in females (Hannon & Flaws, 2015). Because these processes are well conserved in mammalian species, results from studies performed on rodents are considered to be of importance to other species as well (Parks *et al.*, 2000).

Effects on the male reproductive system

DEHP crosses the placental barrier in rats (Stroheker *et al.*, 2006) and may therefore affect foetuses *in utero*. The placental barrier does decrease the foetal exposure significantly, but does not prevent it completely. Therefore the effect of phthalates on foetuses is of interest and has been studied.

Gray *et al.* (2000) showed that phthalates DEHP, BBzP and DiNP after perinatal (*in utero* and neonatal) exposure all alter the sexual differentiation of male rats through their anti-androgenic effect. The phthalates caused reduced testosterone levels which in turn lead to reproductive malformations, reduced testis weight, shortened anogenital distance, retained nipples and undescended testes. Parks *et al.* (2000) similarly to Gray *et al.* (2000) demonstrate that the phthalate DEHP inhibits testicular testosterone production in male rats after exposure during sexual differentiation (*in utero* and neonatal exposure). Male rats displayed reduced testosterone levels, reduced testicular weight, reduced anogenital distance as well as histopathological effects (Leydig cell hyperplasia) on the testes.

DEHP may also antagonise the development of accessory sex organs in rats of twenty days of age. DEHP has been shown to cause a decrease in the relative weights of the prostate, the bulbocavernosus and levator ani muscles and seminal vesicles in male rats (Stroheker *et al.*, 2005). This is also explained by the anti-androgenic effect of DEHP.

The anti-androgenic effect of phthalates does not seem to result from an antagonistic effect on the androgen receptors (AR). Instead, the anti-androgen mechanism appears to be "AR-independent" (Parks *et al.*, 2000). The phthalates appear to target the testes and inhibit the production of testosterone and insulin-like 3 (insl3) (Parks *et al.*, 2000; Wilson *et al.*, 2004),

proposedly though inhibiting fetal Leydig cell maturation so that the production of testosterone and insl3 mRNA is reduced (Wilson *et al.*, 2004).

The anogenital distance, as mentioned above, is used as a measure of demasculinisation of rats exposed to anti-androgenic chemicals (Borch *et al.*, 2006). *In utero* exposure to DiBP, which has similar properties to DBP, showed that DiBP has similar anti-androgenic effects as DBP and DEHP in rats, including reduced anogenital distance, reduced testosterone production and histopathology of the testes including Leydig cell hyperplasia (Borch *et al.*, 2006).

The majority of studies on toxic effects of phthalates are carried out on rats, but there is some research performed on other species. Higuchi *et al.* (2003) studied the effects of exposure of DBP on male rabbits, both *in utero*, and before and after puberty. In the group of rabbits exposed *in utero*, there was a reduction in the number of ejaculated sperm, the weight of the testicles as well as weight of accessory sex glands. Serum testosterone levels were also lower in both the *in utero* group and the adolescent group, and there was an increased amount of abnormal sperm. A few cases of cryptorchidism were also seen, both in the *in utero* group and the adolescent group. The authors conclude that DBP induces lesions in the reproductive system of rabbits, the most sensitive stage being the intrauterine period.

Ljungvall *et al.* (2005) studied the effects of repeated intramuscular administration (twice a week for five weeks) of low dose DEHP to prepubertal male pigs. Two weeks after exposure, there were no differences in testosterone levels compared to the control group. However, at 7.5 months of age (4.5 months after the last exposure) the testosterone concentrations were higher in the group of pigs previously exposed to DEHP, compared to the control group. In other words, the authors found no immediate effects of DEHP on testosterone levels, but delayed effects of DEHP were shown. An increase in the number of testosterone-producing cells was also seen at 7.5 months of age (Ljungvall *et al.*, 2005). In a different study on the immediate and post-pubertal effects of oral DEHP exposure to piglets, DEHP caused precocious maturity of the bulbourethral glands (Ljungvall *et al.*, 2008). These two studies combined demonstrate that prepubertal exposure to DEHP may cause delayed effects on the reproductive system in male pigs.

A study on mice looked at the consequences of oral administration of DBP to male mice from four to fourteen days of age (Moody *et al.*, 2013). The study showed that DBP had effects on testis growth, caused delayed spermatogenesis, lowered serum testosterone and testicular androgen activity as well as a reduced anogenital distance. Conclusively, DBP has anti-androgenic activity in the mouse, and the neonatal-prepubertal testicles in mice are vulnerable to DBP.

Not many studies are performed on the effects of phthalates on dogs. Pathirana *et al.* (2011) carried out an in vitro study on testicular cells from dogs. They concluded that certain phthalate esters (MBP, MEHP) had effects on the secretion of testosterone in testicular interstitial cells from dogs. The phthalates also had effect on the secretion of insulin-like

peptide 3 (INSL3). hCG-induced testosterone and INSL3-secretion were inhibited by both MBP and MEHP, however the basal testosterone release was increased by MEHP. Because pre-pubertal dogs have low LH-levels, the authors discuss that you might anticipate an increase in testosterone concentrations for these individuals and that this could possibly lead to hastened puberty. In sexually mature dogs where LH-levels are higher, the authors discuss that the hCG-mediated effect on testosterone might be of greater importance.

To conclude, phthalates have been shown to affect the male reproductive system in different ways, including effects on testosterone levels, the weight of testes and accessory sex glands, the anogenital distance, formation of female-like nipples and the descent of testes (Gray *et al.*, 2000; Parks *et al.*, 2000; Borch *et al.*, 2006; Stroheker *et al.*, 2005; Higuchi *et al.*, 2003; Moody *et al.*, 2013; Pathirana *et al.*, 2011).

Effects on the female reproductive system

In addition to their effects on the male reproductive system, phthalates have been shown to target the ovaries, affecting the folliculogenesis and steroidogenesis, which can lead to effects on the female reproductive health (Hannon & Flaws, 2015). For example, MEHP (the active metabolite of DEHP) affects granulosa cell estradiol production in the ovaries and significantly decreases estradiol production in rats, as shown in an in vitro study by Lovekamp & Davis (2001). The same study also suggests that this is due to MEHP down-regulating the levels of aromatase transcript. Aromatase is the rate-limiting enzyme for production of estradiol.

DEHP and its metabolites may also affect oocytes in different manners. Exposure to MEHP for 24 hours has been shown to significantly decrease the number of vital oocytes in female mice using an in vitro system (Bonilla & Mazo, 2010). DEHP also caused modifications in the DNA methylation in fetal mouse oocytes that were shown to be heritable (Li *et al.*, 2014).

In utero exposure to MEHP has been shown to cause reproductive effects in mice (Moyer & Hixon, 2012). Female mice exposed to MEHP *in utero* displayed for example delayed estrous onset, prolonged estrous, a decrease in the overall reproductive lifespan, altered estrous cyclicity and mammary gland hyperplasia. As for hormone levels, MEHP exposure caused increased serum estradiol and FSH levels in these mice when they reached adulthood. Maternal body weight or pup growth was however not affected by this exposure (Moyer & Hixon, 2012). A different study on exposure to DEHP via inhalation to prepubertal rats also showed effects on the estrous cycle and hormone levels (Ma *et al.*, 2006). Ma *et al.* (2006) showed that female rats exposed to DEHP in the prepubertal period displayed an earlier onset of the first estrous cycle, irregular estrous cycles and an increase in serum estradiol-, LH- and cholesterol levels. Yet another study on DEHP showed that oral exposure to pre-pubertal female rats caused reduced serum progesterone and estradiol levels (Svechnikova *et al.*, 2007).

Ema *et al.* (2000) showed that DBP caused a significant increase in the incidence of early embryonic loss in pregnant rats, which may be mediated by DBP impairment of uterine

function, causing an adverse effect on uterine decidualization. Similar studies revealed similar effects of MBP (Ema *et al.*, 2001) and BBzP (Ema *et al.*, 1998); MBP and BBzP both caused early (pre- and post-implantation) embryonic loss in rats, mediated at least in part by suppressed uterine decidualization. Ema *et al.* (2000) also conclude that there is an indication of DBP being more harmful on embryos during the early post-implantation period compared to pre-implantation.

Gray *et al.* (2006) studied the effects of chronic DBP exposure to female rats, and found that oral administration of DBP from weaning, through puberty, mating and pregnancy disrupts pregnancy maintenance and induces infertility. Mid-pregnancy abortions were induced and the percentage of females delivering live pups was reduced. DBP treatment caused a significant decrease in ovarian hormone production and reduced serum progesterone levels. The authors speculate that the failure to maintain pregnancy could be due to insufficient ovarian progesterone levels, following the effects of DBP. In the study by Ema *et al.* (1998) a similar trend toward decreased serum progesterone levels was seen in rats after exposure to BBzP.

In summary, several different phthalates have been associated with toxic effects on the female reproductive system, including effects on the levels of progesterone and estradiol, the onset of puberty and the estrous cycle, as well as the maintenance of pregnancy (Ema *et al.*, 2000; Ema *et al.*, 2001; Ema *et al.,* 1998; Gray *et al.*, 2006; Lovekamp & Davis, 2001; Moyer & Hixon, 2012; Ma *et al.*, 2006; Svechnikova *et al.*, 2007).

MATERIAL AND METHODS

Selection of Dogs

Sixteen clinically healthy dogs living in Uppsala, Sweden were selected to participate in the study. The dogs participating in the study were found by emailing out to veterinary students at the Swedish University of Agricultural Sciences. Eight of these dogs lived in homes with over 50 % plastic flooring, while the other eight dogs lived in homes without plastic flooring. The material of the flooring was confirmed through contact with respective landlords in order to avoid confusing plastic flooring (PVC) with similar materials such as linoleum. Each group consisted of four males and four females, aiming to select dogs of medium size and of relatively similar weights, approximately 15-25 kg, to achieve a matching between the two groups.

Collection of Samples

One urine sample of at least 4 ml was collected together with the owner by midstream voided catch at one point of time from each dog. The equipment used to collect the sample consisted of metal and plastic which was free from phthalates. The dogs had stayed in their home environment before the sample was collected, and had not been taken out for a walk for at least two hours.

The dog owners filled in a questionnaire to provide more information about their dogs' home environment. The owners were asked about the flooring in their homes, material of food bowls and toys, how often their home was vacuumed and if their dog had a history of disease in the reproductive system. The owners also signed an approval for their dog to participate in the study.

After each urine sample was collected, it was immediately put in a cooler and transported to a freezer. The urine samples were then stored in a freezer at -20 °C until all samples had been collected. When all samples had been collected they were sent for analysis to a laboratory at the section for Occupational and Environmental Medicine, Department of Laboratory Medicine, Faculty of Medicine, Lund University, Sweden.

Analysis

Each sample was analysed for ten urinary phthalate metabolites originating from five different phthalates. The metabolites and their parent compounds are included in Table 1. The phthalate metabolites in urine were analysed using liquid chromatography tandem mass spectrometry. A detailed description of this technique can be found in Bornehag *et al.* (2015).

Table 1. *The phthalate metabolites included in the analysis and their parent compounds*

Parent compound		Metabolite	
Acronym	*Diester name*	*Acronym*	*Monoester name*
DEP	Diethyl phthalate	MEP	Mono-ethyl phthalate
DBP	Dibutyl phthalate	MnBP	Mono-*n*-butyl phthalate
BBzP	Butylbenzyl phthalate	MBzP	Mono-benzyl phthalate
DEHP	Di(2-ethylhexyl) phthalate	MEHP	Mono-(2-ethylhexyl) phthalate
		MHEHP	Mono-(2-ethyl-5-hydroxylhexyl) phthalate
		MOEHP	Mono-(2-ethyl-5-oxohexyl) phthalate
		MCEPP	Mono-(2-ethyl-5-carboxypentyl) phthalate
DiNP	Diisononyl phthalate	MHiNP	Mono-(4-methyl-7-hydroxyloctyl) phthalate
		MOiNP	Mono-(4-methyl-7-oxo octyl) phthalate
		MCiOP	Mono-(4-methyl-7-carboxyheptyl) phthalate

The metabolite concentrations were adjusted to urine density using the formula below (as described in Carnerup *et al.* (2006)):

$$C_c = \frac{C_o \times (1.0 - 3)}{p - 1}$$

C$_{(corr)}$ is the metabolite concentration corrected for urine density, C$_{(obs)}$ is the observed metabolite concentration and p is the specific urine density of the sample. 1.032 is the mean urine density of the samples in this study.

The data was then analysed statistically. A normal distribution could not be assumed due to the limited sample size and therefore the non-parametric (NPAR1WAY) procedure of SAS (SAS Institute Inc., Cary, NC, USA, version 9.02.01) was used to perform one-sided Wilcoxon two-sample exact tests. Homogeneity of variances was confirmed by Levene's test. Differences in metabolite concentrations corrected for urine density were tested between type of flooring (plastic n=7, or non-plastic flooring n=8), toys (plastic n=6, or non-plastic toys n=9) and sex (male n=7, or female n=8). Due to the small sample size for the other characteristics of the dogs' home environment (Table 2), no statistical calculations were performed on these variables.

RESULTS

Dog Characteristics

Both the plastic and the non-plastic flooring group consisted of 4 female and 4 male dogs after purposely selecting this to achieve an even distribution of sexes. One male dog from the group with plastic flooring had a urine density that could not be quantified, and it was therefore excluded from all calculations carried out on the corrected phthalate metabolite concentrations. For these calculations the group with plastic flooring therefore consisted of 4 female and 3 male dogs.

The aim was to select dogs of similar sizes (around 15-25 kg). The weight of the dogs participating in the study ended up ranging from 13-32 kg, with a mean weight of 23.2 kg. Age varied from 1 to 13 years, with a mean of 5.4 years. Only one of the dogs had a history of disease in the reproductive organs (prostatitis).

There were no differences in body weight or age (p>0.05) between the seven dogs in the group with plastic flooring, and the eight dogs in the group with non-plastic flooring. Similarly, there were no differences in body weight or age between dogs using plastic toys and dogs not using plastic toys.

The owners were asked about their dogs' home environment (Table 2) to identify other risk factors for increased phthalate metabolite concentrations. In the group with plastic flooring, 5/7 dogs had 100% plastic flooring, 1/7 had 75-100% plastic flooring and 1/7 had 50-75% plastic flooring in their homes. The homes of the dogs with plastic flooring were all owned by the same landlord (Foundation of Ultuna Student Housing, Uppsala, Sweden), however the age of the buildings as well as the type and brand of flooring varied.

Table 2. *Characteristics of the study population (n=15)*

Characteristic	Group with plastic	Group with non-

	flooring, $n = 7$[a]	plastic flooring, $n = 8$
Type of flooring		
Plastic	7	0
Linoleum	0	4
Wood	0	4
Other	0	0
Does the dog have plastic toys?		
Yes	4	2
Does the dog have a plastic food bowl?		
Yes	1	1
Frequency of vacuuming the home?		
1-2 times per month	3	1
Once a week	4	4
Several times per week	0	3

[a]The dog with immeasurable urine density is not included in this table.

Metabolite Concentrations

Urine from each of the sixteen dogs was analysed for the ten phthalate metabolites included in Table 1. All dogs had measurable levels of each metabolite in their urine. The mean, median and range of metabolite concentrations, before correction for urine density, can be found in Table 3. The metabolite concentrations after correction for urine density are found in Table 4. Sex did not have any influence on the corrected urine concentrations of phthalate metabolites ($p>0.05$).

Table 3. *Phthalate metabolite concentrations before correction for urine density, values in ng/ml*

	All dogs ($n=16$)		
	Mean ± standard deviation	Median	Range
MEP	22.6 ± 20.5	14.8	4.5-67.3
MnBP	60.1 ± 47.3	40.9	7.1-160
MBzP	75.6 ± 154	14.3	2.7-586
MEHP	18.4 ± 22.4	8.6	1.3-81.6
MHEHP	71.7 ± 79.8	27.2	6.9-241
MOEHP	5.7 ± 6.8	2.3	0.8-26.5
MCEPP	22.5 ± 32.0	8.8	2.3-132
MHiNP	22.9 ± 31.1	11.1	1.3-114
MOiNP	4.7 ± 8.4	1.5	0.1-27.2
MCiOP	56.3 ± 96.7	14.1	1.0-332

As seen in Table 4, the metabolite concentrations were generally higher in the group with plastic flooring, although not significantly so for all metabolites. Figure 1 demonstrates this with box plots, showing the two groups next to each other. MBzP is the metabolite found in highest concentrations.

Table 4. *Phthalate metabolite concentrations after correction for urine density, values in ng/ml*

	All dogs (*n*=15)			Plastic flooring (*n*=7)		Non-plastic flooring (*n*=8)		
	Mean ± stdv[a]	Median	Range	Mean ± stdv	Median	Mean ± stdv	Median	*P*-value[b]
MEP	24.5 ±18.4	17.5	4.4-58.2	24.2 ± 19.5	23.6	24.7 ± 18.7	15.1	0.39
MnBP	60.7 ± 41.2	48.1	19.3-155	79.5 ± 49.2	82.9	44.3 ± 25.5	35.0	0.08
MBzP	72.6 ± 134	17.6	2.6-506	144 ± 175	105	9.8 ± 7.4	6.7	0.005
MEHP	20.1 ± 20.5	6.3	1.3-58.0	31.9 ± 22.0	34.8	9.7 ± 12.7	5.3	0.03
MHEHP	63.6 ± 71.9	31.6	8.3-276	101 ± 91.2	94.5	30.8 ± 23.8	23.5	0.03
MOEHP	5.3 ± 5.0	3.2	0.7-16.0	7.9 ± 5.7	6.9	3.0 ± 3.0	1.7	0.05
MCEPP	21.8 ± 24.0	12.8	2.0-93.8	36.1 ± 28.1	25.3	9.2 ± 9.5	5.4	0.005
MHiNP	25.2 ± 33.9	7.2	2.8-117	25.7 ± 29.8	8.9	24.7 ± 39.1	6.7	0.39
MOiNP	5.3 ± 8.2	1.2	0.2-25.3	6.1 ± 8.4	1.4	4.6 ± 8.5	0.9	0.31
MCiOP	60.8 ± 96.0	10.0	2.7-342	62.1 ± 75.0	10.7	59.6 ± 116	9.3	0.35

[a]Standard deviation.
[b]One-sided Wilcoxon two-sample exact test for differences between flooring groups. *P*-values <0.05 are considered significant.
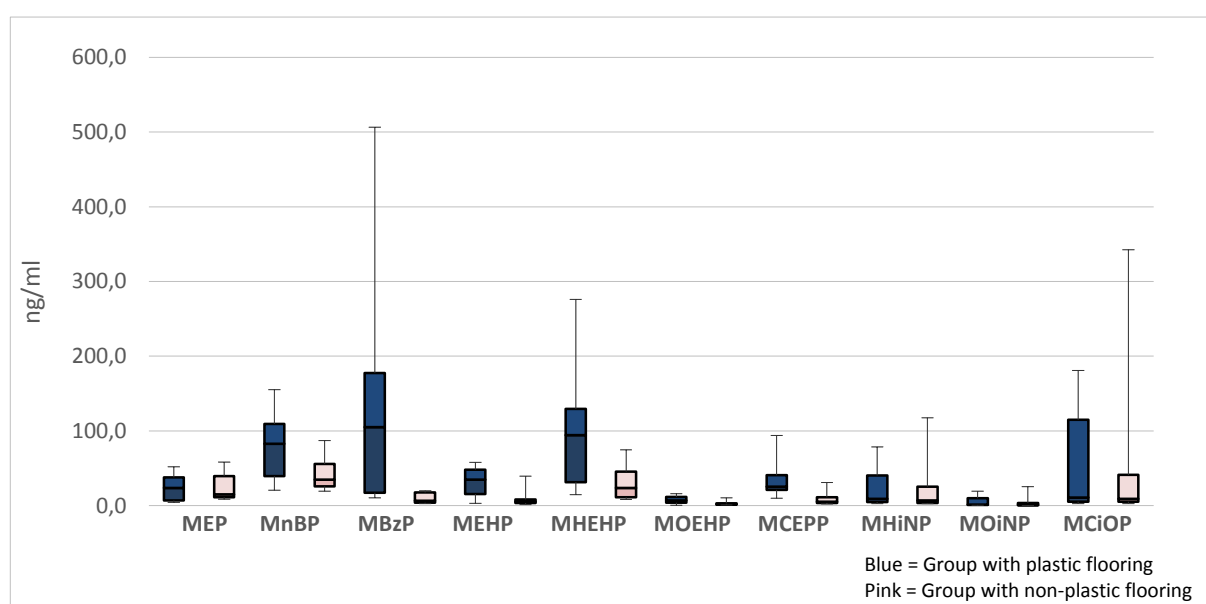

Figure 1. *Urine concentrations of the ten phthalate metabolites corrected for urine density, values in ng/ml (blue = group with plastic flooring, pink = group with non-plastic flooring).*

Five of the ten metabolites were found in significantly higher levels in the group of dogs with plastic flooring, compared to the group of dogs with non-plastic flooring ($p<0.05$, Table 4). These were the metabolites of the phthalates BBzP (MBzP) and DEHP (MEHP, MHEHP, MOEHP, MCEPP). For the metabolites of DEP, DBP and DiNP the difference between the groups was not significant. Figures 2, 3, 4, 5 and 6 show the metabolites where a statistically significant difference ($p<0.05$) was found between the two groups.
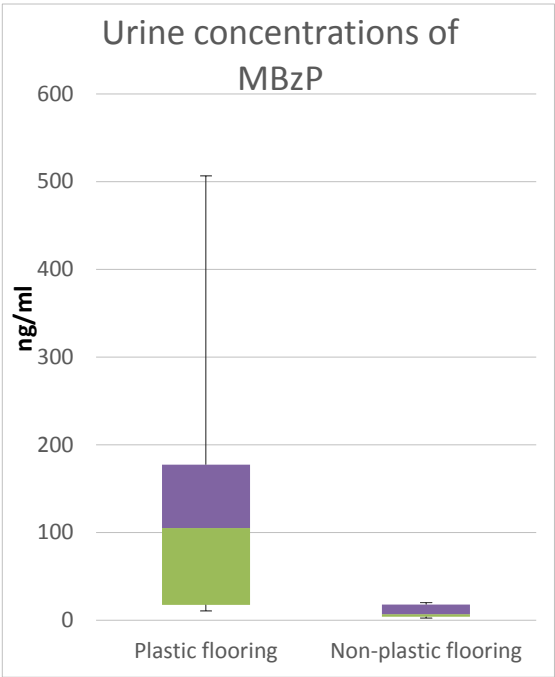

Figure 2. *Urine concentrations of MBzP, metabolite of the phthalate BBzP, corrected for urine density.*
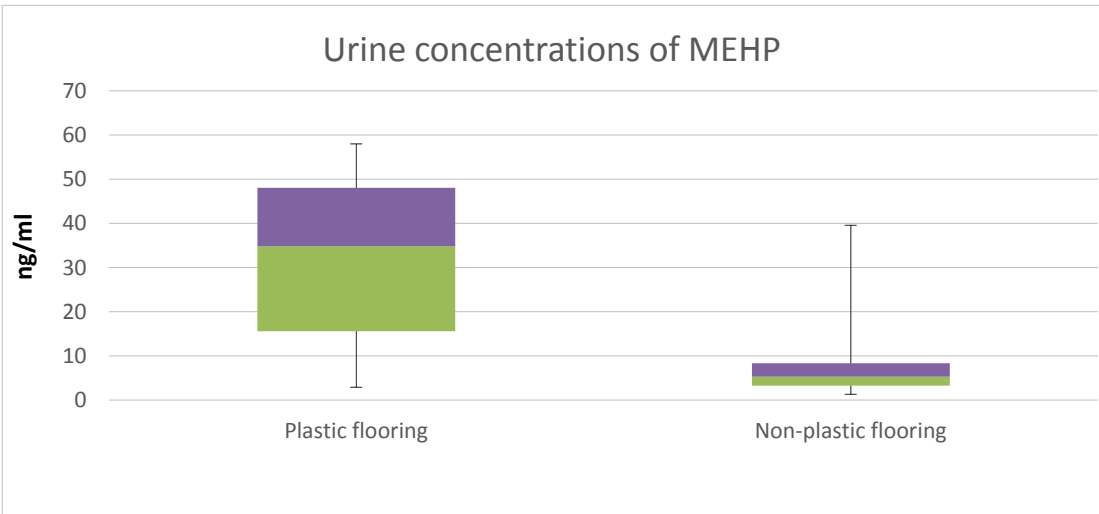

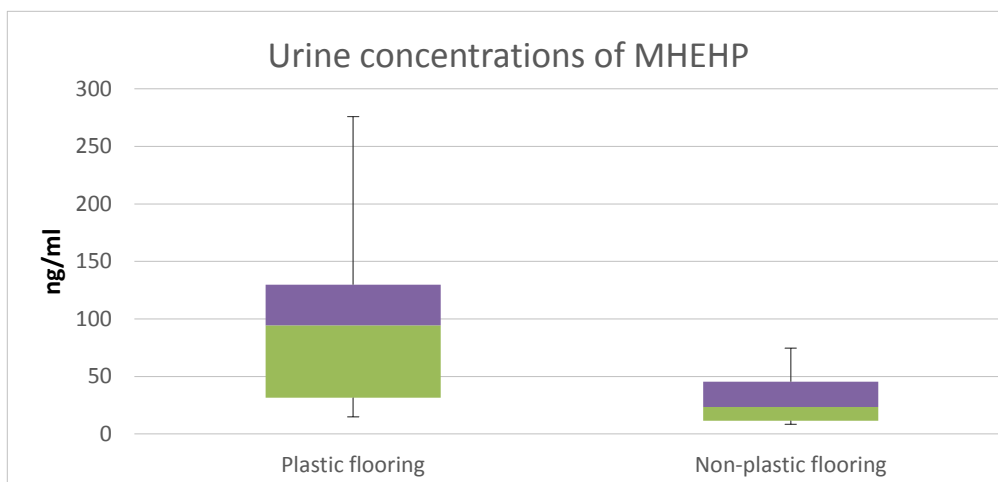Figure 3. *Urine concentrations of MEHP, metabolite of the phthalate DEHP, corrected for urine density.*

Figure 4. *Urine concentrations of MHEHP, metabolite of the phthalate DEHP, corrected for urine density.*
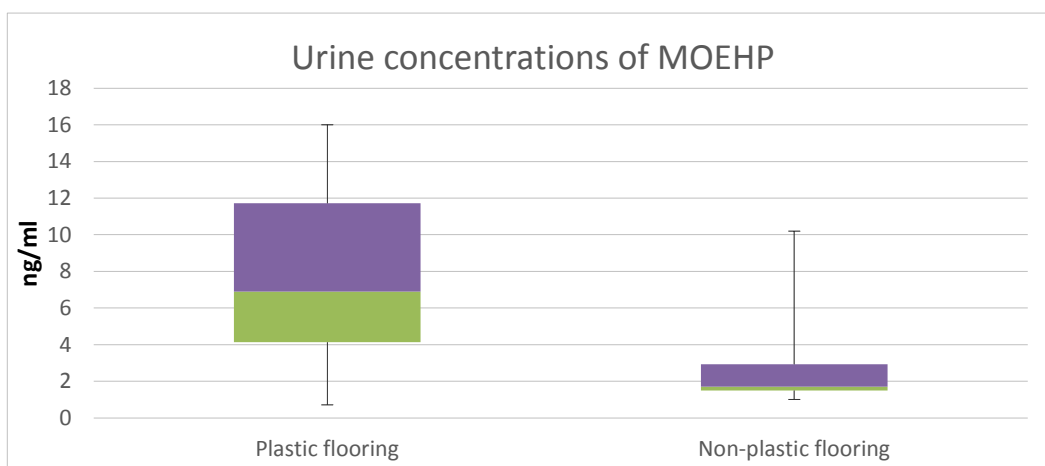

Figure 5. *Urine concentrations of MOEHP, metabolite of the phthalate DEHP, corrected for urine density.*
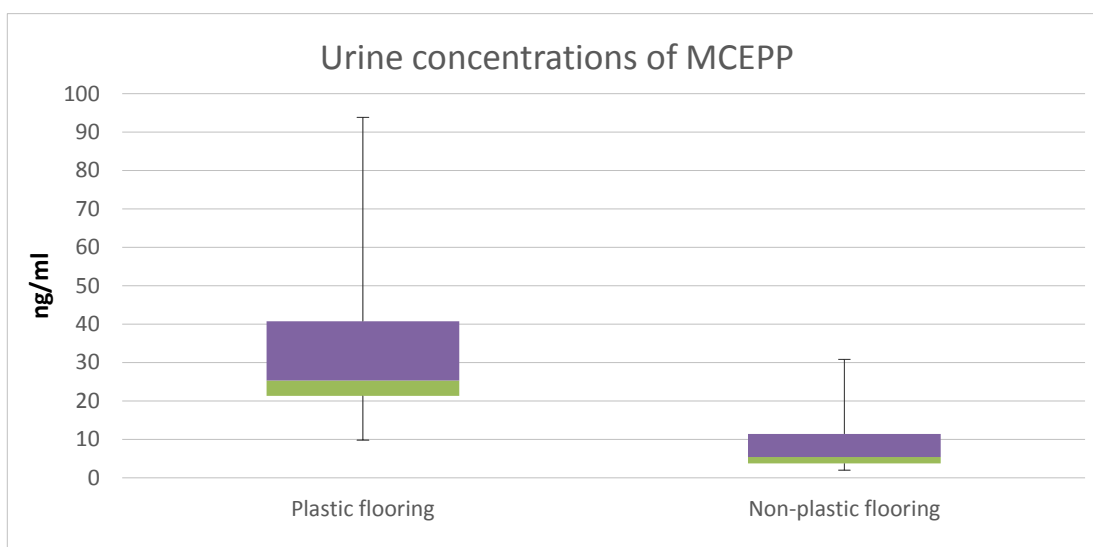

Figure 6. *Urine concentrations of MCEPP, metabolite of the phthalate DEHP, corrected for urine density.*

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There were significant differences in urine concentrations of the metabolites MEP (metabolite of DEP), MBzP (metabolite of BBzP) and MHEHP (metabolite of DEHP) between dogs using plastic toys and dogs not using plastic toys (p=0.01, p=0.01 and p=0.02, respectively). Higher urine concentrations were found in the group of dogs using plastic toys. For MEP, the median values of the corrected urine concentrations were 43.7 ng/ml for dogs using plastic toys, and 12.3 ng/ml in the group not using plastic toys. The corresponding medians for MBzP were 94.2 and 10.3 ng/ml, respectively. For MHEHP, the median in the group using plastic toys was 72.6 ng/ml and 18.2 ng/ml in the group not using plastic toys.

DISCUSSION

This study consists of a small sample of dogs, which has to be considered when looking at the results. The main aim was to quantify phthalate concentrations in urine from dogs, and to investigate differences in urine metabolite concentrations due to differences in the dogs' home environments, primarily plastic flooring compared to non-plastic flooring. The dogs participating in the study were selected to achieve an even distribution of sexes and a relatively similar weight in the two groups, to make the two groups as comparable as possible. The results also showed that there was no difference in body weight or age between the group with plastic flooring and the group with non-plastic flooring. The time of day for which the urine sample was collected varied between the dogs and there was a variation in how many hours had passed since the dog had last been taken out for a walk (at least 2 hours). To compensate for this the metabolite concentrations were corrected for urine density.

All dogs included in the study had measurable levels of each of the ten phthalate metabolites, which leads to the conclusion that dogs are exposed to and absorb phthalates from the environment. The results showed that the concentrations were generally higher for all metabolites in the group with plastic flooring, compared to the group with non-plastic flooring. This difference between the groups was statistically significant ($p<0.05$) for five of the ten metabolites. These were the metabolite of the phthalate BBzP and the four metabolites derived from DEHP. This suggests that dogs living in homes with plastic flooring are at risk of being exposed to a larger amount of phthalates, compared to dogs living in homes without plastic flooring. These results correspond well to a study carried out by Carlstedt *et al.* (2013), where the urine concentration of MBzP (metabolite of BBzP) measured in children was significantly higher in infants exposed to PVC flooring compared to infants not exposed to PVC flooring. Carlstedt *et al.* (2013) conclude that their results indicate that PVC flooring is an important source for human uptake of BBzP. Similarly, the results in this study indicate that plastic flooring is an important source also for canine uptake of BBzP. Flooring is one of two products in Sweden and Denmark where the highest levels of BBzP can be found (KemI, 2014), which could explain why the metabolite MBzP is one of the metabolites where a significant difference was found between the two groups.

In this study, the results showed a significant difference between dogs using plastic toys and dogs not using plastic toys for urine concentrations of the metabolites MEP, MBzP and MHEHP. Other studies have previously identified the parent compounds for these metabolites

in canine toys, for example DEHP (Nohrborg, 2015; Wooten & Smith, 2013) of which MHEHP is a metabolite, and BBzP and DEP (Wooten & Smith, 2013) of which MBzP and MEP are metabolites. One of the studies also showed estrogenic and anti-androgenic activity of some of the phthalates found in dog toys in *in vitro* assays (Wooten & Smith, 2013). These results indicate that plastic toys could possibly have an influence on urine metabolite concentrations of phthalates in addition to plastic flooring, and should be considered as a source of exposure to phthalates for dogs. However, this study was primarily designed to find differences between dogs living in homes with plastic flooring and dogs living in homes without plastic flooring. Because of this, the sample sizes when testing the effect of plastic toys were unbalanced. Some of the dogs with plastic toys also lived in homes with plastic flooring, which could affect the results. Future studies should take the use of plastic toys into account as a risk factor when studying phthalate metabolite concentrations in dog urine.

Because dogs are kept as companion animals living in the same environment as their owners, dogs could possibly be used as an indicator for human exposure to phthalates. Studying the urine excretion of phthalate metabolites in dogs may offer a non-invasive and practical tool for assessing the level of phthalate exposure that humans are subjected to in their home environment. Latini (2005) states that frequent monitoring of human phthalate metabolite concentrations in body fluids such as urine would be "highly advisable" as a tool for health risk assessment, and as a guide when establishing regulations for the use of phthalates. It is possible that measuring phthalate metabolite concentrations in dog urine could be a helpful part in this.

It is of interest to determine if the levels of urinary metabolites found in this study correspond to levels found in human urine samples. However, comparisons are complicated by the fact that the type of urine samples used differs (spot urine sample, morning urine sample or 24 hour urine sample) and that it varies if the metabolite concentrations have been corrected for urine density. Carlstedt *et al.* (2013) studied the concentration of phthalate metabolites in spot urine samples from 83 Swedish children (2-6 months old) of which 35 % had PVC flooring in the infant's bedroom. The uncorrected median value for urine concentrations of MBzP (for all children participating in the study, PVC flooring or other) was 10.5 ng/ml in the study by Carlstedt *et al.* (2013), whereas the uncorrected median for all dogs in our study was 12.0 ng/ml. For MEP the uncorrected median was 19.6 ng/ml (Carlsted *et al.*, 2013) and in our study 14.6 ng/ml. For MnBP the uncorrected median was 39.1 ng/ml (Carlstedt *et al.*, 2013) and in our study 37.6 ng/ml. In summary, the median concentrations for the metabolites MBzP, MEP and MnBP in this study are similar to the median concentrations found in Swedish children (Carlstedt *et al.*, 2013).

In a different study, Frederiksen *et al.* (2011) analysed the urine levels of phthalate metabolites in Danish children and adolescents aged 6-21 years. In that study, the uncorrected median concentrations of MBzP was 17 ng/ml in the 24 hour urine sample and 32 ng/ml in the morning urine sample, compared to the uncorrected median in this study which was 12.0 ng/ml and the corrected median of 17.6 ng/ml. Yet another study by Boas *et al.* (2010) on phthalate metabolites in spot urine samples from 845 Danish children (ages 4-9 years) show

concentrations in about the same region for several metabolites of DEP, BBzP and DEHP. For example, the median concentration of MEP in the study by Boas *et al.* (2010) was 21 ng/ml, in our study the uncorrected median for all dogs was 14.6 ng/ml and the corrected median 17.5 ng/ml. The concentrations in the studies by Carlstedt *et al.* (2013), Frederiksen *et al.* (2011) and Boas *et al.* (2010) have not been corrected for urine density like the concentrations in this study. If they had been corrected for urine density a more accurate comparison could have been made with our corrected values. Still, the uncorrected values are in about the same interval in several cases. This suggests that the level of exposure to phthalates might be similar for dogs and young children.

In the study by Frederiksen *et al.* (2011) the concentrations of phthalate metabolites in the 24 hour urine sample were used to estimate a daily phthalate intake. The estimated daily intake was then compared to the Tolerable Daily Intake (TDI) of four phthalates. The TDIs for phthalates have been set by the Scientific Committee of Food (SCF) and evaluated by the European Food Safety Authority (EFSA) in 2005 (EFSA 2005a, 2005b, 2005c, 2005d). The TDI for DEHP is set to 0.05 mg per kg body weight per day and the TDIs for BBzP, DBP and DiNP are 0.5, 0.01 and 0.15 mg per kg body weight per day, respectively. Frederiksen *et al.* (2011) found that the estimated daily intakes of the two isoforms of DBP exceeded the TDI in several of the children. In one child the TDI for DEHP was exceeded. A second study that estimated the daily intake of DEHP in German children found that TDI was exceeded in three children (1%) (Wittassek *et al.*, 2007). Similar studies could be performed on dogs to calculate an estimated daily phthalate intake. Assuming a similar TDI for dogs as humans, it could be speculated that some dogs might exceed the recommended daily intake of phthalates for some of the metabolites, seeing as the concentrations found in this study in dogs are in the same interval as concentrations found in humans, however more studies are needed.

If this level of exposure to phthalates affects the health of dogs is still unclear. It is known that phthalates have adverse effects on the male and female reproductive system in primarily rodents. There are also a few studies carried out on dogs, such as the in vitro study on testicular cells from dogs by Pathirana *et al.* (2011) showing effects of MBP and MEHP on the secretion of testosterone. The concentrations of phthalates used in these experimental studies are generally much higher than TDI-levels. For example, effects are shown in studies after maternal exposure during gestation by oral gavage to 750 mg/kg/day of DEHP (Parks *et al.*, 2000; Gray *et al.*, 2000), BBzP and DiNP (Gray *et al.*, 2000). Stroheker *et al.* (2005) showed effects starting from a dose of 100 mg DEHP/kg bw/day. However, phthalates may possibly be more toxic or have different mechanisms at lower doses because of their non-monotonic dose response (Beausoleil *et al.*, 2013; Hannon & Flaws, 2015). Therefore, further studies on the effects of long-term exposure to lower concentrations that correlate to concentrations found in samples from normal humans or dogs would be of interest.

CONCLUSION

In summary, this study has shown measurable levels of ten different phthalate metabolites in urine samples from sixteen pet dogs. The metabolite of BBzP (MBzP) was found in the

highest concentrations. The metabolites of BBzP and DEHP were found in significantly higher concentrations in the group of dogs living in homes with plastic flooring, compared to dogs living in homes without plastic flooring, which suggests that exposure to a home environment with plastic flooring leads to higher phthalate metabolite concentrations in urine. The study also identified plastic toys as a possible risk factor for having higher phthalate metabolite concentrations. Since dogs living in homes with plastic flooring had significantly higher concentrations of some of the metabolites that are commonly used in PVC-flooring, it suggests that dogs could possibly be used as indicators for indoor environments. The concentrations found in this study are in around the same range as concentrations found in urine samples from humans. More studies are needed in order to investigate if this exposure to phthalates has a negative effect on the reproductive health and fertility of dogs.

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