Systematic Comparison of the Male Reproductive Tract in Fetal and Adult Wistar Rats exposed to DBP and DINP in utero during the Masculinisation Programming Window

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Highlights

- DINP exposure in the MPW (e15.5-18.5) does not result in effects known for DBP
- DINP exposure in the MPW has no effect ADG on e21.5.
- Leydig cell aggregates on e17.5 and e21.5 were not increased.
- DINP exposure in the MPW does not induce focal testicular dysgenesis.

Systematic Comparison of the Male Reproductive Tract in Fetal and Adult Wistar Rats exposed to DBP and DINP in utero during the Masculinisation Programming Window

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Abstract

This study investigates possible effects of in utero exposure of rats to a low dose (125 mg/kg bw/day) and a high dose (750 mg/kg bw/day) of Diisononyl phthalate (DINP) during the masculinisation programming window (MPW) which is embryonic days 15.5 to 18.5 (e15.5 - e18.5). Dibutyl phthalate (DBP) was used at a high dose level (750 mg/kg bw/day) as an established positive control substance for anti-androgenic effects on the developing male reproductive tract.

We focussed on the MPW and measured a multitude of biological endpoints at various life stages and applied state of the art histopathology staining techniques to refine the characterization of potential changes to the testis, beyond what is currently available with DINP. If DINP can mediate testicular dysgenesis (TDS) disorders, this exposure window would be sufficient to induce androgen impacts and alter male reproductive tract development as shown earlier in this validated experimental model with DBP.

Overall, the results of this systematic comparison provide convincing evidence on the differences between the effects occurring with DBP and DINP. In contrast to what was seen with DBP, DINP did not cause cryptorchidism or hypospadias, had no effect on anogenital distance/anogenital index (AGD/AGi) and Leydig cell aggregates on e17.5 and e21.5 did not increase. With DINP no reduction of intratesticular testosterone, no effects on sperm motility and sperm count and no effect on adult testosterone or luteinizing hormone (LH) levels were seen. Our results demonstrate that DINP does not cause the adverse reproductive effects known to occur with DBP, a well-established endocrine disruptor.

Keywords

- Diisononyl phthalate (DINP)
- Dibutyl phthalate (DBP)
- Endocrine disruption
- Sperm parameters
- Leydig cell aggregate quantification
- Testicular Dysgenesis Syndrome

Abbreviations

AGD: Anogenital distance

AGi: Anogenital distance normalized to body weight

AUC_{inf}: Area under the concentration curve extrapolated to infinity

BSA: bovine serum albumin

C_{max}: maximum concentration

DBP: Dibutyl phthalate

DINP: Diisononyl phthalate

e day: embryonic day (e1=GD1; gestational day 1)

3β-HSD: 3-β-hydroxysteroid dehydrogenase; Leydig cell marker

HMW: high molecular weight phthalate; all with straight carbon chain length of the esterified alcohols ≥7

GD: gestational day (GD1=e1; embryonic day 1)

NCS: normal chicken serum

NGS: normal goat serum

LMW: low molecular weight phthalate; straight chain length of the esterified alcohols between 3 and 6 carbon atoms

MPW: masculinisation programming window

PBS: phosphate buffered saline

SC: Sertoli Cell

SMA: α-smooth muscle actin; peritubular myeloid cell marker

Sox-9: gene mainly expressed in Sertoli cells; Sertoli cell marker

TBS: Tris buffered saline

TDS: Testicular Dysgenesis Syndrome

1. Introduction

Formation of a testis and its subsequent production of androgens in early fetal life are prerequisites for masculinisation (Scott et al., 2009). Any disruption of these events can have adverse consequences and lead to common reproductive disorders that manifest already at birth, in puberty or in adulthood - this has been summarised in the testicular dysgenesis syndrome (TDS) hypothesis (Skakkebaek et al., 2001). A rat model of TDS has been developed and validated involving in utero exposure to Dibutyl phthalate (DBP) (Fisher et al., 2003; Kilcoyne et al., 2014; van den Driesche et al., 2012). DBP is a low molecular weight phthalate (LMW) with a straight carbon chain length in the esterified alcohols of 4 carbons (Table 1). DBP is classified and labelled accordingly as toxic to reproduction and is an established positive control substance for androgen disruption during this period of male fetal development. In utero exposure to DBP at a time which includes the so-called masculinisation programming window (MPW; embryonic day e15.5-e18.5), results in impaired androgen production, late-onset focal testicular dysgenesis and downstream male reproductive tract malformations (Carruthers et al., 2005; MacLeod et al., 2010; van den Driesche et al., 2017; Welsh et al., 2008). Recently it was demonstrated that DBP exposure specifically only in the MPW is necessary and sufficient to induce suppression of fetal testosterone, reduced anogenital distance (AGD), focal testicular dysgenesis

(manifesting as malformed seminiferous tubules and abnormal distribution of somatic cells in focal areas) and TDS disorders such as cryptorchidism, hypospadias, reduced adult testis size, and compensated adult Leydig cell failure (van den Driesche et al., 2017). Reduced androgen production in this small critical window of time during development is considered central to these changes, but exactly how, and its necessity for causing all manifestations is not established.

[Table 1]

Exposure of rats to other LMW phthalates (e.g. Diisobutyl phthalate (DIBP) and Di-(2-ethylhexyl) phthalate (DEHP)) has been shown to disrupt sexual development in male offspring following exposure during the MPW in a manner similar to DBP (Saillenfait et al., 2006, 2008, 2011; Wolfe et al., 2005). Based on the collective data for the LWM phthalates, it is considered that phthalates with straight carbon chain lengths in the esterified alcohols between 3 and 6 form the active cluster for toxicity to reproduction, with testicular toxicity being the most sensitive endpoint (Fabjan et al., 2006). The ability of the high molecular weight (HMW) phthalate Diisononyl phthalate (DINP) with straight carbon chain lengths of ≥7 in the esterified alcohols to similarly disrupt development of the male reproductive tract and lead to TDS disorders is not well supported by existing data (ECHA, 2013, ECHA RAC 2018; NICNAS, 2015).

DINP is a member of the category of HMW phthalates and is a complex mixture of diesters. There are two technically inter-changeable types of DINP available in

commerce: 1,2-Benzenedicarboxylic acid esterified with branched alcohols consisting of C8-C10 (C9 rich) alkyl side chains (CAS No 68515-48-0), also referred to as DINP1, and DINP2 (CAS No 28553-12-0), esterified with alcohols of strictly C9 branched and linear alkyl side chains (EU RAR, 2003; Koch et al., 2012). In DINP1 the alcohols typically consist of dimethyl heptanols branched at diverse positions and 15-25% isodecanols (Waterman et al., 1999). In DINP2 the predominant C9-isomers are identified as methyl octanols and dimethyl heptanols, and 10% of the linear n-nonanol. For DINP, liver toxicity in rodents is identified to be the most sensitive endpoint (EFSA, 2005, 2019; ECHA RAC 2018) and accordingly, EFSA has based their derived tolerable daily intake (TDI) on this endpoint. It has been suggested that liver toxicity is indirectly linked to transient reductions of testicular testosterone (ECHA RAC 2018; Dekant, 2020), but DINP is shown to be less potent than DBP (Clewell et al., 2013b; Furr et al., 2014) in reducing testicular testosterone. Available studies involving exposure to either DINP1 or DINP2 during the MPW collectively show marked differences in the extent, severity and incidence of effects when compared to similar doses of DBP. The studies on DINP have consistently reported a lack of induction of gross male reproductive tract malformations such as cryptorchidism and hypospadias (Boberg et al., 2011; Clewell et al 2013b; Gray et al., 2000). Likewise, DINP has been shown to have no impact on general reproductive tract development manifested as decreased relative weights in androgen sensitive tissues: levator ani/bulbocavernosus muscles (LABC), seminal vesicles, ventral prostrate, glans penis, bulbourethral gland, and epididymis (Boberg et al., 2011; Gray et al., 2000; Clewell et al., 2013b).

Despite the collective evidence demonstrating DINP does not induce TDS disorders, the reporting of DINP's ability to impact testosterone following exposure to the

developing fetus (Borch et al., 2004; Clewell et al., 2013a; Furr et al., 2014; Hannas et al., 2011;), albeit at much higher doses than DBP, spurred regulatory questions regarding the potential of DINP to disrupt male reproductive tract development (CPSC, 2018; ECHA, 2013; EFSA, 2019). The ECHA Risk Assessment Committee (RAC) undertook a thorough in-depth assessment of the DINP database and concluded (ECHA RAC 2018) no classification for DINP for either effects on sexual function and fertility, or for developmental toxicity is warranted (according to European CLP (classification, labelling and packaging of substances) Regulation (EC) No 1272/2008, the EU implementation of the Globally Harmonized System (GHS)). Despite this official regulatory conclusion from ECHA determining absence of DINP mediated adverse effects on reproduction and development, some views continue to suspect DINP of causing adverse effects on male reproductive development through an endocrine mode of action.

While the morphological signs of TDS disorder emerge in the rat after the MPW, the mechanistic changes necessary for mediating the disorder are induced (i.e. programmed) by exposure in the MPW (van den Driesche et al., 2017). That is to say that DBP induction of comparable testosterone deficiency due to exposure after the MPW (GD19.5-20.5) does not induce TDS disorders, nor does more prolonged DBP exposure (GD 13.5-21.5) induce any greater incidence of TDS disorders than just treatment during the MPW (van den Driesche et al. 2017). All of the existing studies assessing DINP effects on *in vivo* testosterone levels in fetal testes measure impacts at a gestational time point subsequent to the MPW. Because impacts on androgen production during -and not after- the MPW (Carruthers et al., 2005; Foster et al., 2001; Welsh et al., 2008; van den Driesche et al., 2017) are central to TDS disorders, assessing if DINP impacts testosterone during this finite window (e15.5-

18.5) in an *in vivo* model is important for reconciling the dataset for DINP. Additionally, we were interested in assessing if changes induced during the MPW alone manifested into focal areas of testicular dysgenesis, similar to what has been seen with DBP (van den Driesche et al., 2012, 2017).

In this study we investigated effects of in utero exposure to a low dose (125 mg/kg bw/day) and a high dose (750 mg/kg bw/day) of DINP during the MPW (i.e. e15.5-e18.5 only) and compared the results with a high dose (750 mg/kg bw/day) DBP exposure using similar read-outs as published by van den Driesche et al., 2017. The high dose level of DINP (750 mg/kg bw/day) was considered appropriate based on the observation of significant reductions of maternal body weight changes as signs of moderate maternal toxicity at this dose in published studies (Hellwig et al., 1997; Masutomi et al., 2003); and the recognition that impacts on testes testosterone levels and steroidogenic gene transcription have been reported at this dose of DINP and lower (Borch et al., 2004; Clewell et al., 2013a; Furr et al., 2014; Hannas et al., 2011, 2012). Saturation of DINP metabolism and significant changes of kinetics occur between the DINP dose levels of 250 mg/kg bw/day and 750 mg/kg bw/day, where despite a 3-fold increase in dose, C_{max} and AUC_{inf} did not increase (Clewell et al., 2013a). Deviation from linearity and saturation of metabolism at 750 mg/kg bw/day was also noted in the recently developed PBPK model for DINP (Campbell et al., 2020). We narrowed the exposure window to e15.5-e18.5 to focus on the critical question of DINP's potential anti-androgenic impacts on the developing male reproductive tract and measured both hormone levels and impacts on steroidogenesis at various life stages and applied state of the art histopathology staining techniques to refine the characterization of potential changes to the testis beyond what the current DINP dataset provides. If indeed DINP can mediate TDS

disorders, this exposure window, as validated with DBP which is used here as a positive control in this experimental model, would be sufficient to induce androgen impacts and alter male reproductive tract development.

2. Materials and Methods

2.1. Ethics Statement

We treated the animals used in this study humanely and according to the Animal (Scientific Procedures) Act 1986 and approval by the UK Home Office. Studies were conducted under Project License (PPL P5B09956A) following review by the University of Edinburgh Animal Research Ethics Committee.

2.2. Animals, study design and treatments

[Figure 1]

Time-mated female Wistar rats (Harlan, UK) were housed for a minimum of 1 week in ventilated cages (2000P cages; Tecniplast, Buguggiate, Italy) prior to use in experimental studies and were kept under standard conditions with ad libitum access to sterile water and fed a soy-free breeding diet (RM3(E); SDS, Dundee, Scotland).

Housing conditions were carefully controlled (lights on at 07:00, off at 19:00, temperature 19–21° C, humidity 45-65%, GOLD shavings and LITASPEN standard bedding (SPPS, Argenteuil, France)). The dams were allocated randomly to receive vehicle control (corn oil; Sigma-Aldrich Company Ltd) treatment, 750 mg/kg bw/day Dibutyl phthalate (DBP; Sigma-Aldrich Company Ltd, Dorset, UK; 99% pure (GC area%) according to the supplier), 125 mg/kg bw/day or 750 mg/kg bw/day Diisononyl phthalate (DINP2; BASF SE, Germany, purity: ≥99.5% (GC area%)) in 1 ml/kg corn oil, daily by oral gavage. The dose of DBP was based on our previous study (van den Driesche et al., 2017) The high dose DINP was based on considerations of maternal toxicity (Hellwig et al., 1997; Masutomi et al., 2003), toxicokinetics (Campbell et al., 2020; Clewell et al., 2013a) and dose effect levels in prior studies reporting effects on molecular endpoints assessed herein (Borch et al., 2004; Clewell et al., 2013a; Furr et al., 2014; Hannas et al., 2011, 2012). All treatments were performed in a single animal facility at the University of Edinburgh and treatments were administered between 09:00-10:30 during the masculinisation programming window treatment (MPW; e15.5-e18.5). Male fetuses were collected at e17.5 (within the MPW), e21.5 (at the end of gestation) or at postnatal day 90 (adulthood). Pregnant dams were killed by CO_2 inhalation followed by cervical dislocation. Fetuses were removed, decapitated and placed in ice-cold PBS (Sigma-Aldrich). Post-natal animals were housed with their natural mothers from birth or in cages with a maximum of 6 animals after weaning and were killed by CO_2 inhalation followed by cervical dislocation. Fetal testes were removed by microdissection and fixed in Bouin's fixative for 1 hour, transferred to 70% (v/v) ethanol and then processed into paraffin blocks using standard procedures. Testes from the fetuses used for intratesticular testosterone measurements and adult testes and epididymides used for sperm head counts were frozen on dry ice.

2.3. Adult phenotypical analysis

When the adult male rats were collected, their body weight was measured and they were subjected to a detailed inspection to determine testicular descent and the normality of the penis. Hypospadias and cryptorchidism were examined as described previously (Welsh et al., 2008; van den Driesche et al., 2017). Anogenital distance (AGD) was measured prior to opening of the abdomen, using digital callipers (Faithfull Tools, Kent, UK), as was the length of the penis after being dissected out. Anogenital Index (AGi) was calculated to normalize to pup or adult body weight (i.e. AGi was calculated by dividing AGD by the cube root of body weight). The testes, ventral prostate and seminal vesicles were also dissected out and weighed.

2.4. Adult sperm motility and sperm head count

The testis and cauda epididymis were taken from all groups and sperm motility, sperm morphology, sperm head count (cauda epididymis) and sperm head count (testis) were determined. Sperm motility examinations were carried out in a randomized sequence using the sperm from the cauda epididymis. For sperm motility the numbers of observations are: vehicle control (27 from 7 litters), 750 mg/kg bw/day DBP (DBP-750; 17 from 6 litters), 125 mg/kg bw/day DINP (DINP-125; 36 from 7 litters), 750 mg/kg bw/day DINP(DINP-750; 28 from 7 litters). Numbers for sperm head counts were the same for vehicle control and DINP groups, but different for the DBP-750 group (26 from 6 litters) because a number of adults treated with 750 mg/kg bw/day DBP had epididymal agenesis or missing epididymis. Sperm motility was assessed using methodology described previously (Slott et al., 1991),

and sperm head counts were similarly done according to previously published studies (Meistrich, 1989). In brief, the tunica albuginea was opened with a scalpel and the parenchym of the testis was isolated and weighed. The samples were refrigerated in a solution of 0.1 % Triton X-100 (Merck) and stored at -20° C until further processing. After thawing, the samples were homogenized in randomized order for 30 seconds with a Polytron at 20 000 U/min, the homogenates were collected through a Nylon sieve and the residual content in the sieve was washed twice with water. The homogenization resistant spermatids were kept on a magnetic stirrer running at low velocity until counting. 3 µl of the solution were counted in the MAKLER chamber (20-fold magnification, counting of 100 squares) and the mean value of 3 chambers counted per animal was taken.

2.5. Triple immunofluorescence for Smooth Muscle Actin, 3β-HSD and Sox-9

Fetal testes were removed by microdissection and fixed in Bouin's fixative for 1 hour, transferred to 70% (v/v) ethanol and then processed into paraffin blocks using standard procedures. Sampling at the different time points was as follows:

E17.5: Control: n = 15 from 6 litters, DBP-750: n = 15 from 6 litters, DINP-125: n = 15 from 6 litters, DINP-750: n = 15 from 5 litters.

E21.5: Control: n = 15 from 6 litters, DBP-750: n = 15 from 5 litters, DINP-125: n = 15 from 5 litters, DINP-750: n = 15 from 6 litters.

We aimed for 15 males for each timepoint by selecting 3 males per litter. In cases where there were only 2 males available in a litter, we completed the number of males to 15 from another litter.

In order to visualize focal dysgenesis, specific antibodies were used for co-immunolocalisation of 3β-HSD (Leydig cell marker; antibodies-online.com), Sox-9 (Sertoli cell marker; Chemicon International, UK) and α-smooth muscle actin (α-SMA, peritubular myoid cell marker; Sigma-Aldrich, UK). Slides were washed between all incubation steps in Tris Buffered Saline (TBS) pH 7.6 (3 x 5 min) and all incubations were carried out in a humidity box (Fisher Scientific, UK). Sections were dewaxed and rehydrated using standard procedures, followed by a peroxidase block in 3% (v/v) H_2O_2 in methanol for 30 minutes. Next, the sections were blocked in normal goat serum (NGS; Vector Laboratories Inc, USA) diluted 1:5 in TBS containing 5% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich; NGS/TBS/BSA), followed by incubation with the first primary rabbit antibody, anti-Sox-9, which was diluted 1:1,000 in NGS/TBS/BSA overnight at 4° C. The next day, sections were incubated with peroxidase-conjugated goat F(ab) anti-rabbit secondary antibody (GARP; Abcam UK), diluted 1:200 in NCS/TBS/BSA for 30 minutes at room temperature (RT), and followed by incubation with Tyr-Cy3 (Perkin Elmer-TSA-Plus Cyanine3 System; Perkin Elmer Life Sciences, USA) according to the manufacturer's instructions. From here onwards slides were kept in the dark. Next, the sections were subjected to antigen retrieval by boiling in a pressure cooker in 0.01 mol/l citrate buffer (pH 6.0) for 5 minutes and left to cool for 20 minutes. This was followed by another block in NGS/TBS/BSA and overnight incubation at 4° C with the second primary antibody, rabbit anti-3β-HSD, diluted 1:1,000 in NGS/TBS/BSA. On the third day, slides were incubated with peroxidase-conjugated goat F(ab) anti-rabbit secondary antibody (Abcam) diluted 1:200 in NGS/TBS/BSA for 30 minutes at RT, followed by incubation with Tyr-fl (Perkin Elmer-TSA-Plus Fluorescein System; Perkin Elmer Life Sciences) according to the manufacturer's instructions. Sections were again blocked against peroxidase in 3%

(v/v) H_2O_2 in TBS plus 0.01% (v/v) Tween-20 (Sigma-Aldrich) for 20 minutes followed by blocking in NGS/TBS/BSA and overnight incubation at 4° C with the third primary antibody, mouse anti-SMA, diluted 1:10,000 in NGS/TBS/BSA. On the last day, sections were incubated with peroxidase-conjugated goat F(ab) anti-mouse secondary antibody (Abcam) diluted 1:200 in NGS/TBS/BSA for 30 minutes at RT, and followed by incubation with Tyr-Cy5 (Perkin Elmer-TSA-Plus Cyanine5 System; Perkin Elmer Life Sciences, Boston, MA, USA) according to the manufacturer's instructions. Finally, the slides were mounted with Permafluor (Thermo Scientific, UK) and fluorescent images were captured using a Nikon A1R confocal laser microscope.

2.6. Hormone measurements

Adult male animals were sacrificed by CO_2 inhalation followed by cervical dislocation and straight after that blood was collected from the heart and stored on wet ice. Plasma was collected by centrifugation of all samples collected at the day of sacrifice. Fetal testes were homogenised in 250 µL ice cold PBS using a handheld tissue homogeniser (Thomas Scientific). The homogenates were centrifuged at full-speed and the supernatant was used for intratesticular testosterone measurements as described below. Fetal intratesticular testosterone, adult plasma testosterone and LH measurements were done by the Specialised Assay Laboratory at the MRC Centre for Reproductive Health, Edinburgh, UK. The testosterone ELISA was performed by coating 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) with 100 µl of donkey anti-rabbit IgG (Jackson ImmunoResearch Inc, West Grove, USA) per well at a dilution of 1:500 in ELISA coating buffer (100 mM Na Bicarbonate, pH 9.6) covered and incubated in a fridge at 4º C overnight. Before use the plates were washed 2 times

with wash buffer (0.05 M Tris/HCl + 0.05 % Tween 20, pH 7.4). Next, 250 µl of blocking buffer (PBS pH 7.4 containing 0.5% BSA) was added to each well and incubated for 1 hour at room temperature whilst shaking before being washed 2 times with wash buffer. Standards, samples and controls (20 µl per well) were added to each well, followed by 80 µl of testosterone-HRP conjugate (Astra Biotech GmbH, Berlin, Germany) at 1:20,000 in assay buffer (PBS pH 7.4 containing 0.1% BSA and 250 ng/ml cortisol), followed by 50 µl of rabbit anti-testosterone-19 antibody (AMS Biotechnology, USA) 1:200,000 in assay buffer. Plates were incubated at room temperature for 2 hours on a microtiter plate shaker (IKA®, Schuttler MTS4, IKA Labortechnik, Staufen, Germany), then washed 5 times with assay wash buffer and 120 µl of substrate solution (3,3,5,5-Tetramethylbenzidine, Millipore Corporation, Temecula, CA, USA) was added to each well. Plates were incubated at room temperature without shaking in the dark. After 20 min., the reaction was stopped by adding 80 µl of 2N H_2SO_4 solution (Sigma-Aldrich Company Ltd., Dorset, UK). Finally, plates were read on a plate reader at 450 nm. Plasma LH levels in adult rats were measured as described previously (McNeilly et al., 2000).

Standard curves were prepared with a total of 8 different concentrations (24.3, 8.1, 2.7, 0.9, 0.3, 0.1 0.03 and 0 ng/ml). Samples, standards and controls were included in duplicate. Inter- and intra-assay coefficients of variation (CV) were calculated from two controls of low and high testosterone in duplicate in each of eight assays. The inter-assay CV for low and high pools respectively were 11.4% and 9.1%; the intra-assay CV were 8.9% and 5.6%. The lower limit of detection was calculated at 0.2 ng/ml.

2.7. Measurement of Leydig cell aggregation

Measurement of Leydig cell aggregate size in the fetal testis was done as described previously (Mahood et al., 2007; van den Driesche et al., 2012). Briefly, testes from the different treatment groups (n=15 from 5-6 litters per treatment group) were serially sectioned and three representative sections from each testis then selected and immunostained for 3β-HSD (antibodies-online.com; 1:500) on a Leica BOND-MAX automatic immunostaining machine using the BOND Polymer Refine Detection (Leica, UK). The three sections chosen were those corresponding to approximately 25, 50, and 75% intervals through the serially sectioned testis. Sections immunostained for 3β-HSD were not counterstained, so as to provide sufficient homogeneity, high contrast, and low background to allow computer-assisted thresholding and subsequent computer-assisted counting of Leydig cell (3β-HSD-immuno-positive) aggregates and determination of Leydig cell aggregation area. Slides were scanned using a Zeiss Axioscan Z1 whole slide scanner at 20x magnification using a Hitachi HV-F202SCL camera, following intensity-based tissue detection a 0.2% onion skin focusing strategy was applied and slides scanned. Fiji image analysis software was used for quantification (Schindelin et al., 2012). A custom macro was designed that used thresholding to identify the whole tissue section and quantify total pixel area. A Gaussian blur (sigma 2) filter was applied to smooth the images and reduce noise. Thresholding was then used within the tissue regions to identify aggregates of immuno-positive cells. The 3β-HSD staining permitted the identification of cluster borders and the measurement of the total area of each cluster volume was quantified. Leydig cell aggregates were expressed as percentage of total Leydig cell area. Leydig cell aggregates were then assigned arbitrarily to one of two groups: small aggregates, accounting for ≤5% of the total Leydig cell aggregate area per testis, and large aggregates, which individually accounted for ≥5.1% of total Leydig cell aggregate area

per testis. The mean of the measured Leydig cell aggregates per testis per animal was then calculated and used for analysis.

2.8. Gene expression analysis

Total RNA was extracted from e17.5 and e21.5 frozen fetal testes samples (n=9 from 3 litters per treatment group) using the RNeasy Micro Kit (Qiagen, UK) as per manufacturer's instructions. The RNA concentration of each sample was measured via Nanodrop and each sample was diluted to 100 ng/µl in RNAse-free water. cDNA was then prepared from these dilutions using the Superscript VILO cDNA Synthesis Kit (Life Technologies). Quantitative real-time RT-PCR was performed on StepOne Plus (Life Technologies). Expression of rat *StAR*, *Cyp11a1*, and *Cyp17a1* RNA was determined using the Roche Universal Probe Library (*StAR* forward primer: 5′-TCACGTGGCTGCTCAGTATT-3′, reverse primer: 5′-GGGTCTGTGATAAGACTTGGTTG-3′, probe number 83 cat. no. 04689062001; *Cyp11a1* forward primer: 5′-TATTCC- GCTTTGCCTTTGAG-3′, reverse primer 5′-CACGATCTCCTC- CAACATCC-3′, probe number 9 cat. no. 04685075001; *Cyp17a1* forward primer: 5′-CATCCCCCACAAGGCTAAC-3′, reverse primer: 5′-TGTGTCCTTGGGGACAGTAAA-3′, probe number 67 cat. no. 04688660001; Roche Applied Sciences). Two negative control samples were included, one containing all required elements but no RNA sample, and one containing an RNA sample but excluding reverse transcriptase enzyme. The expression of each gene was normalized using a ribosomal 18S internal control (Applied Biosystems cat. no. 4308329). All samples were run in triplicate and compared to adult testis control cDNA (Ambion).

2.9. Statistics

Values are expressed as mean ± SEM. Comparison of treatment effects used one-way ANOVA followed by the Bonferroni post test when multiple treatment groups were compared. These analyses used GraphPad Prism (version 8; GraphPad Software Inc., San Diego, CA). The presented data used each litter as the unit, rather than the animal.

3. Results

No generalized adverse effects of the DBP or DINP treatments were observed in the exposed females. The litter size or sex ratio was not significantly affected by the treatments (data not shown).

3.1. Reproductive tract analysis in adult rats exposed to DBP or DINP during the MPW

Similar to previously published results (van den Driesche et al., 2017), gestational exposure to 750 mg/kg bw/day DBP during the MPW resulted in significant effects in adult males on anogenital distance (AGD), anogenital index (AGi), average testis weight, penis length, penis weight, ventral prostrate weight and seminal vesicle weight (Table 2). In contrast, neither high dose (750 mg/kg bw/day) nor low dose (125 mg/kg bw/day) DINP exposure during the MPW resulted in any adverse effects on the male reproductive tract in adulthood (Table 2). Likewise, only adult animals exposed to 750 mg/kg bw/day DBP during the MPW, and not exposed to either dose of DINP, showed

a significant reduction in both sperm motility and sperm head count (P<0.001; Figure 2C and 2D, respectively).

[Table 2]

[Figure 2]

3.2. Occurrence of hypospadias and cryptorchidism in adulthood following in utero exposure to either DBP or DINP

Of the adult animals that were exposed in utero to 750 mg/kg bw/day DBP during the MPW, 81% presented with cryptorchidism (Figure 2A) and 23% with hypospadias (Figure 2B). No cryptorchidism or hypospadias was observed in any adult animals that were exposed to either dose of DINP during the MPW (Figures 2A, B).

3.3 Effects of in utero exposure to DBP and DINP during the MPW on testosterone and luteinizing hormone production

Previously we observed induction of “compensated Leydig cell function” in adult males exposed during the MPW to 750 mg/kg bw/day DBP, as indicated by a statistically significant increase in circulating luteinizing hormone (LH) levels and concomitant significantly increased LH/testosterone ratio (van den Driesche et al., 2017). In the current study, while exposure to 750 mg/kg bw/day DBP resulted in decreased testosterone together with increased LH production and concomitant increase in the LH/testosterone ratio in adults, the effects did not reach statistical significance. Small changes in adult circulating testosterone levels without any effect on LH production and no effects on the LH/testosterone ratio (Figure 2E-F) seem not meaningful.

However, only when the data were analysed using individual animal values of the adults rather than litter means, a small, but statistically significant positive effect of in utero exposure to 750 mg/kg bw/day DBP on circulating LH levels was observed, resulting in an increased LH/testosterone ratio ($P < 0.05$; data not shown). Similar to the litter mean data, this effect was not observed in either 125 or 750 mg/kg bw/day DINP treatment groups when the data were analysed as individual animals (data not shown).

3.4. Effects of in utero exposure to DBP or DINP during the MPW on the fetal testis

We isolated fetuses exposed in utero to either vehicle control, 750 mg/kg bw/day DBP, 125 or 750 mg/kg bw/day DINP at embryonic day e17.5 and e21.5. Similarly to the effects observed in adulthood (Table 2), only exposure to 750 mg/kg bw/day DBP during the MPW had significant effects on AGD ($P<0.001$; Figure 3A), average testis weight ($P<0.001$; Figure 3B), and intratesticular testosterone ($P<0.001$; Figure 3C) at GD21.5, which confirms previously published data (van den Driesche et al., 2012, 2017). No effects were observed after in utero exposure to either DINP dose. At e17.5 (towards the end of the MPW) only exposure to 750 mg/kg bw/day DBP, caused a significant reduction in intratesticular testosterone production ($P<0.001$; Figure 3D). No effects were observed on intratesticular testosterone production on e17.5 following exposure to DINP.

[Figure 3]

The quantitative mRNA expression of the steroidogenic synthesis genes *StAR, Cyp11a1* and *Cyp17a1* was significantly reduced after exposure to 750 mg/kg bw/day DBP at e17.5 ($P<0.05$; Figure 4A-C), however at e21.5 this reduction had returned to control levels (Figure 4D-F). Exposure to either 125 mg/kg bw/day or 750 mg/kg bw/day DINP did not have any effect on *StAR, Cyp11a1* and *Cyp17a1* expression at either e17.5 or e21.5 (Figure 4).

[Figure 4]

3.5. In utero exposure to DBP, not DINP, during the MPW causes fetal testicular dysgenesis

The fetal testicular morphology was investigated using triple immunofluorescence for 3β-HSD (fetal Leydig cell marker), Sox-9 (Sertoli cell marker) and SMA (peritubular myoid cell marker). Similarly, to what we have described previously (van den Driesche et al., 2017), in utero exposure to 750 mg/kg bw/day DBP during the MPW results in large dysgenetic areas, which consist of a mixture of fetal Leydig cell aggregations and ectopically localized Sertoli cells (Figure 5B, asterisk). Fetal testes isolated from fetuses that were exposed to either 125 or 750 mg/kg bw/day DINP during the MPW looked morphologically similar to control testes at e21.5 (compare Figures 5C, D with 5A).

[Figure 5]

The size of fetal Leydig cell aggregates was systematically analyzed at both e17.5 and e21.5 after exposure to vehicle control, 750 mg/kg bw/day DBP and 125 or 750 mg/kg

bw/day DINP, using similar methods as previously described (Mahood et al., 2007; van den Driesche et al., 2012). The fetal testis was serial sectioned and sections at roughly 25%, 50% and 75% of the testis were used for automatic immunostaining procedures (Figure 6A, B). This allowed for quantification of the 3β-HSD immunostained areas using software analysis (see Methods) and thereby a systematic determination of testicular dysgenesis in the fetal testis samples. As shown in Figures 6C and 6D, in utero exposure to 750 mg/kg bw/day DBP resulted in a significant reduction in the percentage of small fetal Leydig cell aggregates and a significant increase in large fetal Leydig cell aggregates (which is a measurement for testicular dysgenesis). At e17.5 and e21.5 either dose of DINP did not result in any significant differences in the percentage of small or large Leydig cell aggregates when compared with vehicle control exposed animals (Figure 6C, D). Interestingly, we did notice a small, but significant effect when we compared the 125 with 750 mg/mg bw/day DINP exposure groups at e21.5, with the higher DINP dose reflecting a reduction in small and an increase in large fetal Leydig cell clusters at e21.5 when compared with the low dose DINP exposure group (Figure 6D).

[Figure 6]

4. Discussion

The results of our study support that DINP does not elicit the events necessary and sufficient for inducing TDS as seen with DBP such as reduced reproductive organ size (testes, prostate, seminal vesicles, penis), cryptorchidism, hypospadias, nor reduced AGD. In order to set our results into perspective some aspects merit a more detailed discussion.

4.1. Exact Timing of the exposure

Consistent with previously published findings (van den Driesche et al. 2012, 2017), our study demonstrates that exposure to DBP within the MPW only results in focal dysgenesis that manifests histologically after the MPW and after cessation of DBP exposure. This dysgenesis is associated with reduced androgen production in the MPW (Figure 3), and associated with effects on the male reproductive tract (Table 2 and Figure 2) including hypospadias (via disruption of genital tubercle development); cryptorchidism (via disruption of testis descent); and reduction in AGD and AGi that is maintained into adulthood (via disruption of androgen mediated proliferation/cell migration of perineal tissue). The temporal mismatch between inhibition of steroidogenesis in the MPW and the manifestation of the abnormal testicular histopathology days later, indicates that DBP induces focal dysgenesis via a 'programming' that occurs during the MPW. However, it is well established that the temporal window of importance for androgen in mediating male reproductive development extends beyond the MPW and DBP has been shown to reduce testosterone following exposures initiated after the MPW (van den Driesche et al., 2012). In this study we aimed to untangle the events mediated during the MPW from those initiated or impacted later in development by focusing attention on the ability of DINP to induce focal dysgenesis via the 'programming' and steroidogenesis impacts that occur during the MPW. Studies assessing effects of DINP on male reproductive tract development (Figure 7) have reported effects on testosterone (Borch et al., 2004; Clewell et al., 2013a; Furr et al., 2014; Hannas et al., 2011) and steroidogenesis gene expression (Adamsson et al., 2009; Hannas et al., 2011, 2012;

Li et al., 2015) at doses of 750 mg/kg bw/day and lower, and Leydig cell aggregates at doses as low as 300 mg/kg bw/day (Clewell et al., 2013b). If indeed DINP is a lower potent inducer of testicular dysgenesis it was reasonable that exposure to 750 mg/kg bw/day during this necessary and sufficient window of exposure would lead to detectable molecular effects at e17.5 and cellular effects at e21.5. Failure to observe any effects of DINP using the sensitive techniques employed herein, suggests that DINP is unable to induce TDS through a similar 'programming' mechanism as DBP.

DEHP and DBP have been shown to also impact rat development in OECD guideline developmental toxicity studies (i.e. OECD TG414) in which exposures stop prior to the MPW, i.e. exposures during GD6-15 per the former OECD TG414 as published in 1981 (the guideline was modified in 2001 to include exposures extending beyond organogenesis). Effects in these developmental toxicity studies are not specific to the male reproductive tract but also include malformations such as neural tube defects and cleft palate. Studies with DINP (Figure 7, depicted in blue) do not induce these effects. In our opinion this points out a toxicodynamic distinction between LMW phthalates from DINP on the fetus worth emphasizing.

[Figure 7]

4.2. Steroidogenesis

Phthalates leading to adverse antiandrogenic effects are shown to interfere with the steroidogenic pathways of the testes (Plummer et al., 2007). Testosterone biosynthesis starts from cholesterol via a multistep process involving cholesterol transport to the mitochondria of the steroidogenic cell and enzymatic conversion into

the active steroid hormone (Thompson et al., 2004). If impacts on steroid biosynthesis precede the testosterone reductions leading to disrupted male development, the timing of steroidogenic impacts would have to be prior to the critical reduction in fetal testosterone that occurs during the MPW and has been associated with induction of TDS disorders (van den Driesche et al., 2017). The steroidogenic acute regulatory protein, StAR, is involved in the transport of cholesterol, the precursor of testosterone, in the cell into the mitochondria (Johnson et al., 2007). In this study, the quantitative mRNA expression of *StAR,* and of*Cyp11a1* and *Cyp17a1 (*involved in cholesterol conversion to testosterone) was significantly reduced after exposure to DBP at e17.5 ($P<0.05$; Figure 4A-C), however at e21.5 this reduction had returned to control levels (Figure 4D-F). The return to control levels at e21.5 may reflect a “rebound” after the DBP treatment window stopped at e18.5, which gave the fetal testes time to recover the gene expression levels that were measured at e21.5. In contrast, DINP did not demonstrate an impact on fetal testicular mRNA levels of genes involved in steroidogenesis (*Cyp11a1*, *Cyp17a1*) or cholesterol transport (*StAR*) on either e17.5 or e21.5. DINP has been reported in the literature to impact expression of these genes following in utero exposure on e14-e18 (Hannas et al., 2011, 2012). Possible differences in experimental design may be responsible for the different outcomes. Hannas et al., 2011, 2012, exposed the more sensitive Sprague Dawley rats slightly longer (e14-e18) and employed testes extraction at e18. Two other studies have also reported DINP-mediated effects on steroidogenesis gene expression. Adamsson et al., 2009, reported a statistically significant increase in Cyp11a1 and no change on StAR, whereas Li et al., 2015, reported a decrease in both genes. The exposure paradigms for both of these studies varied from one another and from our study design (Figure

7). The dose used herein was comparable to the highest dose in Adamsson et al., 2009, while Li et al., 2015, dosed up to 1000 mg/kg bw/day. Neither Adamsson et al., 2009, nor Li et al., 2015, observed a change in corresponding steroidogenesis protein levels following exposure to DINP.In the experimental model herein, the contrast between the outcomes of DBP, which reduces the expression of the three genes significantly on e17.5, and DINP, which has no effect on gene expression, provides compelling support that DINP does not reduce mRNA levels of steroidogenic genes in fetal testes during the critical MPW.

The data on intratesticular testosterone levels mirrored that of the mRNA expression data for steroidogenesis genes. Exposure to DBP showed a statistically significant reduction in intratesticular testosterone at e17.5 which was maintained, however with lesser magnitude, at e21.5. The reduced magnitude of effect on intratesticular testosterone reduction at e21.5 compared to e17.5 (Figure 3C, D) is consistent with a ‘rebound’ effect following cessation of exposure (van den Driesche et al., 2012. 2017). In contrast, DINP showed no effect on intratesticular testosterone at either timepoint (Figure 3 C, D). There are many possible explanations for why the ability of DINP to impact testosterone may not have been observed. A difference in androgen sensitivity among rat strains has been reported, with Sprague Dawley rats being more sensitive to steroidogenic impacts than the Wistar rat strain (Wilson et al., 2007). However, the studies reporting a reduction in testosterone following DINP exposure (Borch et al., 2004; Clewell et al., 2013a; Furr et al., 2014; Hannas et al., 2011; Li et al., 2015) versus those that do not report a dose responsive effect on testosterone (Adamsson et al., 2009; Boberg et al., 2011; Clewell et al., 2013b) do not correlate to strain (Figure 7), meaning the strain used is an unlikely explanation

for the lack of effect shown here. The length of time between cessation of the DINP exposure and the testosterone measurement could also be an important consideration for why no effect on testosterone was observed. Adamsson et al., 2009, who did not detect a change in intratesticular testosterone, measured on e19.5 following DINP exposure e13.5-17.5, proposed the 'rebound' effect as responsible for the lack of observed effect on intratesticular testosterone. The 'rebound' effect could account for the lack of effects on testosterone found in this study at GD21.5 (Figure 3), but would not explain the lack of effect during the MPW at e17.5. Also note that DBP exposure during the MPW is shown here (Figure 3D) and elsewhere (van den Driesche et al., 2017) to reduce intratesticular testosterone days after exposure has ceased (although to a lesser extent). Therefore, the 'rebound' effect does not account for all of the observations in this study. DINP has been demonstrated to be a lower potency steroidogenesis inhibitor compared to DBP and other LMW phthalates to impact steroidogenesis in an ex vivo model of testicular testosterone production, where testes are outside of homeostatic controls for a period of hours before testosterone levels are assessed (Furr et al., 2014; Hannas et al., 2011). Therefore, the dose, duration of exposure, or nature of measurement may explain why no effect on testosterone was observed herein. However, taking a very simplistic view, studies with longer duration of exposure to higher (Boberg et al., 2011) or comparable doses of DINP (Clewell et al., 2013b) than used here did not observe dose dependent changes in intratesticular testosterone; while impacts on intratesticular testosterone (Clewell et al., 2013a) and ex vivo testicular testosterone production (Hannas et al., 2011) have been reported in studies at doses lower than 750 mg/kg bw/day. As discussed further below, an alternative explanation for our findings is that DINP exposure during the MPW does not inhibit steroidogenesis or it

does not inhibit steroidogenesis to an extent great enough to overcome homeostatic controls which are necessary for eliciting downstream TDS disorders.

As mentioned previously, DBP has been shown to reduce intratesticular testosterone levels following exposure during the MPW (e15.5-18.5) as well as following exposure after the MPW (e19.5-20.5), however, the fetal testosterone deficiency induced after the MPW is not associated with induction of TDS disorders (van den Driesche et al., 2017). Therefore, the timing of androgen reduction matters. The studies that assess effects of DINP on male reproductive tract development intertwine the evaluation of effects mediated during the MPW (i.e. programming event) with those that are initiated or impacted later in development due to the duration of the exposures employed (see Figure 7). Interpretation of these data is further complicated by a lack of standardization of what developmental time point measurements are taken. Interpreting the meaning of reported outcomes on DINP without carefully considering the dynamics and temporality of biology during fetal development confounds interpretation and may be obscuring the meaning of reported observations. None of the existing studies on DINP (Figure 7) limited exposure to the MPW only, nor measured intratesticular testosterone during the MPW. The absence of a DINP-mediated effect on intratesticular testosterone levels on e17.5 observed herein, is consistent with the body of evidence reporting a lack of induction of gross male reproductive tract malformations such as cryptorchidism and hypospadias following DINP exposures (Boberg et al., 2011; Clewell et al., 2013b; Gray et al., 2000). Absence of an impact on testosterone at e17.5 and e21.5 following exposure during the MPW is also consistent with the body of evidence indicating DINP does not induce changes to AGD. AGD is a sensitive indicator of in utero androgen levels and

a shortened AGD serves as a useful correlative biomarker for reduced fetal androgen levels in the MPW (Schwartz et al., 2019; Welsh et al., 2008; van den Driesche et al., 2017). The majority of studies on DINP report no statistically significant change on AGD following in utero exposure (Clewell et al., 2013a; Gray et al., 2000; Li et al., 2015; Masutomi et al., 2003). Of the two studies that report a statistically significant finding, Boberg et al., 2011, reported a small but statistically significant AGD decrease in males exposed to 900 mg/kg bw/day DINP. The biological and toxicological relevance of this reported finding are questionable however, due to the small magnitude (~5%) of the change, reported challenges in reproducing the statistical significance upon reanalysis of the raw data (Chen et al., 2017), and published methodology modifications (Boberg et al., 2016) indicating AGD was measured by different technicians on different days (as rapid pup growth and technician variability can affect AGD measurements, randomization of animals across days and technicians is important for informing significance of outcomes). Clewell et al., 2013b, reported a statistically significant decrease of AGD (both scaled for body weight and absolute) on postnatal day 14 at the highest DINP dose (11,400 ppm), but not at postnatal days 2 and 49. AGD is highly dependent on animal size, and the postnatal day 14 animal weights for males in this dose group were also statistically different than controls. Given that anogenital differences induced by anti-androgenic influences in utero should be apparent at birth, the difference observed at postnatal day 14 without a correlative difference at postnatal day 2 suggest the postnatal day 14 observation was unlikely due to an anti-androgenic effect. The findings reported here that DINP does not impact testosterone levels on e17.5 add more collective weight to the conclusion that DINP is not anti-androgenic during the MPW.

4.3. Leydig cell aggregates

As expected from the results of a previous study with 500 mg/kg bw/day (van den Driesche et al., 2012), in utero exposure to 750 mg/kg bw/day DBP resulted in statistically significant decreases in small and increases in large Leydig cell aggregates on both time points (e17.5 and e21.5) compared to controls. However, in contrast with DBP, in utero exposure to DINP did not result in any significant differences in the percentage of small or large Leydig cell aggregates when compared with vehicle control exposed animals (Figure 6C, D). The relevance of the small, but significant effect noted when comparing the 125 to the 750 mg/mg bw/day DINP exposure group at e21.5 remains unclear. This reduction in small and the corresponding increase in large fetal Leydig cell clusters at e21.5 could also be within the variation of the method used (Figure 6D).

4.4 Effects on Sperm

The findings reported herein are similar to those in previous studies on DINP reporting a lack of statistically significant or dose-related effects of DINP on testicular descent, penile development and AGD in adult animals following in utero exposure to DINP during the MPW (Boberg et al., 2011; Clewell et al., 2013b; Gray et al., 2000). Neither the current study (Figure 2) nor the study by Boberg et al., 2011, found an effect on sperm count in adult males (see also Chen et al., 2017 for reanalysis of the statistics reported in Boberg et al., 2011). In contrast, no effect was observed on sperm motility in this study, whereas Boberg et al., 2011 reported a decrease in sperm motility at doses of 600 mg/kg bw/day and higher. However, the

sperm motility in the untreated controls did not reach the minimum value of 70% motility which OECD (Guidance Document No. 43, 2008; Seed et al., 1996). A key difference in our study design is the window of exposure. We stopped exposure at e18.5 whereas Boberg et al., 2011, extended the exposure window to postnatal day 17. Exposure to DBP during the MPW alone is shown herein to be sufficient to impact sperm count and motility (Figure 2 C, D). However, considering the timeline for germ cell development and postnatal development of sperm, timing of exposure could be important for explaining the differences seen with DINP between studies. For example, during normal testis development at e21.5 germ cells are migrating outwards to gain contact with basal lamina where they differentiate into spermatogonia. As shown by Lara et al., 2017, DBP shows a much greater disruption of this migration and increased germ cell aggregation following exposure from e19.5-20.5 or e13.5-20.5, with significantly more germ cells migrating and reduced aggregation observed following exposure during the MPW alone. This is consistent with germ cell aggregation being an effect induced later in gestation, supporting that timing of exposure as possibly important for detecting effects on sperm. Considering the low percentage of motile sperm in the controls of the Boberg et al., 2011, study, the known sensitivity of sperm to experimental conditions (Seed, et al., 1996) and importance of optimized computer assisted counting measurements (Schleh et al, 2013), it is also possible that the reported outcomes by Boberg et al., 2011, are a nuance of the experimental approach.

5. Conclusion

Overall, the results of this systematic comparison provide convincing evidence on the differences between the effects occurring with the LMW DBP (positive control) and the HMW DINP. In contrast to what was seen with DBP, DINP did not cause cryptorchidism or hypospadias. There was no effect on AGD/AGi. Leydig cell aggregates on e17.5 and e21.5 did not increase. There was no reduction of intratesticular testosterone, no effects on sperm motility and sperm count and no effects on adult testosterone and LH levels were seen. In this experimental model, DINP does not cause adverse reproductive effects and endocrine effects like DBP. Our findings are similar to those in previous studies on DINP reporting a lack of statistically significant or dose-related effects of DINP on testicular descent, penile development and AGD in adult animals following in utero exposure to DINP during the MPW (Boberg et al. 2011; Clewell et al., 2013b; Gray et al., 2000).

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Drs. Angelika Langsch, Christine Palermo and Rainer Otter are employed by producers of DINP.

The authors of the University of Edinburgh, UK, declare no conflict of interest.

References:

Adamsson A, Salonen V, Paranko J, Toppari J Effects of maternal exposure to di-isononylphthalate (DINP) and 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,p_-DDE) on steroidogenesis in the fetal rat testis and adrenal gland, 2009. Reproductive Toxicology. 28: 66–74. https:/doi.org/10.1016/j.reprotox.2009.03.002

Animal (Scientific Procedures) Act 1986. http://www.legislation.gov.uk/ukpga/1986/14/contents). Accessed: October 2020

Boberg J, Christiansen S, Axelstad M, Kledal TS, Vinggaard AM, Dalgaard M, et al., 2011. Reproductive and behavioural effects of diisononyl phthalate (DINP) in perinatally exposed rats. Reproductive Toxicology 31: 200-209. https://doi.org/10.1016/j.reprotox.2010.11.001

Boberg J, Christiansen S, Axelstad M, Kledal TS, Vinggaard AM, Dalgaard M, et al., 2016. Corrigendum to “Reproductive and behavioural effects of diisononyl phthalate (DINP) in perinatally exposed rats” [Reprod. Toxicol. 31(2) (2011)200-209]. Reproductive Toxicology 63: 183-184. https:// doi: 10.1016/j.reprotox.2016.07.001

Borch J, Ladefoged O., Hass U, Vinggaard AM, 2004. Steroidogenesis in fetal male rats is reduced by DEHP and DINP, but endocrine effects of DEHP are not modulated by DEHA in fetal, prepubertal and adult male rats. Reproductive Toxicology, 18: 53–61. https://doi:10.1016/j.reprotox.2003.10.011

Campbell Jr JL, Otter R, Anderson WA, Longnecker MP, Clewell RA, North C and Clewell III HJ, 2020. Development of a physiologically based pharmacokinetic model of diisononyl phthalate (DiNP) in pregnant rat and human. Journal of Toxicology and Environmental Health Part A, 83: 631-648. https://doi.org/10.1080/15287394.2020.1798831

Carruthers CM and Foster PMD, 2005. Critical window of male reproductive tract development in rats following gestational exposure to Di-n-butyl phthalate. Birth Defects Research (Part B) 74: 277–285. https://doi.org/10.1002/bdrb.20050

Chen M, Alyea R, Morfeld P, Otter R, Kemmerling J, Palermo C., 2017 Reproducibility discrepancies following reanalysis of raw data for a previously published study on diisononyl phthalate (DINP) in rats. Data in Brief. 13: 208-213. http://dx.doi.org/10.1016/j.dib.2017.05.043

Clewell RA, Sochaski M, Edwards K, Creasy DM, Willson G, Andersen ME, 2013a. Disposition of diisononyl phthalate and its effects on sexual development of the male fetus following repeated dosing in pregnant rats. Reproductive Toxicology 35: 56-69. https://doi.org/10.1016/j.reprotox.2012.07.001

Clewell RA, Thomas A, Willson G, Creasy DM, Andersen ME, 2013b. A dose-response study to assess effects after dietary administration of diisononyl phthalate

(DINP) in gestation and lactation on male rat sexual development. Reproductive Toxicology, 25:70-80. http://dx.doi.org/10.1016/j.reprotox.2012.07.008

CPSC 2018. 16 CFR Part 1307. Docket No. CPSC–2014–0033. Prohibition of Children's Toys and Child Care Articles Containing Specified Phthalates. https://www.govinfo.gov/content/pkg/FR-2017-10-27/pdf/2017-23267.pdf Accessed: October 2020

Dekant W, 2020. Grouping of phthalates for risk characterization of human exposures. Toxicology Letters, 330: 1-6. https://doi.org/10.1016/j.toxlet.2020.05.003

ECHA, 2013. Evaluation of new scientific evidence concerning DINP and DIDP in relation to entry 52 of Annex XVII to REACH Regulation (EC) No 1907/2006. ECHA-13-R-07-EN, ISBN: 978-92-9244-001-5 https://echa.europa.eu/documents/10162/31b4067e-de40-4044-93e8-9c9ff1960715 Accessed: October 2020

ECHA RAC, 2018. Committee for Risk Assessment RAC Opinion proposing harmonised classification and labelling at EU level of 1,2-Benzenedicarboxylic acid, di-C8-10-branched alkylesters, C9- rich; [1] di-"isononyl" phthalate; [2] [DINP], EC Number: 271-090-9 [1] 249-079-5 [2] CAS Number: 68515-48-0 [1] 28553-12-0 [2], CLH-O-0000001412-86-201/F, Adopted 9 March 2018. https://echa.europa.eu/documents/10162/56980740-fcb6-6755-d7bb-bfe797c36ee7. Accessed: October 2020

EFSA: Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) on a request from the Commission related to Di-isononylphthalate (DINP) for use in food contact materials, Question N° EFSA-Q-2003-194, Adopted on 30 July 2005, The EFSA Journal (2005) 244:1-18. https://doi.org/10.2903/j.efsa.2005.244. Accessed: October 2020

EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP), Scientific Opinion; adopted September 2019: Silano V, Baviera JMB, Bolognesi C, Chesson A, Cocconcelli PS, Crebelli R, et al.. Update of the risk assessment of di-butylphthalate (DBP), butyl-benzyl-phthalate (BBP), bis(2-ethylhexyl)phthalate (DEHP), di-isononylphthalate (DINP) and diisodecylphthalate (DIDP) for use in food contact materials. EFSA Journal 2019;17(12): 5838. https://doi.org/10.2903/j.efsa.2019.5838

EU RAR 2003. European Union Risk Assessment Report: 1,2-benzenedicarboxylic acid, di-C8-10-branched alkyl esters, C9-rich and di-“isononyl” phthalate (DINP). https://echa.europa.eu/documents/10162/83a55967-64a9-43cd-a0fa-d3f2d3c4938d Accessed: October 2020

Fabjan E, Hulzebos E, Mennes W, Piersma AH, 2006. A category approach for reproductive effects of phthalates. Critical Reviews in Toxicology 36: 695–726. http://dx.doi.org/10.1080/10408440600894914

Fisher JS, Macpherson S, Marchetti N, and Sharpe RM, 2003. Human 'testicular dysgenesis syndrome': a possible model using in-utero exposure of the rat to dibutyl phthalate. Human Reproduction 18(7): 1383-1394. https://doi.org/10.1093/humrep/deg273

Foster PMD, Mylchreest E, Gaido KW and Sar M, 2001. Effects of phthalate esters on the developing reproductive tract of male rats. Human Reprod Update 7(3): 231-235. https://doi.org/10.1093/humupd/7.3.231

Furr JR, Lambright CS, Wilson VS,Foster PM, and Gray Jr. LE, 2014. A short-term in vivo screen using fetal testosterone production, a key event in the phthalate adverse outcome pathway, to predict disruption of sexual differentiation. Toxicological Sciences 140(2): 403–424. https://doi.org/10.1093/toxsci/kfu081

Gray Jr., LE et al., 2000. Perinatal exposure to the phthalates DEHP, BBP and DINP but not DEP, DMP or DOTP alters sexual differentiation of the male rat. Toxicological Sciences 58: 350-365. https://doi.org/10.1093/toxsci/58.2.350

Hannas BR, Lambright CS, Furr J, Howdeshell K, Wilson VS and Gray Jr. LE, 2011. Dose-response assessment of fetal testosterone production and gene expression levels in rat testes following in utero exposure to Diethylhexyl phthalate, Diisobutyl phthalate, Diisoheptyl phthalate, and Diisononyl phthalate. Toxicological Sciences 123(1): 206-216. https://doi:10.1093/toxsci/kfr146

Hannas BR, Lambright CS, Furr J, Evans N, Foster PMD, Gray EL, and Wilson VS, 2012. Genomic biomarkers of phthalate-induced male reproductive developmental toxicity: A targeted RT-PCR array approach for defining relative potency. Toxicological Sciences 125: 544-557. https://doi:10.1093/toxsci/kfr315

Hellwig J, Freudenberger H and Jäckh R, 1997. Differential prenatal toxicity of branched phthalate esters in rats. Food and Chemical Toxicology 35: 501-512 https://doi.org/10.1016/S0278-6915(97)00008-2

Johnson, KJ, Hensley, JB, Kelso, MD, Wallace, DG, and Gaido, KW, 2007. Mapping gene expression changes in the fetal rat testis following acute dibutyl phthalate exposure defines a complex temporal cascade of responding cell types. Biology of Reproduction 77: 978–989. https://doi.org/10.1095/biolreprod.107.062950

Kilcoyne KR, Smith LB, Atanassova N, Macpherson S, McKinnell C, van den Driesche S, et al., 2014. Fetal programming of adult Leydig cell function by androgenic effects on stem/progenitor cells. Proceedings of the National Academy of Sciences 111(18): E1924-E1932. https://doi.org/10.1073/pnas.1320735111

Koch HM∗, Haller A, Weiß T, Käfferlein HU, Stork J, Brüning T, 2012. Phthalate exposure during cold plastisol application - a human biomonitoring study. Toxicology Letters 213: 100– 106. https://doi.org/10.1016/j.toxlet.2011.06.010

Lara NLM, van den Driesche S, Macpherson S, Franca LR, Sharpe RM, 2017. Dibutyl phthalate induced testicular dysgenesis originates after seminiferous cord formation in rats. Scientific Reports, 7(1):2521. https://doi.org/10.1038/s41598-019-43786-3

Li L, Bu T, Su H, Chen Z, Liang Y, Zhang G, Zhu D, Shan Y, Xu R, Hub Y, Li J, Huc G, Lian Q, Gel RS, 2015. In utero exposure to diisononyl phthalate caused testicular dysgenesis of rat fetal testis. Toxicology Letters 232: 466–474 http://dx.doi.org/10.1016/j.toxlet.2014.11.024

MacLeod DJ, Sharpe RM, Welsh M, Fisken M, Scott HM, Hutchison GR, Drake AJ, van den Driesche S., 2010. Androgen action in the masculinisation programming window and development of male reproductive organs. Int J Andrology 33: 279-287. https://doi.org/10.1111/j.1365-2605.2009.01005.x

Mahood IK, Scott HM, Brown R, Hallmark N, Walker M, and Sharpe RM, 2007. In Utero Exposure to Di(n-butyl) Phthalate and Testicular Dysgenesis: Comparison of Fetal and Adult End Points and Their Dose Sensitivity. Environmental Health Perspectives 115(suppl 1): 55–61. http://dx.doi.org/10.1289/ehp.9366

Masutomi N, Shibutani M, Takagi H, Uneyama C, Takahashi N, Hirose M, 2003. Impact of dietary exposure to methoxychlor, genistein, or diisononyl phthalate during the perinatal period on the development of the rat endocrine/reproductive systems in later life. Toxicology 192: 149–170. https://doi.org/10.1016/S0300-483X(03)00269-5

McNeilly JR, Saunders PTK, Taggart M, Cranfield M, Cooke HJ and McNeilly AS, 2000. Loss of oocytes in Dazl knockout mice results in maintained ovarian steroidogenic function but altered gonadotropin secretion in adult animals. Endocrinology 141(11): 4284-4294. https://doi.org/10.1210/endo.141.11.7764

Meistrich, ML,1989. Evaluation of reproductive toxicity by testicular sperm head counts. International Journal of Toxicology 8(3): 551-567. https://doi.org/10.3109/10915818909014538

NICNAS, 2015 (National Industrial Chemicals Notification and Assessment Scheme): Diisononylphthalate https://www.nicnas.gov.au/chemical-information/factsheets/chemical-name/diisononyl-phthalate-dinp.
Accessed: October 2020

OECD, 2008. Series on Testing and Assessment, No. 43. Guidance document on mammalian reproductive toxicity testing an assessment. ENV/JM/MONO(2008)16. http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2008)16&doclanguage=en. Accessed: October 2020

Plummer, S, Sharpe, RM, Hallmark, N, Mahood, IK, and Elcombe, C, 2007. Time-dependent and compartment-specific effects of in utero exposure to di(n-butyl) phthalate on gene/protein expression in the fetal rat testis as revealed by

transcription profiling and laser capture microdissection. Toxicological Sciences 97: 520–532. https://doi.org/10.1093/toxsci/kfm062

Saillenfait AM, Sabate JP, Gallissot F, 2006. Developmental toxic effects of diisobutyl phthalate, the methyl-branched analogue of di-n-butyl phthalate, administered by gavage to rats. Toxicolology Letters 165: 39–46. http://dx.doi.org/10.1016/j.toxlet.2006.01.013

Saillenfait AM, Sabate JP, Gallissot F, 2008. Diisobutyl phthalate impairs the androgen-dependent reproductive development of the male rat. Reproductive. Toxicology 26: 107–115. http://dx.doi.org/10.1016/j.reprotox.2008.07.006

Saillenfait AM, Roudot AC, Gallissot F, Sabate JP, 2011. Prenatal developmental toxicity studies on di-n-heptyl and di-n-octyl phthalates in Sprague-Dawley rats. Reproductive Toxicology 32: 268–276. http://dx.doi.org/10.1016/j.reprotox.2011.08.001

Schindelin J, Arganda-Carreras I, Frise, E et al., 2012. Fiji: an open-source platform for biological-image analysis. Nature Methods 9(7): 676-682. http://dx.doi.org/10.1038/nmeth.2019

Schleh C, Leoni A, 2013. How to optimize the benefits of computer assisted sperm analysis in experimental toxicology. Journal of Occupational Medicine and Toxicology 8: 6. https://doi.org/10.1186/1745-6673-8-6

Schwartz CL, Christiansen S, Vinggaard AM, Axelstad M, Hass U, Svingen T, 2019. Anogenital distance as a toxicological or clinical marker for fetal androgen action and risk for reproductive disorders. Archives of Toxicology 93(2): 253-272. https://doi.org/10.1007/s00204-018-2350-5

Scott HM, Mason JJ, Sharpe RM, 2009. Steroidogenesis in the fetal testis and its susceptibility to disruption by exogenous compounds. Endocrine Reviews 30(7): 883–925. https://doi.org/10.1210/er.2009-0016

Seed J, Chapin RE, Clegg ED, Dostal LA, Foote RH, Hurtt ME., Klinefelter GR, Makris SL, Perreault SD, Schrader S, Seyler D, Sprando R, Treinen KA, Veeramachaneni DNR and Wise LD, 1996. Methods for assessing sperm motility, morphology, and counts in the rat, rabbit, and dog: a consensus report. Reproductive Toxicology 10(3): 237-244. https://doi.org/10.1016/0890-6238(96)00028-7

Skakkebaek NE, Meyts RD, Main KM, 2001. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. Human Reproduction 16(5): 972–978. https://doi.org/10.1093/humrep/16.5.972

Slott VL, Suarez JD and Perreault SD, 1991. Rat sperm motility analysis: Methodological considerations. Reproductive Toxicology 5: 449-458. https://doi.org/10.1016/0890-6238(91)90009-5

Thompson DJ; Ross SM, Gaido KW, 2004. Di(n-butyl) phthalate impairs cholesterol transport and steroidogenesis in the fetal rat testis through a rapid and reversible mechanism. Endocrinology 145: 1227 – 1237.

https://doi.org/10.1210/en.2003-1475. Accessed: October 2020

van den Driesche S, Kolovos P, Platts S, Drake AJ, Sharpe RM, 2012. Inter-Relationship between Testicular Dysgenesis and Leydig Cell Function in the Masculinization Programming Window in the Rat. PLoS ONE 7(1): e30111.

https://doi:10.1371/journal.pone.0030111

van den Driesche S, Kilcoyne KR, Wagner I, Rebourcet D, Boyle A, Mitchell R, et al., 2017. Experimentally induced testicular dysgenesis syndrome originates in the masculinization programming window. JCI Insight 2: e91204.

https://doi.org/10.1172/jci.insight.91204

Waterman SJ, Ambroso JL, Keller LH, Trimmer GW, Nikoforov AI, and Harris SB, 1999. Developmental toxicity of Di-isodecyl and Di-isononyl phthalates in rats. Reproductive Toxicology 13(2): 131–136.

https://doi.org/10.1016/S0890-6238(99)00002-7

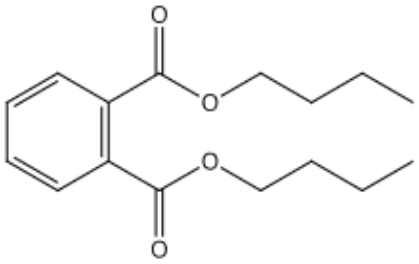
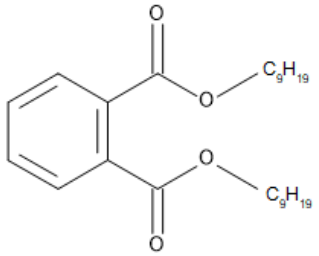
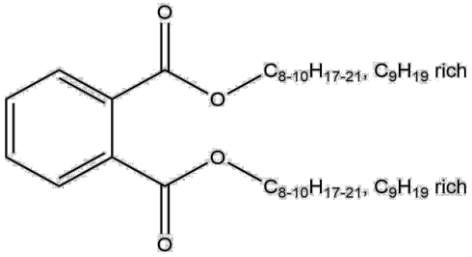
Welsh M, Saunders PTK, Fisken M, Scott HM, Hutchison GR, Smith LB and Sharpe RM, 2008. Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. The Journal of Clinical Investigation 118: 1479–1490. https://doi.org/10.1172/JCI34241.

Wilson VS, Howdeshell KL, Lambright CS, Furr J, Gray Jr. LE, 2007. Differential expression of the phthalate syndrome in male Sprague–Dawley and Wistar rats after in utero DEHP exposure. Toxicology Letters 170: 177–184 https://doi.org/10.1016/j.toxlet.2007.03.004

Wolfe GW, Layton KA, 2005. Multigeneration reproduction toxicity study in rats: Diethylhexylphthalate: Multigenerational reproductive assessment by continuous breeding when administered to Sprague-Dawley rats in the diet. TherImmune Research Corporation (Gaithersburg, Maryland), TRC Study No 7244-200. https://ntrl.ntis.gov/NTRL/dashboard/searchResults/titleDetail/PB2005107575.xhtml

Table

Table 1: Structures and abbreviations of phthalates in this study

Chemical Name	Abbreviation	CAS No	Structure
Dibutyl phthalate	**DBP**	84-74-2	
Diisononyl phthalate	**DINP** (DINP2)	28553-12-0	
1,2-Benzenedi-carboxylic acid, di-C8-10, branched alkyl esters, C9-rich	**DINP** (DINP1)	68515-48-0	

Table

Table 2. Effects of in utero exposure to vehicle control, 750 mg/kg bw/day DBP (DBP-750), 125 mg/kg bw/day DINP (DINP-125) and 750 mg/kg bw/day DINP (DINP-750) from e15.5-18.5 on adult reproductive phenotype

	Control	DBP-750	DINP-125	DINP-750
Body weight (g)	408.03 ± 9.70	383.30 ± 11.38	399.23 ± 6.90	417.97 ± 4.64
AGD (mm)	44.80 ± 0.94	35.67 ± 1.48 [a]	43.598 ± 0.55	44.36 ± 0.44
Anogenital index	6.04 ± 0.09	4.91 ± 0.19 [a]	5.92 ± 0.08	5.93 ± 0.05
Average testis weight (g)	1.96 ± 0.04	0.97 ± 0.13 [a]	1.99 ± 0.02	1.94 ± 0.04
Penis length (mm)	10.94 ± 0.22	9.17 ± 0.21 [a]	10.66 ± 0.07	10.51 ± 0.19
Penis weight (g)	0.148 ± 0.006	0.109 ± 0.004 [a]	0.138 ± 0.003	0.134 ± 0.005
Ventral prostrate weight (g)	0.48 ± 0.03	0.40 ± 0.02 [a]	0.49 ± 0.03	0.49 ± 0.02
Seminal vesicle weight (g)	0.73 ± 0.03	0.33 ± 0.03 [a]	0.71 ± 0.02	0.69 ± 0.03

Results are expressed as litter means ± SEM. n = 7 for control, DINP 125 mg/kg bw/day and DINP 750 mg/kg bw/day groups, and n = 6 for the DBP 750 mg/kg bw/day group.
[a] $p<0.001$ (ANOVA – Bonferroni)

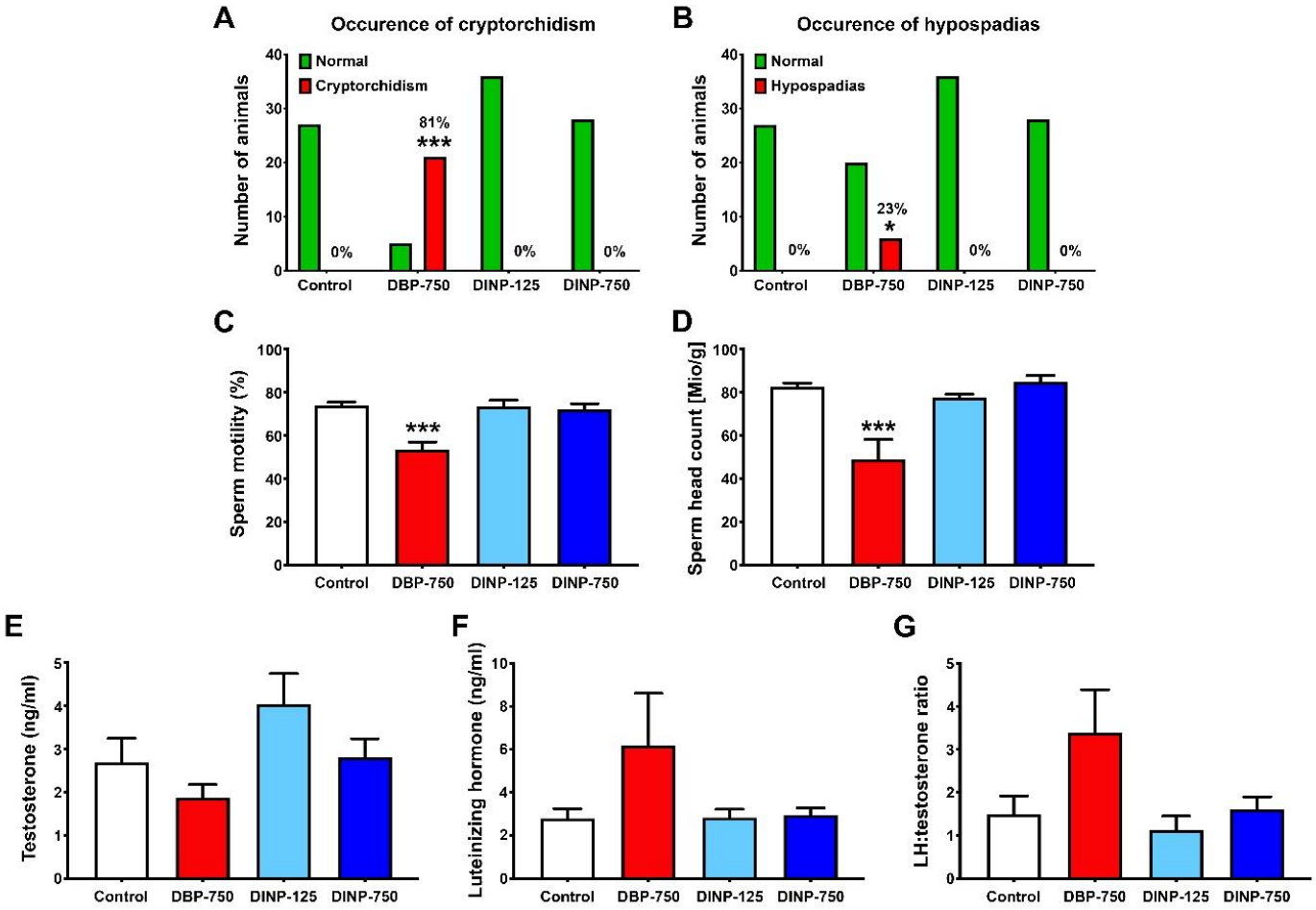


Figure 2. In utero exposure to Dibutyl phthalate (DBP, 750 mg/kg bw/day) in the masculinisation programming window (e15.5-e18.5), but not to low (125 mg/kg bw/day) or high (750 mg/kg bw/day) doses of Diisononyl phthalate (DINP) induces TDS-like symptoms in adulthood. Values are Litter Means ± SEM (n = 7 for control, n=6 for DBP-750, n = 7 for DINP-125, n=7 for DINP-750). *p<0.05, ***p<0.001 in comparison with respective controls.

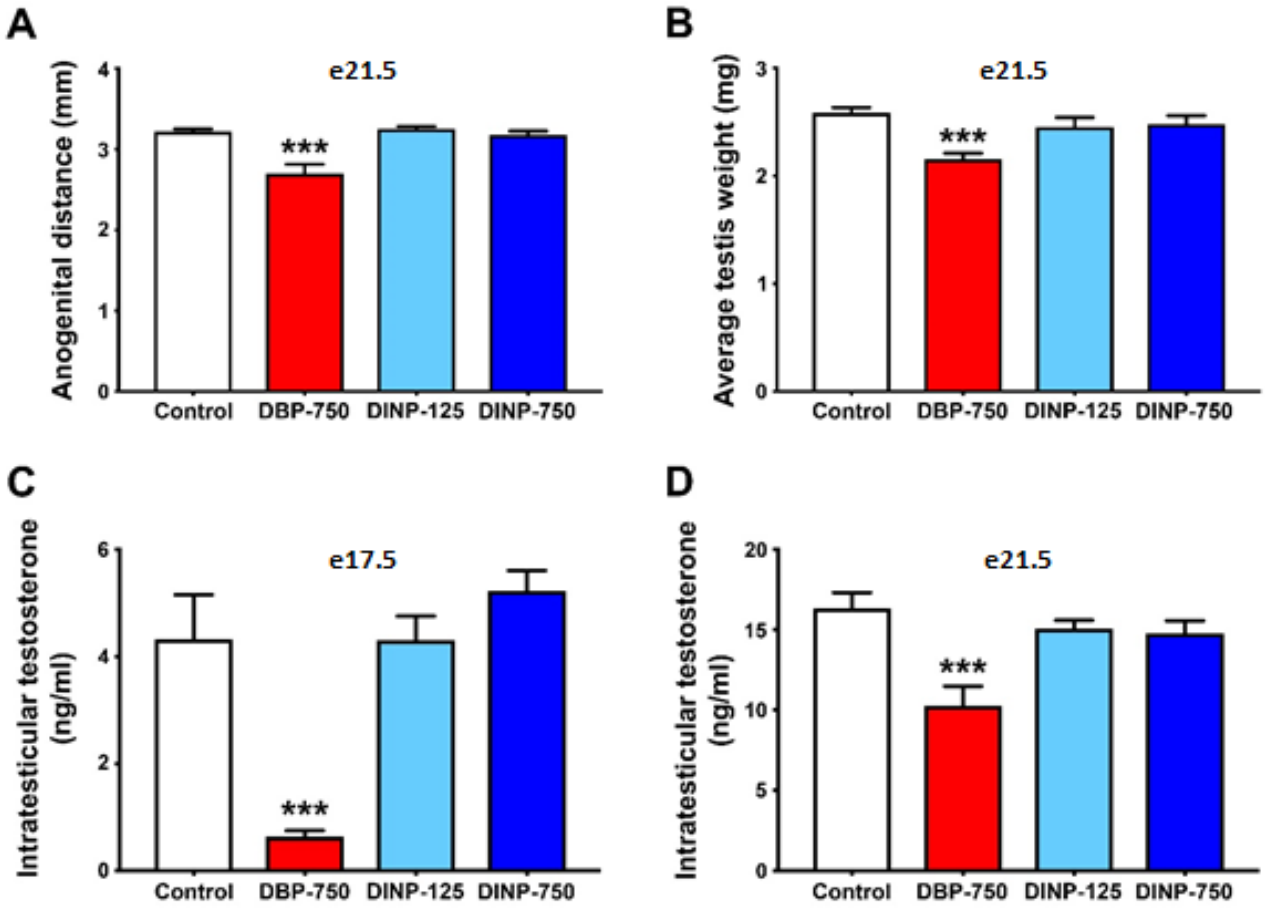


Figure 3. In utero exposure to Dibutyl phthalate (DBP, 750 mg/kg bw/day) in the masculinisation programming window, but not to low (125 mg/kg bw/day) or high (750 mg/kg bw/day) doses of Diisononyl phthalate (DINP) induces effects on testosterone and anogenital distance (AGD) in male fetuses. Effects from in utero exposure vehicle (Control), DBP (750 mg/kg bw/day), DINP (125 or 750 mg/kg bw/day) on (A) AGD at e21.5 (Values are Litter Means ± SEM n = 7 for control, n=6 for DBP-750, n = 6 for DINP-125, n = 6 for DINP-750), (B) average testis weight at e21.5 (Values are Litter Means ± SEM n = 7 for control, n=6 for DBP-750, n = 6 for DINP-125, n = 6 for DINP-750), (C), intratesticular testosterone at e17.5 (Values are Litter Means ± SEM n = 7 for control, n=6 for DBP-750, n = 6 for DINP-125, n = 6 for DINP-750, and (D) intratesticular testosterone at e21.5 (values are Litter Means ± SEM (n = 4 for control, n=7 for DBP-750, n = 4 for DINP-125, n = 6 for DINP-750 at e17.5). ***p<0.001 in comparison with respective controls.

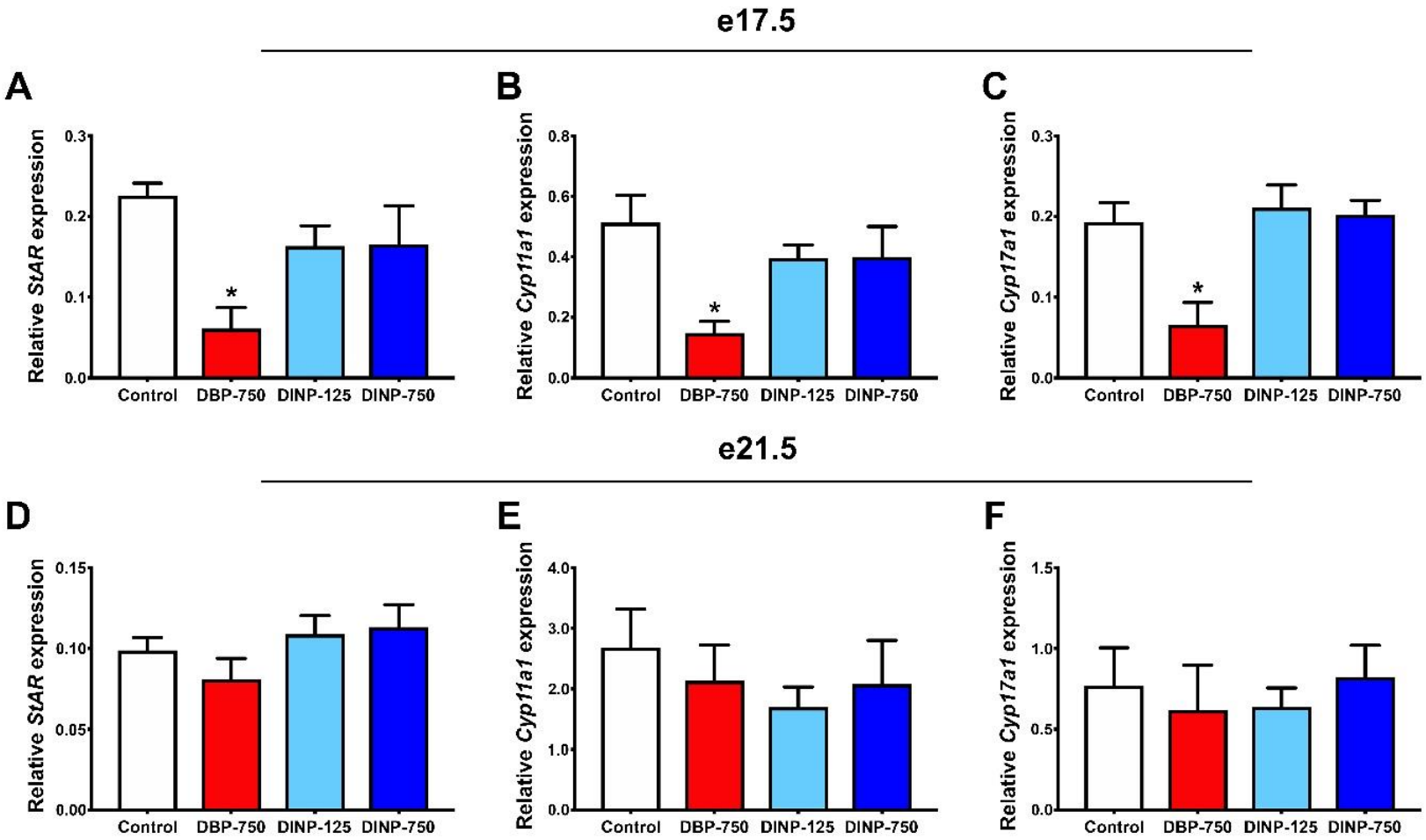


Figure 4. Quantitative mRNA expression of steroidogenic genes in e17.5 and e21.5 testes after exposure to vehicle (Control), Dibutyl phthalate (DBP-750 mg/kg bw/day), Diisononylphthalate (DINP-125 or 750 mg/kg bw/day). (A, D) Steroidogenic acute regulatory protein (*St*AR), (B, E) cytochrome P450, family 11, subfamily a, polypeptide 1 (*Cyp11a1*), (C, F) cytochrome P450, family 17, subfamily a, polypeptide 1 (*Cyp17a1*). Dams were exposed from embryonic day (e) 15.5-17.5 (A-C) or e15.5-18.5 (D-F). Values are Litter Means ± SEM (n = 3 litters per treatment group). *p<0.05 in comparison with respective controls.

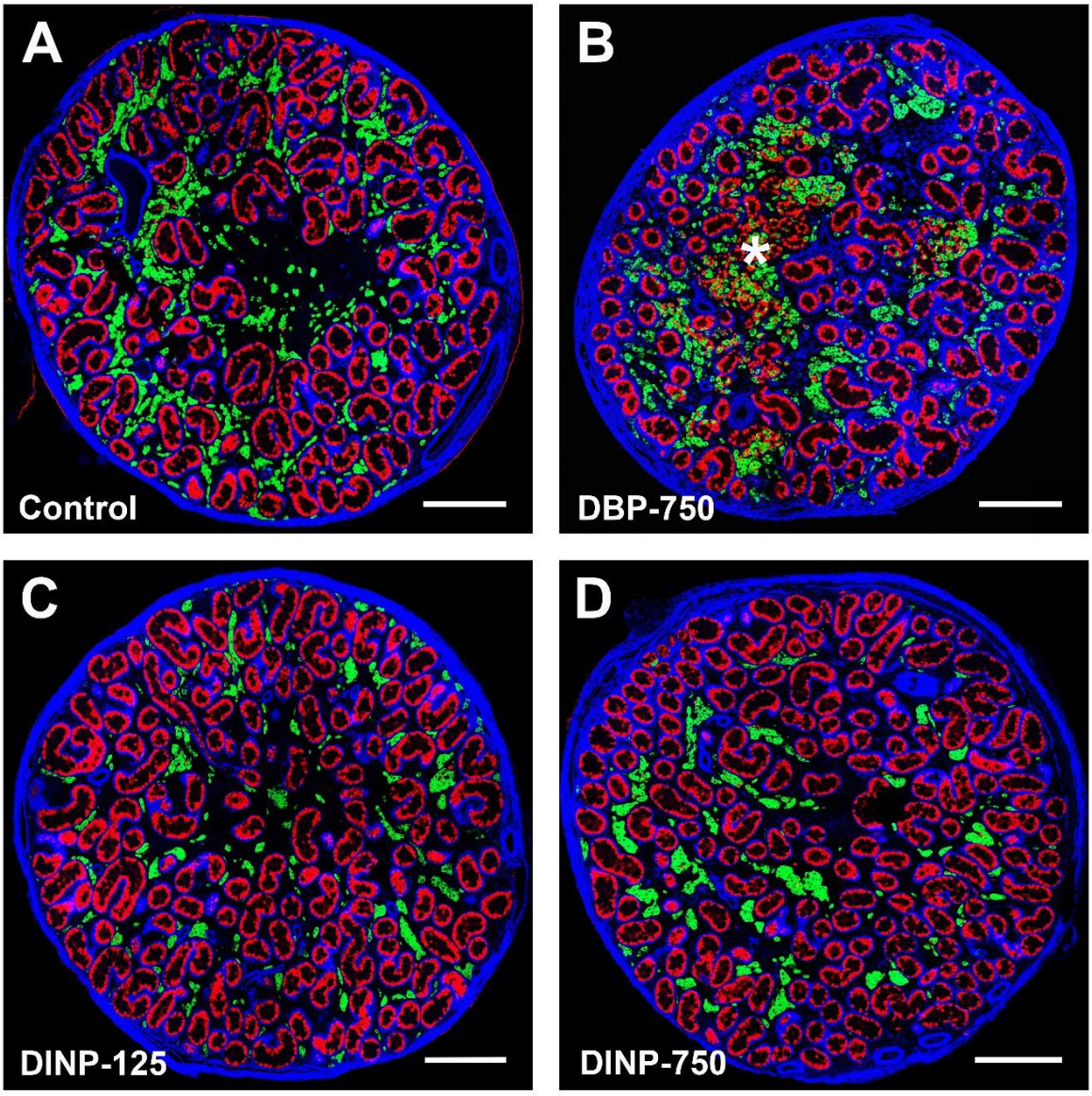


Figure 5. Triple immunofluorescense labelling of fetal testes at e21.5 following exposure during the male programming window to vehicle (Control), Dibutyl phthalate (DBP-750 mg/kg bw/day), Diisononylphthalate (DINP- 125 or -750 mg/kg bw/day.

3β-HSD (green; fetal Leydig cell marker), Sox-9 (red; Sertoli cell marker), SMA (blue; α-smooth muscle actin, peritubular myoid cell marker). The asterisk in panel B illustrates focal dysgenesis in the DBP 750 mg/kg bw/day (DBP-750) treatment group. Scale bar = 200 μm.

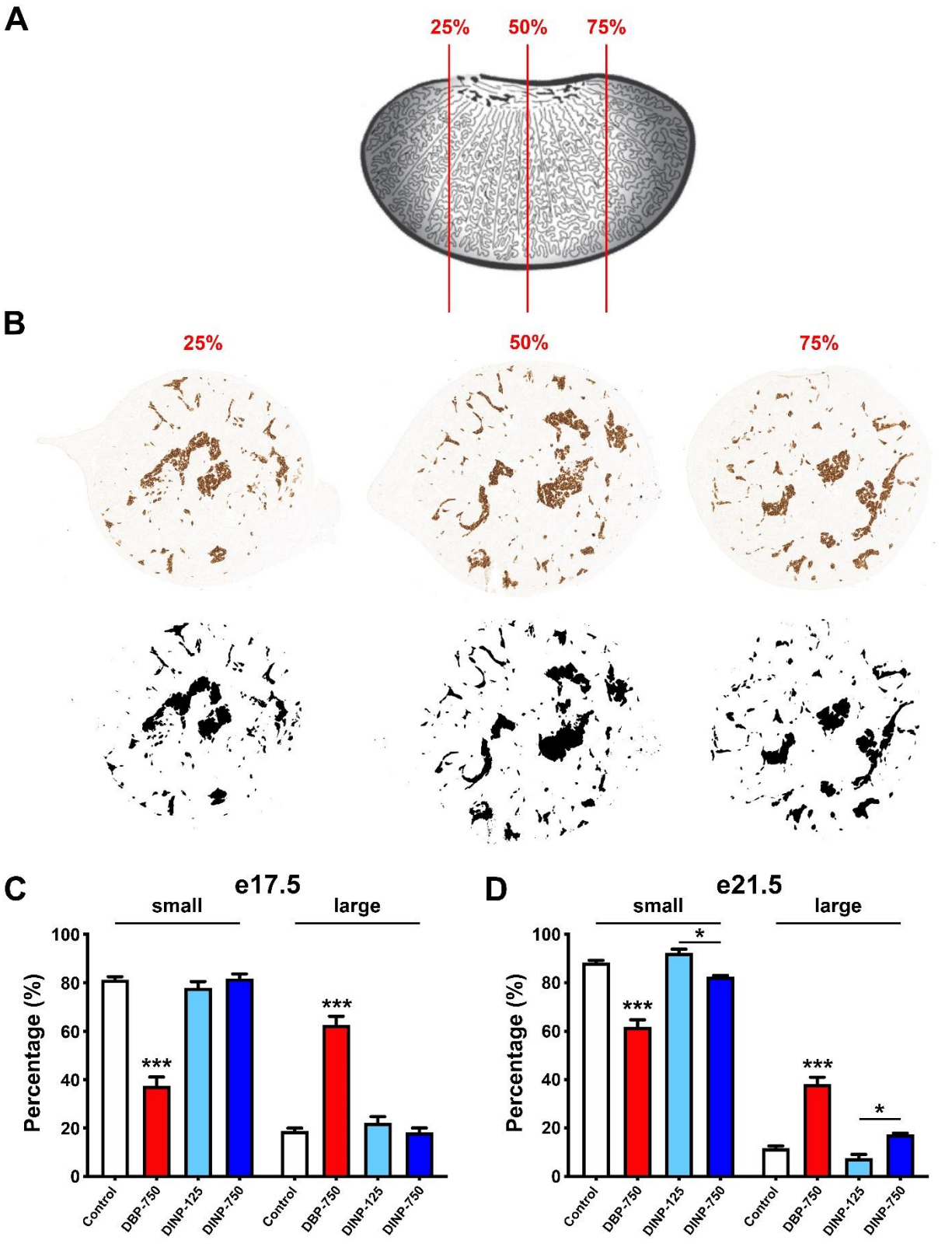


Figure 6: Quantitative evaluation of Leydig cell aggregates in e17.5 and e21.5 testes after exposure during the male programming window (MWP) to vehicle (Control), Dibutyl phthalate (DBP, 750 mg/kg bw/day), Diisononylphthalate (DINP, 125 or 750 mg/kg bw/day).
(A) Schematic diagram to illustrate the collection of the sections at 25, 50 and 75%, which were included in the systematic analysis in order to get a representative average for each testis.
(B) Examples of the immunostaining with 3β-HSD (1st row), corresponding transformed, background corrected pictures for automatic counting (2nd row). For technical details see the Materials and Methods.

(C) Quantitative Assessment of Leydig cell aggregates. Values are Litter Means ± SEM (n = 6 for control, n=6 for DBP 750 mg/kg bw/day (DBP-750), n = 6 for DINP 125 mg/kg bw/day (DINP-125), n = 5 for DINP 750 mg/kg bw/day (DINP-750) at e17.5 and e21.5). *$p<0.05$ when DINP-125 and DINP-750 groups are compared, ***$p<0.001$ in comparison with controls.

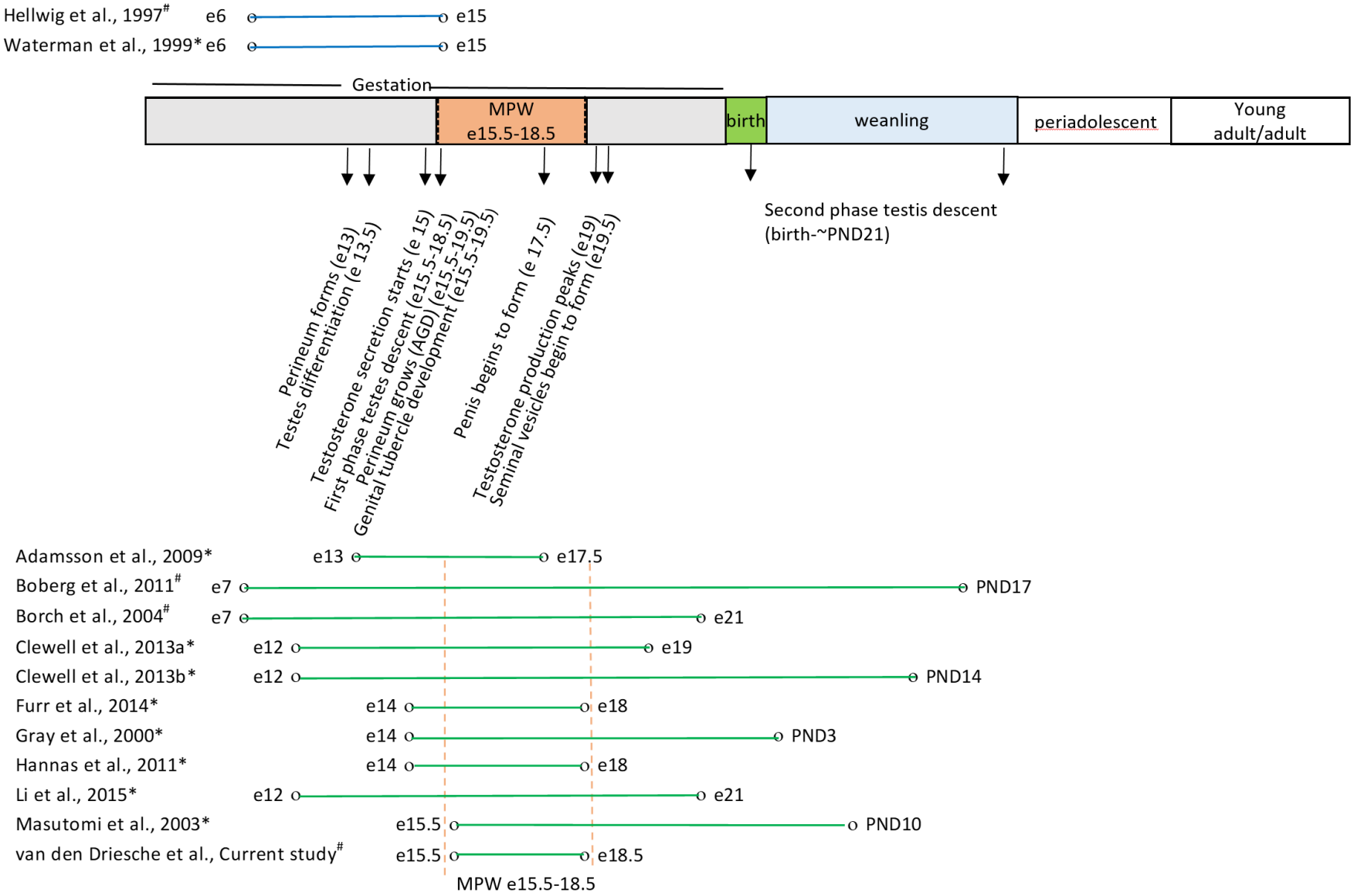


Figure 7. Developmental Toxicity Studies with DINP. The duration and timing of exposure for published studies on DINP are depicted here in relation to select male rat developmental milestones. Studies involving exposures encompassing the male programming window (MPW) are depicted in green. Studies involving exposure prior to the MPW are depicted in blue. # denotes study was performed in Wistar rats; * denotes study was performed in Sprague Dawley rats. Postnatal day (PND), AGD (anogenital distance), e (embryonic day)

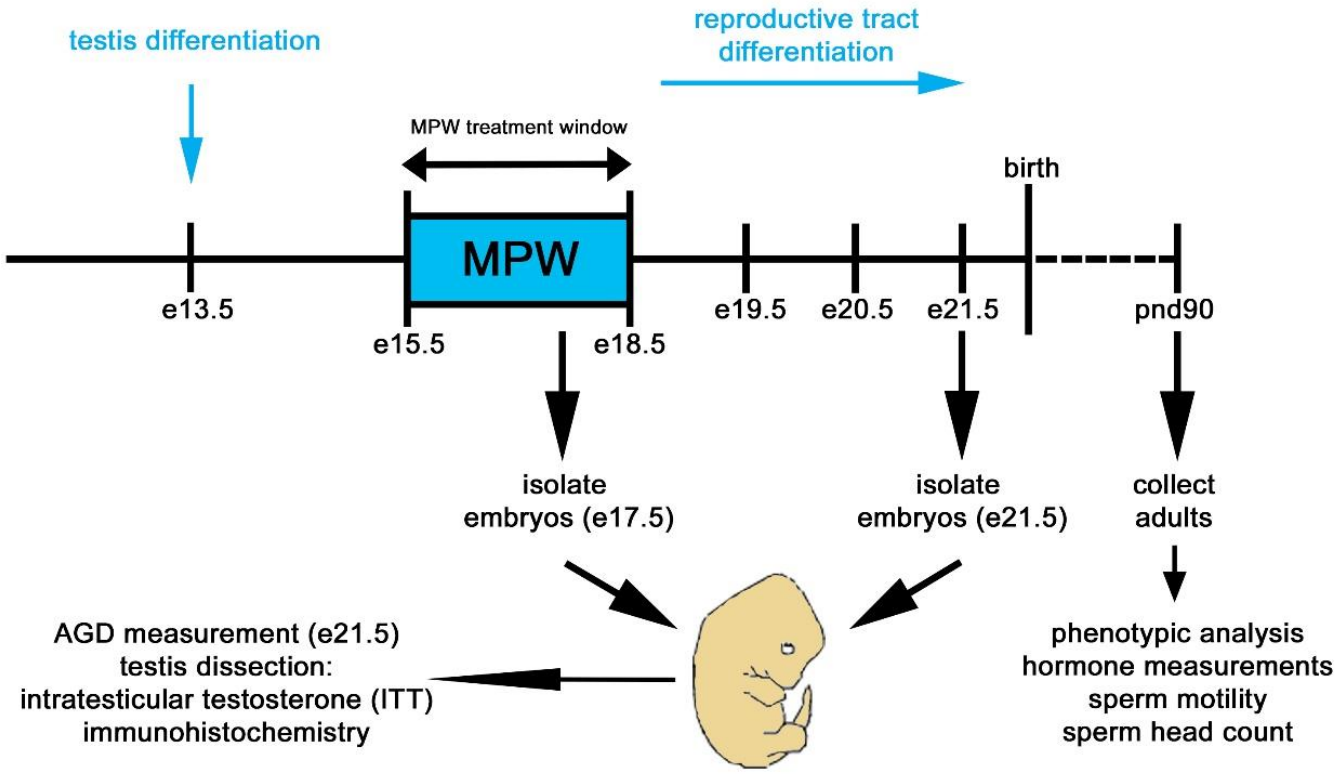


Figure 1: Study design: Indicated with blue arrows are testis differentiation in the rat (starting at embryonic day e13.5 and reproductive tract differentiation (from e18.5 onwards). The masculinisation programming window (MPW, e15.5–e18.5) is indicated with the blue box. Three different experiments were performed. The treatment window that was used in the two fetal studies was during the MPW, from e15.5–e17.5 for e17.5 fetal isolations; and from e15.5-e18.5 for e21.5 fetal isolations. The following observations were made: anogenital distance (AGD) was measured (in e21.5 fetal isolations only), testes were dissected and weighed (testes of e17.5 fetal isolations were not weighed), 1 testis was fixed for subsequent immunohistochemistry and 1 testis was frozen on dry ice for subsequent intratesticular testosterone (ITT) measurement. In a third experiment, pregnant rats were treated from e15.5–e18.5 and male animals were collected in adulthood at postnatal day (pnd) 90. Blood was taken for hormone measurements, the animals were phenotypically analysed, sperm motility was determined and testis samples were collected for sperm head counts.

*Declaration of Interest Statement

Declaration of interests

☒ The authors from the University of Edinburgh declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Drs. Angelika Langsch, Christine Palermo and Rainer Otter are employed by producers of DINP