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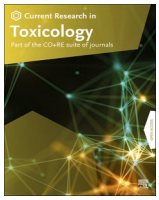

Research Paper

# Using targeted fetal rat testis genomic and endocrine alterations to predict the effects of a phthalate mixture on the male reproductive tract

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ABSTRACT

Administration of phthalates *in utero* disrupts gene expression and hormone levels in the fetal rat testis, which are key events in an Adverse Outcome Pathway (AOP) for the Phthalate Syndrome. These measures can be used to predict the postnatal adverse effects of phthalate esters (PEs) on male rat sexual differentiation. Here, pregnant rats were exposed to dibutyl (DBP)- and diisononyl (DINP) phthalate on gestational days 14 to 18 individually and as a mixture (DBP,250 mg/kg/d; DINP, 750 mg/kg/d; and DBP 250 mg/kg/d plus DINP 750 mg/kg/d). We found that each PE reduced testosterone production (T Prod) and related gene transcripts by about 50 % and that they acted in a dose additive manner, reducing T Prod and gene expression by 75 % as a mixture. Based upon effects on T Prod, DINP was 0.33 times as potent as DBP and thus the DBP + DINP mixture was predicted to be equivalent to 500 mg DBP/kg/d.

Logistic regression models of T Prod predicted that the adverse effects of the DBP + DINP mixture group versus the DBP and DINP individual treatments would reduce anogenital distance (AGD) by 27 % versus 10 %, increase hypospadias in 18 % versus < 1 %, induce epididymal agenesis in 46 % versus 10 %, and increase areolae/nipples in 4.8 % versus < 0.1 % of the, respectively. These predictions were highly consistent with effects from previously published dose response studies on the male reproductive effects of DBP. In summary, these results support the use of this New Approach Method to predict the detrimental effects of PEs and PE mixtures, replacing or reducing the need to run long-term, resource and animal use intensive extended one-generation reproduction studies for this class of chemicals.

## Introduction

Maternal administration of phthalate esters (PEs), including those with di-ortho alkyl groups of three to seven carbons, alter male and female rat reproductive tract differentiation *in utero*. Exposure to PEs during the last week of pregnancy (Carruthers and Foster, 2005) or perinatal life (Dostal et al., 1988) disrupts testis function in the male, whereas PE exposure during the second week of gestation induces uterine and vaginal malformations in females (Hannas et al., 2013). In the male, PEs alter fetal synthesis of testosterone and insulin-like hormone 3 (Insl3), Sertoli cell function, steroid hormone and cholesterol transport, cholesterol synthesis and lipid metabolism. Consequently, male rat offspring display effects that are part of the "Phthalate Syndrome" including malformations of testosterone- and Insl3-dependent tissues, reduced sperm production, and infertility (Foster, 2006). Furthermore, several regulatory agencies, including US Consumer Product Safety Commission (CPSC, 2014), Australia NICNAS, and Health Canada (Canada, 2015) have used reduced fetal T Prod as the critical effect in risk characterization in phthalate cumulative risk assessments.

Dose response studies of fetal testis endocrine function reveal a wide-range of potencies among these PEs with diisononyl phthalate (DINP) being one of the least potent, only reducing fetal androgen production and testis gene expression at high dosage levels as compared to other more potent active phthalates like dibutyl phthalate (DBP) or dipentyl phthalate (DPeP) (Mylchreest et al., 2000; Furr et al., 2014; Gray et al., 2016; Gray et al., 2021). For example, DBP induced epididymal agenesis and other reproductive tract malformations in about 80 % of male CRSD

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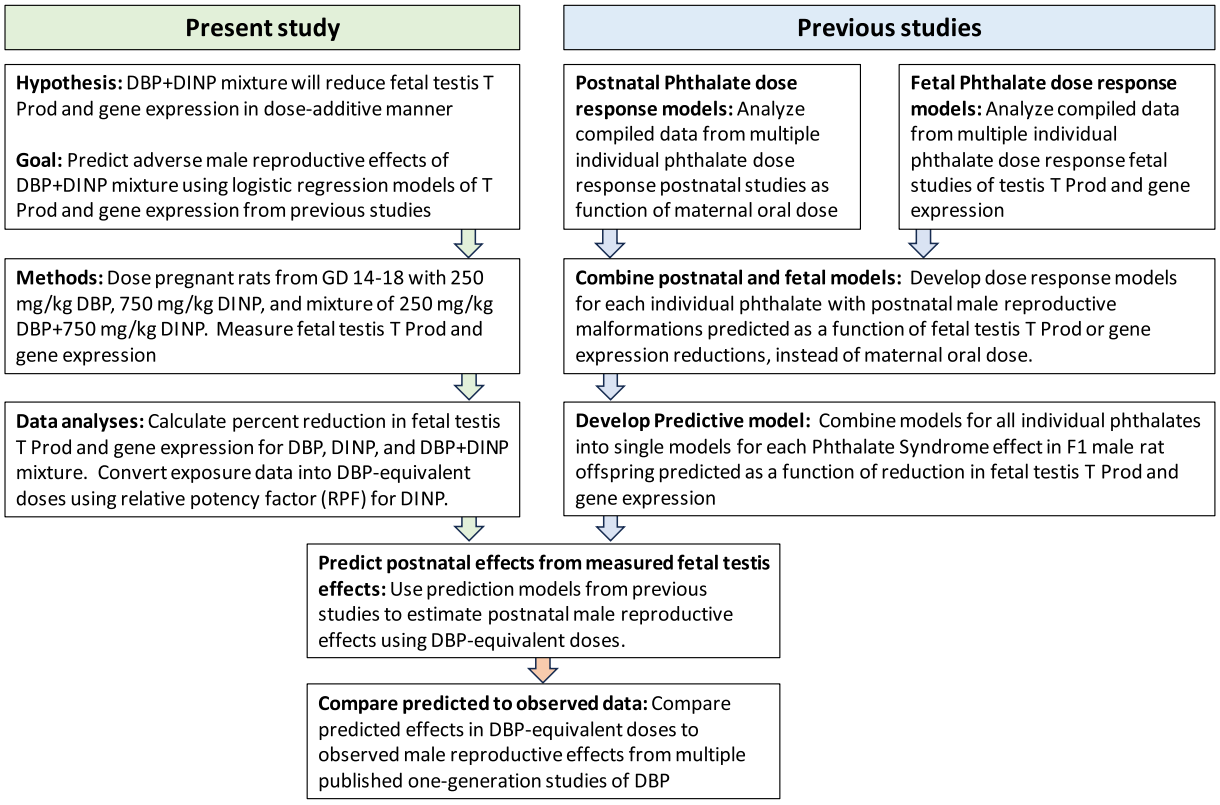



**Fig. 1.** Study Rationale.

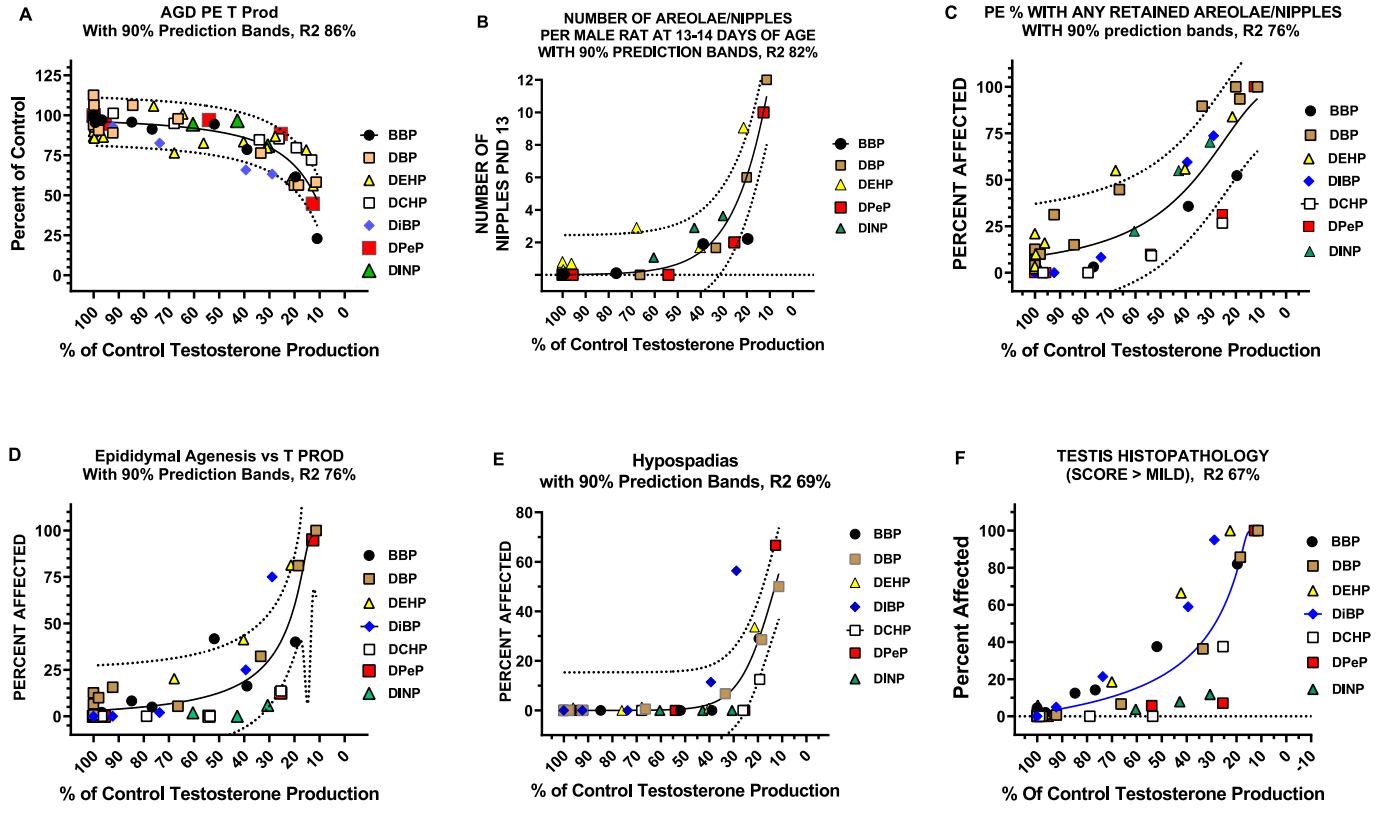



**Fig. 2.** Logistic Regression Prediction Models of testosterone production (T Prod) (Furr et al., 2014; Gray et al., 2021) versus Phthalate Syndrome Abnormalities from published studies (Supplemental file). A: Anogenital distance (AGD) (% of control, female = 0 %). B: Number of female like areolae/nipples per infant male (12 maximum) and C: Percent of infant males with any areolae/nipples. D: Percent of males with epididymal agenesis. E: Percent of males with hypospadias. F: Percent of males displaying mild, moderate or severe histopathological lesions.

**Table 1**
Chemicals used in the current study, and chemical purity, and lot numbers.

| Test chemical or mixture | Dibutyl Phthalate (DBP) |
|---|---|
| **CASRN** | 84-74-2 |
| **Chemical Supplier** | RTI |
| **Catalog Number** | Sample Date 12-15-09; RTI Log # 031609-A-17 |
| **Purity** | 99.9 |
| **Lot** | Chem ID I16; Lot # 91997PJ |
| **Vehicle** | Corn oil |
| **Dosing rate** | mg/kg/d |
| **Doses** | 250 |
| **Test chemical or mixture** | **Diisononyl Phthalate (DiNP)** |
| **CASRN** | 28553-12-0 |
| **Chemical Supplier** | Sigma |
| **Catalog Number** | 376,663 |
| **Purity** | >99 % |
| **Lot** | STBK6586 |
| **Vehicle** | Corn oil |
| **Dosing rate** | mg/kg/d |
| **Doses** | 750 |
| **Test chemical or mixture** | MIXTURE OF DBP AND DINP (250 + 750) |
| **Vehicle** | Corn oil |
| **CASRN** | 8001-30-7 |
| **Vehicle Supplier** | Sigma-Aldrich |
| **Catalog Number** | C8267 |
| **Lot** | MKBV2080V |

rat offspring at 750 mg/kg/d whereas exposure to 750 mg DINP/kg/d induced malformations in only 7.7 % of the CRSD rat offspring (Gray et al., 2000; Gray, 2023).

Several PEs including DINP and DBP are currently included in hazard assessments by regulatory agencies in the US and abroad (Canada, 2020; CPSC, 2014) (ECHA Final Report on DIDP and DINP, 2013). In August 2022 EPA issued a supplemental proposed rule that would add DINP to the list of toxic chemicals subject to the Toxics Release Inventory (TRI) reporting requirements under the Emergency Planning and Community Right-to-Know Act and the Pollution Prevention Act (https://www.epa.gov/chemicals-under-tsca/epa-releases-proposed-rule-requiring-tri-reporting-dinp, last accessed on 3/14/2024). EPA also released an updated hazard assessment which stated that "DINP can reasonably be anticipated to cause serious or irreversible reproductive dysfunctions as well as other serious or irreversible chronic health effects in humans, specifically, developmental, kidney, and liver toxicity." (www.govinfo.gov/content/pkg/FR-2023-07-14/pdf/2023-14642.pdf).

Previously, we reported that the severity of the demasculinizing effects of DPeP and DINP on the androgen-dependent organs and gubernaculum testis were accurately predicted from statistical models of reduced T prod and *Insl3* mRNA, respectively (Gray et al., 2016; Gray 2023). Taken together, those results demonstrated that reductions in fetal T prod and *Insl3* could be used to predict the severity of demasculinizing effects from *in utero* exposure to PEs and PE mixtures. Here, we tested the hypothesis that a mixture of DINP and DBP would alter fetal testis T Prod and gene expression in a cumulative, dose-additive manner. Further, our objective was to then predict the adverse reproductive effects of exposure to the DBP + DINP mixture on the male offspring reproductive system from reductions in fetal T Prod and testis *Insl3* mRNA expression (Fig. 1).

The prediction models combined our data on the effects of PEs on T Prod and *Insl3* expression (Furr et al., 2014; Gray et al., 2021) with F1 male data from our studies and studies from other laboratories that administered PEs to pregnant rats during fetal sexual differentiation. T Prod and *Insl3* expression were used as the dose metric on the X axis rather than the administered dose. These models describe how much of a reduction in fetal T Prod and *Insl3* mRNA are required to produce reproductive tract alterations in the F1 male rat. This approach would use far fewer resources than a one-generation study and provide data useful for regulatory decision makers to identify points of departure for hazard identification of PEs that induce the "phthalate syndrome" *in utero* (Fig. 2).

## Methods

### *Experimental design*

The study is a binary mixture design with four groups including a vehicle control, DBP (250 mg/kg/d), DINP (750 mg/kg/d) and a mixture group (DBP (250 mg/kg/d) plus DINP (750 mg/kg/d). Based upon previous studies (Gray et al., 2021; Gray 2023), we expected the treatments to reduce T Prod by ~ 40–50 % of control for DINP and DBP and by ~ 70 % in the DBP + DINP mixture group. Detailed chemical information is provided in the following Table 1.

### *Animals*

Time-mated Sprague-Dawley rats (Crl:CD(SD)), ~11–12 weeks old, were purchased from Charles River Laboratories (Raleigh, NC, USA) and shipped to USEPA (Research Triangle Park, NC, USA) on gestation day (GD) 2 (GD0 = bred date; GD1 = plug positive date). Dams were housed individually in clear polycarbonate cages (20 × 25 × 47 cm) with heat-treated, laboratory-grade pine shavings (Northeast Products, Warrensburg, NY) and fed NIH07 Rat Chow and treated municipal tap water (Durham, NC tap water, 50 μm particle filtration, granular activated charcoal filtration, and rechlorinated) *ad libitum*. Animals were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and maintained at 20–22 °C, 45–55 % humidity, and a 12:12 h photoperiod (06:00 – 18:00 EST). This study was conducted in accordance with a protocol approved by the approved by the EPA-RTP Institutional Animal Care and Use Committee (IACUC) and the facility is accredited by the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

### *Fetal study (GD 14–18 maternal exposure)*

The fetal study was comprised of two blocks with 15 dams per block. Dams were weight-ranked and randomly assigned to treatment groups using PROC OPTEX in SAS 9.4 to produce four treatment groups with similar mean and variances in initial body weight (n = 7–8 dams per treatment group). In each block, dams were exposed to DBP at 250 mg/kg/d, DINP at 750 mg/kg/d or a mixture of 250 mg DBP/kg/d plus 750 mg DINP/kg/d from GD 14–18. Dosing was administered once daily via oral gavage at 2.5 mL/kg-body weight between 07:00 – 09:00 EST.

On GD18 dams and fetuses were euthanized by decapitation 2–4 h after the final oral dose. The euthanasia order was stratified such that the timing of necropsy was equally distributed across treatment groups. We evaluated maternal weight gain from GD14-18, reproductive output (number of fetuses, resorptions), measured testis T Prod from 3 males per litter and collected and pooled testis mRNA from the remaining males.

### *Testis gene expression in GD18 fetal rats*

GD18 fetal testis mRNA was pooled by litter for analysis of gene expression as previously described (Gray et al., 2021). GD18 fetal testes mRNA samples were evaluated using 96-well custom designed RT2 Profiler PCR Arrays from Qiagen. The arrays interrogated mRNA for 24 genes per litter (19 target genes, two housekeeping genes, a reverse transcriptase control, a DNA contamination control and a positive PCR control gene) with mRNA from four litters from different treatments groups evaluated on each array. The target genes included on these arrays were selected because our studies (Gray et al., 2021; Waterbury et al., 2024) and others (Barlow et al., 2003; Liu et al., 2005; Johnson et al., 2007) consistently demonstrate statistically significant alterations of expression levels of these genes from PE exposure and the array includes mRNA for genes primarily expressed in Sertoli cells, Leydig cells or gonocytes. The two house-keeping genes were selected from the four genes on our original custom arrays with 88 target genes because these

two were consistently unaffected by *in utero* phthalate exposure (Gray et al., 2021; Gray. 2023). PCR reactions were run using RT2 SYBR Green qPCR Master Mix (Qiagen; Hilden, Germany) on a CFX96 Touch Real-Time Detection System (Bio-Rad; Hercules, CA).

*Testis testosterone production*

Testicular testosterone production (T Prod) was determined using an *ex vivo* culture method established at EPA (Furr et al., 2014; Parks et al., 2000; Wilson et al., 2004; Gray et al., 2021). One freshly isolated testis from each of three males per litter was immersed in modified M-199 media without phenol red (Gibco Life Technologies, Product #A31224DK), supplemented with 10 % dextran-coated charcoal stripped fetal bovine serum (Hyclone Laboratories, Logan, UT) for 3 h in a humidified incubator at 37 °C on an orbital shaker, to allow testosterone to diffuse into media. Following incubation, media was removed and stored at −80 °C until measurement of T Prod in media. T Prod in media was quantified using LCMS-MS. Mobile phase components were 0.1 % formic acid in water and acetonitrile. Diluents were analyzed on an AB Sciex 6500 + QTRAP Linear Ion Trap mass spectrometer using electrospray ionization in positive ion, multiple reaction monitoring modes. Chromatographic separation was performed on a Restek Raptor Biphenyl column (2.1 mm × 50 mm, 1.8 μm). Calibrators were analyzed before sample analysis. The coefficient of correlation of the curve was ≥ 0.995 for all analytes (see calibration report). The calculated concentration for each testosterone calibration point was within 80–120 % of the actual spike amount. The calibration was verified by a second source standard prepared similar to the calibration standards at a concentration of 1.00 ng/mL. The acceptance range was 70–130 %. The actual recovery for the second source standard was 82 %.

*Data analyses*

All data analyses were conducted using GraphPad Prism (version 8.4; GraphPad, Inc.; La Jolla, CA, USA) and SAS (version 9.4; SAS Institute; Cary, NC, USA). All maternal and fetal endpoints were analyzed by analysis of variance (ANOVA) using PROC GLM in SAS, followed by pairwise comparison of individual dose levels to vehicle controls using LSMEANS ($\alpha = 0.05$). Litter means were used as the statistical unit for analysis and presentation of testosterone and gene expression for the fetal data.

Fetal testis RT-qPCR gene expression data from samples collected on GD 18 were analyzed using the comparative cycle threshold ($C_T$) method. Delta $C_T$ values were calculated using the equation $2_T^{-\Delta\Delta C}$ and normalized to the mean $C_T$ value with housekeeping genes for each litter on the array. We selected genes that did not display significant treatment effect of exposure (*Actb, B2m*) to normalize the data to delta $C_T$ values. Delta $C_T$ values were then converted to fold-induction by dividing the treated replicate delta $C_T$ by the mean delta $C_T$ of the control replicates for each gene. Because most of the genes displayed similar treatment −related reductions these were averaged to provide a simple metric of the effect of the treatments on gene expression *(Supergene=(Star + Cyp11b1 + MVD + Cyp11a1 + Hmgcr + Cyp11b2 + Inha + Hsd3b3 + Tm7sf2 + Cyp17a1 + Ebp + Lhcgr + Idi1 + Scarb1 + Hmgcs1 + Insl3 + Cyp51)*/17).

Next, we fit previously published T Prod and *Insl3* dose response data for DBP (Furr et al., 2014; Gray et al., 2021) and DINP with logistic regression models in Prism with top constrained to 100 %, the bottom constrained to 0 %, and the slopes as shared value for both data sets to estimate DBP and DINP potency (i.e., $ED_{50}$s). Then a relative potency factor (RPF) for DINP was calculated using the T Prod results as $RPF_{DINP}$ = DBP $ED_{50}$ /DINP $ED_{50}$. For RPF analysis, the $RPF_{DINP}$ for each effect was used to convert DINP maternal dose levels to DBP equivalent doses ($DBP_{EQ}$) by multiplying each DINP dose in mg/kg/d * $RPF_{DINP}$, and the $DBP_{EQ}$ for the DBP + DINP mixture was calculated (Mixture $DBP_{EQs}$ = 250 mg + $RPF_{DINP}$ × 750 mg). The individual DBP, DINP, and DBP + DINP mixture data were plotted as $DBP_{EQ}$ to evaluate dose additivity similar to the approach described by (EPA 2003; Ritz et al., 2006).

*Development of prediction model for maternal weight gain*

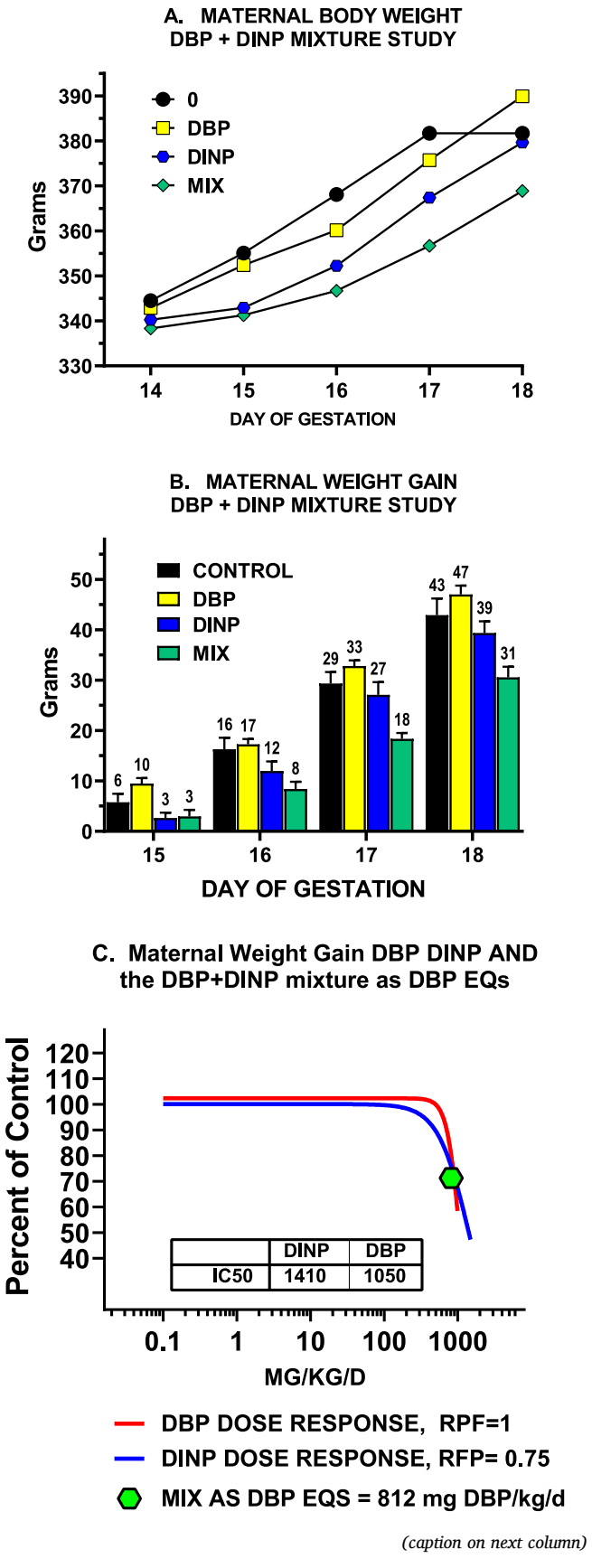
We did not use the fetal endocrine data to calculate an RPF for the effects of DBP or DINP on maternal weight gain. Furthermore, since neither DINP nor DBP treatments significantly affected maternal weight gain we could not calculate a $RPF_{DINP}$ relative to DBP (the reference chemical) from the data in the current study. Rather, an $RPF_{DINP}$ for reduced maternal weight gain was determined using $ED_{50s}$ for maternal weight from our published studies on DBP (Furr et al., 2014; Gray et al., 2021) and DINP (Gray 2023). Then the predicted DBP + DINP mixture maternal weight gain $DBP_{EQ}$ values were compared to the published DBP maternal weight gain dose response curve to determine if the treatment effects were accurately predicted and if the DBP + DINP mixture acted in a dose additive manner. (EPA 2003; Ritz et al., 2006).

*Development of T Prod and Insl3 versus phthalate syndrome prediction models*

Finally, we used 5 parameter logistic regression (5PL) models that describe the relationships between T Prod and *Insl3* and the reproductive abnormalities to predict the postnatal effects of DBP, DINP and the DBP + DINP mixture on male rat AGD, areolae/nipple retention, hypospadias, epididymal and testicular malformations including agenesis, and fluid-filled flacid, atrophic, and necrotic testes and testicular histopathology. Testicular histopathological scores included here were described as mild (score 2) to severe (score 5), but not including minimal (score 1) alterations). The most common histopathological lesion of the testis in phthalate treated adult F1 male rats was seminiferous tubular (ST) degeneration, followed by Sertoli cell vacuolation and interstitial cell hyperplasia and adenoma. In our studies tissues are evaluated by Board Certified Pathologists. The histopathology model included data from testes that were scored as mildly to severely affected having 31 (mild) to 100 % (severe) degenerate STs. Other postnatal reproductive abnormalities were not modeled because there was insufficient dose response data to develop predictive models. We also did not model the changes in the "supergene" because these values were essentially identical to the reductions in T Prod and we did not model the increases in *Testin* due to the lack of dose response data.

These models (Gray 2023) were developed by comparing T Prod and gene expression data from our studies (Furr et al., 2014; Gray et al., 2021) with the postnatal effects of oral maternal administration of multiple phthalates to pregnant rats on the male offspring data from our studies and other published studies (Fig. 2). Studies from the literature that exposed pregnant rats to phthalates during sexual differentiation (including gestational days 14 to 18 at a minimum) and that evaluated adult F1 male rat offspring were included from laboratories with history of publishing high quality quantitative data, with biologically plausible results. Several of the postnatal data sets included multiple studies of a phthalate and multiple phthalates. For endpoints like AGD, data were available from > 20 studies of eight phthalates (DBP, DEHP, BBP, DINP, DPeP, DCHP, DHP, and DiBP), whereas other endpoints like the number of areolae/nipples per infant male had data from fewer studies and only five phthalates. (The studies included in these models and the criteria used to select them are described in the Supplemental file).

We used 5PL rather than 4PL models to interpolate the effects of DBP, DINP and the DBP + DINP mixture on the F1 male rat reproductive tract because the 4PL model assumes that the dose response curve is symmetrical on either side of the $ED_{50}$ whereas the 5PL allows for asymmetry of the dose response curves. The predicted effects were determined by interpolation of the reductions in T Prod for DBP and DINP (46 %) and for the DBP + DINP mixture (76 %) seen in the current study from models of T Prod and the effects of multiple phthalates on the male rat reproductive tract. *Insl3* reductions used for interpolation were




*(caption on next column)*

**Fig. 3.** Effects of DBP, DINP, and the DBP + DINP mixture (MIX) on maternal bodyweight (panel A) and bodyweight gain (panel B, values above bars report mean weight gain in grams). Dose-additive effect of the DBP + DINP mixture on maternal weight gain during dosing on days 14 to 18 of pregnancy (panel C). The dose response data in panel C are from Gray (2023) for DINP and the DBP data are from Gray et al. (2021). The ED50s for reduced maternal weight gain for DINP and DBP were 1410 and 1050 mg/kg/d and the $RPF_{DINP}$ was 0.75. The predicted DBP + DINP mixture effect was calculated by converting the DBP + DINP mixture into DBP equivalent doses ($DBP_{EQ}$s). Mixture DBP EQS = (250 × 1 (DBP RPF) + 750 × 0.75 (DINP RPF)) = 812 mg DBP/kg. The location of the green hexagon in 3C for DINP + DBP as $DBP_{EQS}$ demonstrates that the effect of DINP and DBP acted together in a dose-additive manner. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

36 %, 29 % and 65 % for DBP, DINP and the DBP + DINP mixture, respectively.

*Validation of the predictions: comparison of the predicted effects with observed effects*

For comparison, these predictions were compared to the results from our published data from several studies combined on the postnatal effects of 500 mg DBP/kg/d since the DBP + DINP mixture was equivalent to 500 mg $DBP_{EQs}$ /kg/d (Gray et al., 2001; Hotchkiss et al., 2004; Howdeshell et al., 2007; Rider et al., 2010; Howdeshell et al., 2017).

## Results

*Maternal results*

Maternal body weight was not statistically significantly reduced by DBP, DINP or DBP + DINP treatments (Fig. 3a). However, but GD14-18 maternal weight gain (weight at GD 18 minus weight at GD 14) was statistically significantly reduced ($P < 0.002$) in the DBP + DINP mixture group (28 % reduction compared to control) (Fig. 3b). A $RPF_{DINP}$ for reduced maternal weight gain was determined using data from our published studies on DBP (Furr et al., 2014; Gray et al., 2021) and DINP (Gray 2023). The $RPF_{DINP}$ was 0.75 versus 1.0 for DBP. Maternal weight gain as percent of control was 100 %, 109 %, 92 % and 71 % in the Control, DBP, DINP and the DBP + DINP mixture groups, respectively. The magnitude of the reduction in weight gain on GD 18 was accurately predicted by dose addition using an RPF approach whereas response addition underpredicted the cumulative effect of the DBP + DINP mixture expressed as $DBP_{EQS}$ = 812.5 mg DBP/kg/d {(1x250 mg DBP)+(0.75x750 mg DINP) = 812.5}(Fig. 3c).

*Fetal results*

None of the treatments reduced litter sizes or fetal viability (data not shown).

*Testosterone production*

T prod was reduced ~ 46 % in the DBP (250 mg/kg) and DINP (750 mg/kg) groups and 76 % of the DBP + DINP mixture group as compared to the control group ($p < 0.0001$) (Fig. 4a). The DBP + DINP mixture group also was significantly reduced ($p < 0.0001$) as compared to the control, DBP and DINP groups. RPF analysis indicated that the combination of DBP + DINP acted in a dose additive manner, the combination of DBP (250 mg/kg) + DINP (750 mg/kg) being equivalent to 500 mg DBP/kg/d (Fig. 4b). The reduction in T Prod to 23 % of control by the DBP + DINP mixture was expected to reduce T prod similarly to 500 mg DBP/kg if DBP and DINP acted in a dose additive manner. Fig. 4b shows that the RPF prediction of the DBP + DINP mixture effect was consistent with the effect of 500 mg DBP/kg from our previously published results.

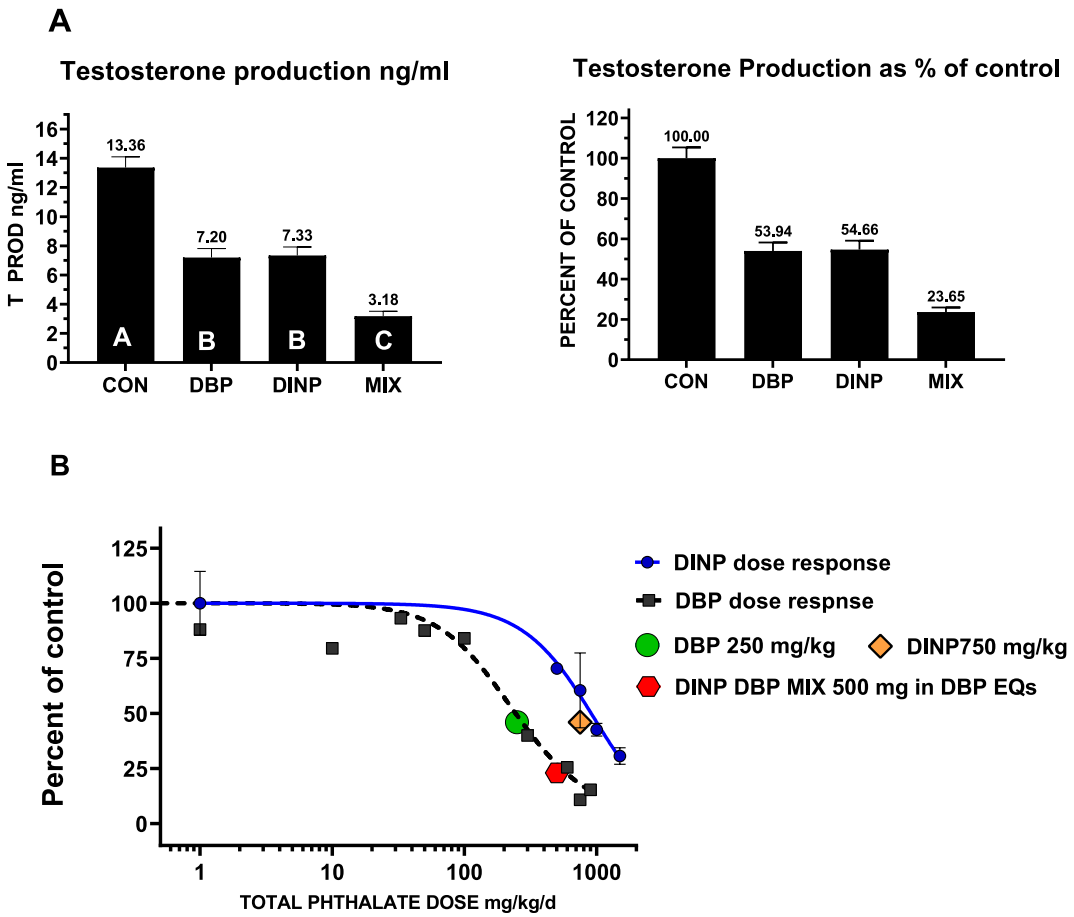



**Fig. 4.** Effects of DBP, DINP, and the DBP + DINP mixture (MIX) on testosterone production on GD18 in ng/ml/3 hr. incubation period and as percentage of control (A). Observed effects of 250 mg DBP (green circle), 750 mg DINP (orange diamond) and the mixture (red hexagon) shown in DBP equivalents on testosterone production in the current study compared to published DBP and DINP dose response data (DBP data (black squares/line) from Gray et al., 2021 and Furr et al., 2014 and DINP data from Gray et al., 2023 (blue circles/line) (B). Histograms with different letters differ significantly from one another $p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

*Gene expression*

Gene expression for 18 of the 19 target genes measured was significantly reduced by each DBP, DINP and the DBP + DINP mixture (Fig. 5). The expression of the two house-keeping genes was not affected by any of the treatments ($F(3,25\ df) > 0.65$). For all target genes, the mixture DBP + DINP group differed significantly from control. In addition, the DBP + DINP mixture group expression levels for *Cyp11a1, Cyp11b1, Cyp17a1, Cyp51, HMRC, Hmgcs1, Hsd3b3, Idi1, Inha, Insl3, MVD, Scarb1, Star, and Tm7sf2* were significantly lower than both the DBP and the DINP groups ($p < 0.0001$) (all statistical contrasts are shown in the Supplemental file). In contrast, *Testin* expression was significantly increased ($F(3, 25\ df) = 11.7$, $p < 0.0001$) by 3.57-fold with DBP ($p < 0.01$ versus control), 2.46-fold with DINP ($p < 0.11$ versus control) and six-fold in the DBP + DINP ($p < 0.01$ versus all groups) groups (Fig. 5).

When genes were clustered into the "*supergene*" (i.e., the mean of 17 similarly affected downregulated genes), the means were reduced by ~ 46 % with each PE alone and 69 % in the DBP + DINP mixture group ($F\ 3,25\ df = 50.1$, $p < 0.0001$), nearly identical to the effects on T Prod (Fig. 4a). In addition, the DBP + DINP mixture group *supergene* expression level was significantly lower than both the DBP and the DINP groups ($p < 0.0001$).

*Predicted Effects of in utero exposure to DBP, DINP and the Mixture of DBP + DINP*

The effects of DBP, DINP and the DBP + DINP mixture groups on T Prod and *Insl3* were used to predict the effects of the potential effects of these treatments on the male rat offspring reproductive tract using logistic regression models that describe the relationships between reduced T Prod and increased reproductive abnormalities in the male offspring.

The T Prod 5PL models provide the following predictions for the incidences of reproductive effects DBP, DINP and DBP + DINP mixture groups: 11 %, 11 % and 47 % with epididymal malformations; 5 %, 5 % and 14 % reduced absolute AGD; <0.1 %, <0.1 % and 4.8 % with retained female-like areolae/nipples in infant males (of 12 maximum); <0.1 %, <0.1 % and 20 % with hypospadias; and 18 %, 18 % and 65 % with histopathological alterations in the testis (Fig. 6a). Given the steep slope of several of the models, and the increasing variability around the threshold, some of these predictions have wide confidence bands (not shown). For the effects with steep slopes, there is greater certainty in the quantitative predictions at T Prod levels in the range of 30–100 % of control whereas the quantitative predictions in the $ED_{20}$ to $ED_{80}$ range of the curves (generally 10–30 % of control) are less precise because small reductions in T Prod in this range result in large increases in the percent affected.

The percent of males expected to display reduced AGD, retained areolae/nipples and reproductive tract malformation in the DBP, DINP and DBP + DINP mixture groups were accurately predicted from our

**Figure 5. Effects of DBP 250, DINP 750 and the mixture administered from gestational day 14 to 18 on fetal testis gene expression**

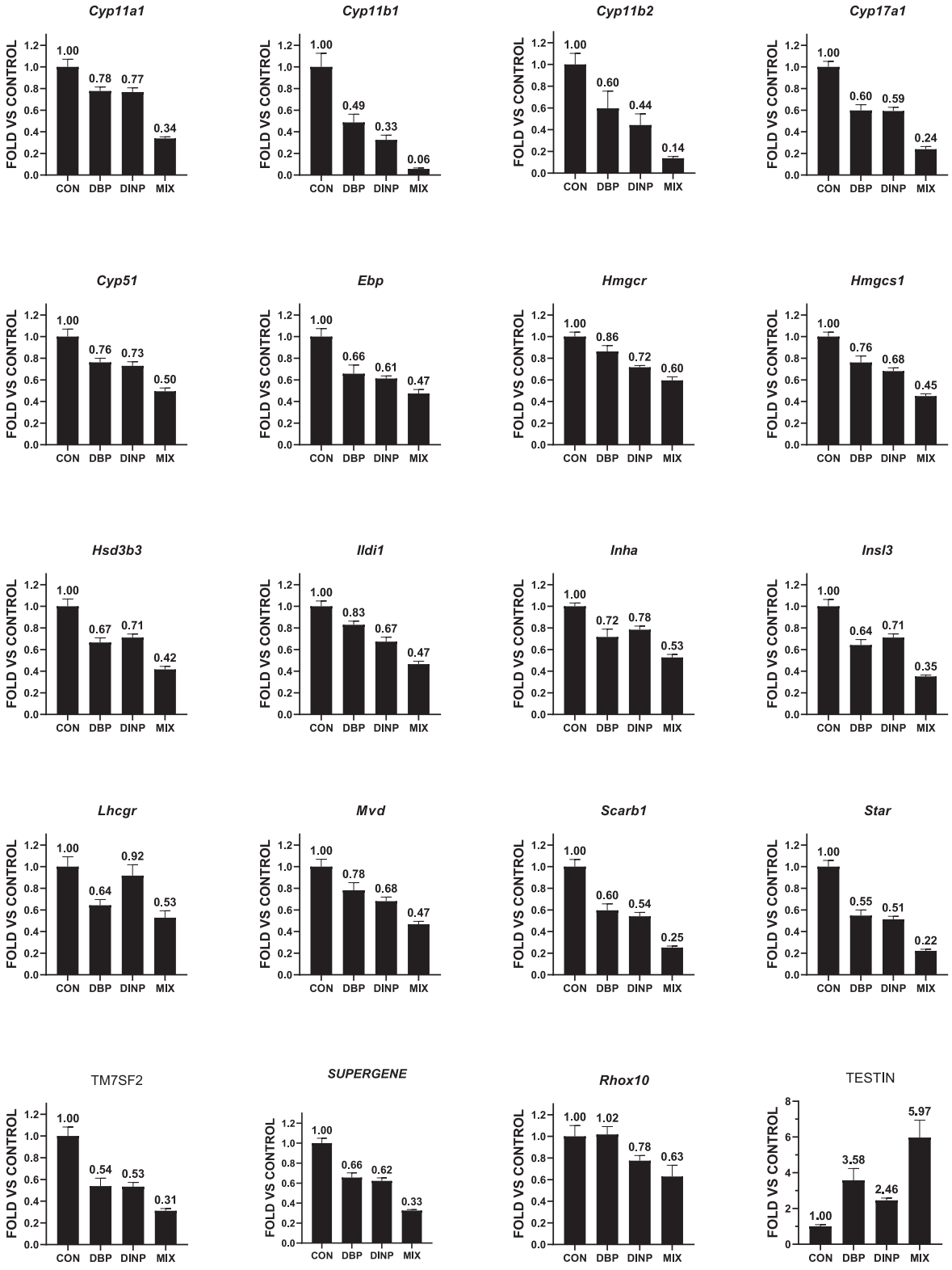



**Fig. 5.** Effects of DBP 250, DINP 750 and the mixture administered from gestational day 14 to 18 on fetal testis gene expression.

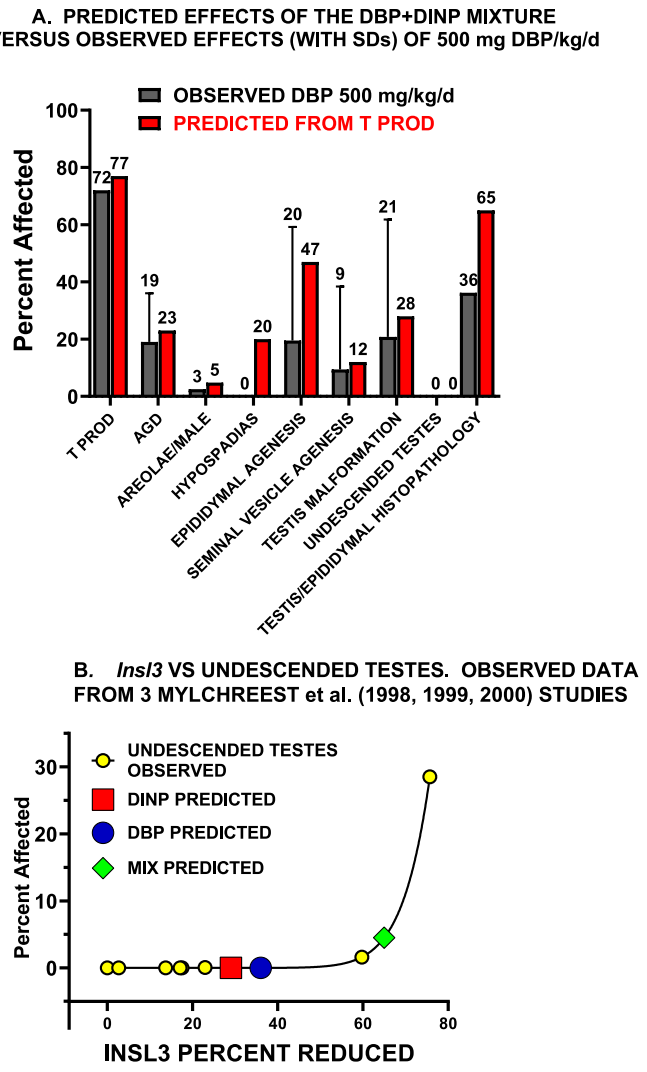



**Fig. 6.** A comparison of the observed effects of DBP at 500 mg/kg/d from one-generation studies with the predicted effects of the DBP + DINP mixture based upon T Prod (6a) and *Insl3* (6b).

published T Prod data on DBP (Gray et al., 2021) when compared to the observed percent affected from five of our studies that administered DBP at 500 mg/kg/d on GD 14–18 to pregnant rats (Fig. 6a). Furthermore, the percent of males expected to display undescended testes in the DBP, DINP and DBP + DINP mixture groups was accurately predicted from our published *Insl3* data on DBP (Gray et al., 2021) when compared to the observed percent affected from studies by Mylcheest et al. (1998, 1999, 2000) (Fig. 6b).

## Discussion

The genomic and endocrine analyses of the data from the current study clearly demonstrate that DINP interacts with DBP in a cumulative, dose-additive manner and supports the contention that it is appropriate to include this phthalate in a common mechanism group when grouping phthalates for cumulative risk assessment. A dose-additive reduction was also observed with GD14-18 maternal weight gain. The fact that the RPFs for DINP versus DBP differed for the fetal testis T Prod and maternal weight gain suggests that these outcomes reflect disturbances of different molecular initiating events and adverse outcome pathways, however, both effects displayed dose-additive responses. For T Prod, we found that the DBP + DINP mixture group was equivalent to administering 500 mg DBP/kg/d as $DBP_{EQS}$ ($RPF_{DINP} = 0.33$) whereas the effect of DBP + DINP mixture on maternal weight gain was equivalent to 812 DBP/kg/d ($RPF_{DINP} = 0.75$).

The mRNA for several genes was similarly reduced including genes related to steroid hormone synthesis and transport, peptide hormone synthesis, cholesterol synthesis and lipid metabolism, gonocyte differentiation, and seminiferous tubule development. While much of the focus on the PS is on Leydig cells and T Prod, multiple fetal testis cell types are disrupted by PE treatments and not just Leydig cells. In fact, in the last few years several publications have reported that fetal mouse Leydig cells lack 17βHSD and androstenedione is major androgen produced by the Leydig cell whereas the T Prod is dependent upon 17bHSD in the Sertoli cells (O'Shaughnessy et al., 2000; Sararols et al., 2021). In addition, it was recently shown that reduced Leydig cell *Inha*, which is reduced by PE treatments, leads to a reduction in gonocytes, multinucleated germ cells within cords, and germ cells outside of cords (Wiley et al., 2023). We included an assessment of *Testin* mRNA in our arrays because it is one of the few transcripts that have been reported to be increased by DBP (Liu et al., 2005; Plummer et al., 2007) and DCHP (Waterbury et al., 2024).

Testin is a Sertoli cell secretory glycoprotein that binds to receptors on the Sertoli cell membrane at Sertoli-germ cell (SG) junctions and levels increase with disruption of the SG junction (Grima et al., 1998). In utero PE exposure increases Testin production which is associated with disruption of the cords an induction of multinuclear germ cells in the fetal testis (Liu et al., 2005; Gaido et al., 2007). The function of these genes in the fetal testis, and other genes, and the integration of these signals is discussed in more detail in Lui et al. (2005, Fig. 6). Previously, we published logistic regression models showing that the magnitude of DPeP-induced abnormalities in the reproductive tract of male offspring were related to quantitative reductions fetal testis T Prod and *Insl3* mRNA levels (Gray et al., 2016) and subsequently used these models to predict the effects of DINP (Gray 2023). The effects modeled included androgen-dependent male reproductive endpoints like AGD, infant areolae/nipple retention, androgen-dependent reproductive organ weights and malformations, and cryptorchidism, as well as *Insl3*-dependent elongation or agenesis of the gubernaculum. In the current study, the T Prod models were expanded to include data from multiple studies of several phthalates. Where possible, the models included DPeP, the most potent PE for male reproductive effects, DINP one of the least potent PEs, as well as DBP, DEHP, DCHP, and DiBP.

These analyses predicted very low incidences of reproductive effects after exposure to the individual phthalate groups with considerably higher levels of effects in the DBP + DINP mixture group. In conclusion, these results are consistent with our hypothesis that measuring T Prod and gene expression in a 5-day *in vivo* assay can be used to replace or reduce the need to run long-term, resource and animal use intensive one-generation reproduction studies for this class of chemicals. Furthermore, the fact that the predicted DBP + DINP mixture effects are similar to the level of reproductive abnormalities seen with 500 mg DBP/kg/d in our studies (Gray et al., 2001; Hotchkiss et al., 2004; Howdeshell et al., 2007; Rider et al., 2010; Howdeshell et al., 2017) (Fig. 6a, b) enhances our confidence that this New Approach Method can be used as an alternative to life-time, resource-intensive one-generation rat reproduction tests for chemicals that disrupt sexual differentiation via this mode of action.

## Disclaimer

The research described in this article has been reviewed by U.S. Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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**L. Earl Gray:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Christy S. Lambright:** Investigation, Methodology, Writing – review & editing, Resources. **Nicola Evans:** Investigation, Writing – review & editing, Resources. **Jermaine Ford:** Investigation, Methodology, Writing – review & editing, Resources. **Justin M. Conley:** Conceptualization, Methodology, Investigation, Writing – review & editing, Visualization, Supervision, Investigation.

## Declaration of competing interest

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

## Data availability

Data will be made available on request.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2024.100180.

## References

Barlow, N.J., Phillips, S.L., Wallace, D.G., Sar, M., Gaido, K.W., Foster, P.M., 2003. Quantitative changes in gene expression in fetal rat testes following exposure to di(n-butyl) phthalate. Toxicol. Sci. 73, 431–441. https://doi.org/10.1093/toxsci/kfg087.

Canada Go, 2020. Phthalate Substance Grouping. In Canada H, ed.

Canada, H., 2015. Proposed Approach for Cumulative Risk Assessment of Certain Phthalates under the Chemicals Management Plan In Canada E, ed., pp. 1–96.

Carruthers, C.M., Foster, P.M., 2005. Critical window of male reproductive tract development in rats following gestational exposure to di-n-butyl phthalate. Birth Defects Res. B 74, 277–285.

CPSC, 2014. Report to the U.S. Consumer Product Safety Commission by the chronic hazard advisory panel on phthalates and phthalate alternatives. In Commission USCPS, ed. US Government, online, pp 1-597.

Dostal, L.A., Chapin, R.E., Stefanski, S.A., Harris, M.W., Schwetz, B.A., 1988. Testicular toxicity and reduced Sertoli cell numbers in neonatal rats by di(2-ethylhexyl) phthalate and the recovery of fertility as adults. Toxicol. Appl. Pharmacol. 95, 104–121.

EPA, 2003. Developing Relative Potency Factors for Pesticide Mixtures: Biostatistical Analyses of Joint Dose-Response. In National Center for Environmental Assessment OoRaDUSEPA, ed. EPA, Cincinnati, OH 45268.

Foster, P.M.D., 2006. Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. Int. J. Androl. 29, 140–147. https://doi.org/10.1111/j.1365-2605.2005.00563.x.

Furr, J.R., Lambright, C.S., Wilson, V.S., Foster, P.M., Gray Jr., L.E., 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. Toxicol. Sci. 140, 403–424. https://doi.org/10.1093/toxsci/kfu081.

Gaido, K.W., Hensley, J.B., Liu, D., Wallace, D.G., Borghoff, S., Johnson, K.J., Hall, S.J., Boekelheide, K., 2007. Fetal mouse phthalate exposure shows that Gonocyte multinucleation is not associated with decreased testicular testosterone. Toxicol. Sci. 97, 491–503. https://doi.org/10.1093/toxsci/kfm049.

Gray, L.E., 2023. Biologically relevant reductions in fetal testosterone and Insl3 induced by in utero exposure to high levels of di-isononyl phthalate (DINP) in male rats. Toxicol. Appl. Pharmacol. 465, 116454 https://doi.org/10.1016/j.taap.2023.116454.

Gray Jr., L.E., Ostby, J., Furr, J., Price, M., Veeramachaneni, D.N., Parks, L., 2000. Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. Toxicol. Sci. 58, 350–365.

Gray, L.E., Ostby, J., Furr, J., Wolf, C.J., Lambright, C., Parks, L., Veeramachaneni, D.N., Wilson, V., Price, M., Hotchkiss, A., Orlando, E., Guillette, L., 2001. Effects of environmental antiandrogens on reproductive development in experimental animals. Hum. Reprod. Update 7, 248–264.

Gray Jr., L.E., Furr, J., Tatum-Gibbs, K.R., Lambright, C., Sampson, H., Hannas, B.R., Wilson, V.S., Hotchkiss, A., Foster, P.M., 2016. Establishing the "Biological Relevance" of Dipentyl Phthalate Reductions in Fetal Rat Testosterone Production and Plasma and Testis Testosterone Levels. Toxicol. Sci. 149, 178–191. https://doi.org/10.1093/toxsci/kfv224.

Gray, L.E., Lambright, C.S., Conley, J.M., Evans, N., Furr, J.R., Hannas, B.R., Wilson, V.S., Sampson, H., Foster, P.M.D., 2021. Genomic and Hormonal Biomarkers of Phthalate-Induced Male Rat Reproductive Developmental Toxicity Part II: A Targeted RT-qPCR Array Approach That Defines a Unique Adverse Outcome Pathway. Toxicol. Sci. 182, 195–214. https://doi.org/10.1093/toxsci/kfab053.

Grima, J., Wong, C.C., Zhu, L.J., Zong, S.D., Cheng, C.Y., 1998. Testin secreted by Sertoli cells is associated with the cell surface, and its expression correlates with the disruption of Sertoli-germ cell junctions but not the inter-Sertoli tight junction. J. Biol. Chem. 273, 21040–21053. https://doi.org/10.1074/jbc.273.33.21040.

Hannas, B.R., Howdeshell, K.L., Furr, J., Gray Jr., L.E., 2013. In utero phthalate effects in the female rat: a model for MRKH syndrome. Toxicol. Lett. 223, 315–321. https://doi.org/10.1016/j.toxlet.2013.03.021.

Hotchkiss, A.K., Parks-Saldutti, L.G., Ostby, J.S., Lambright, C., Furr, J., Vandenbergh, J. G., Gray Jr., L.E., 2004. A mixture of the "antiandrogens" linuron and butyl benzyl phthalate alters sexual differentiation of the male rat in a cumulative fashion. Biol. Reprod. 71, 1852–1861. https://doi.org/10.1095/biolreprod.104.031674.

Howdeshell, K.L., Furr, J., Lambright, C.R., Rider, C.V., Wilson, V.S., Gray Jr., L.E., 2007. Cumulative effects of dibutyl phthalate and diethylhexyl phthalate on male rat reproductive tract development: altered fetal steroid hormones and genes. Toxicol. Sci. 99, 190–202. https://doi.org/10.1093/toxsci/kfm069.

Howdeshell, K.L., Hotchkiss, A.K., Gray Jr., L.E., 2017. Cumulative effects of antiandrogenic chemical mixtures and their relevance to human health risk assessment. Int. J. Hyg. Environ. Health 220, 179–188. https://doi.org/10.1016/j.ijheh.2016.11.007.

Johnson, K.J., Hensley, J.B., Kelso, M.D., Wallace, D.G., Gaido, K.W., 2007. Mapping gene expression changes in the fetal rat testis following acute dibutyl phthalate exposure defines a complex temporal cascade of responding cell types. Biol. Reprod. 77, 978–989. https://doi.org/10.1095/biolreprod.107.062950.

Liu, K., Lehmann, K.P., Sar, M., Young, S.S., Gaido, K.W., 2005. Gene expression profiling following in utero exposure to phthalate esters reveals new gene targets in the etiology of testicular dysgenesis. Biol. Reprod. 73, 180–192. https://doi.org/10.1095/biolreprod.104.039404.

Mylchreest, E., Wallace, D.G., Cattley, R.C., Foster, P.M., 2000. Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to Di(n-butyl) phthalate during late gestation. Toxicol. Sci. 55, 143–151. https://doi.org/10.1093/toxsci/55.1.143.

O'Shaughnessy, P.J., Baker, P.J., Heikkila, M., Vainio, S., McMahon, A.P., 2000. Localization of 17beta-hydroxysteroid dehydrogenase/17-ketosteroid reductase isoform expression in the developing mouse testis–androstenedione is the major androgen secreted by fetal/neonatal leydig cells. Endocrinology 141, 2631–2637. https://doi.org/10.1210/endo.141.7.7545.

Parks, L.G., Ostby, J.S., Lambright, C.R., Abbott, B.D., Klinefelter, G.R., Barlow, N.J., Gray Jr., L.E., 2000. The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. Toxicol. Sci. 58, 339–349.

Plummer, S., Sharpe, R.M., Hallmark, N., Mahood, I.K., 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. Toxicol. Sci. 97, 520–532. https://doi.org/10.1093/toxsci/kfm062.

Rider, C.V., Furr, J.R., Wilson, V.S., Gray Jr., L.E., 2010. Cumulative effects of in utero administration of mixtures of reproductive toxicants that disrupt common target tissues via diverse mechanisms of toxicity. Int. J. Androl. 33, 443–462. https://doi.org/10.1111/j.1365-2605.2009.01049.x.

Ritz, C., Cedergreen, N., Jensen, J.E., Streibig, J.C., 2006. Relative Potency in Nonsimilar Dose-Response Curves. Weed Sci. 54, 407–412.

Sararols, P., Stevant, I., Neirijnck, Y., Rebourcet, D., Darbey, A., Curley, M.K., Kuhne, F., Dermitzakis, E., Smith, L.B., Nef, S., 2021. Specific Transcriptomic Signatures and Dual Regulation of Steroidogenesis between Fetal and Adult Mouse Leydig Cells. Front. Cell Dev. Biol. 9, 695546 https://doi.org/10.3389/fcell.2021.695546.

Waterbury CR, Conley JM, L.E. G, Wehmas LC. 2024. Targeted RNA-sequencing of testes from fetal rats exposed to dicyclohexyl phthalate informs potency and adverse outcome pathway development. *The Toxicologist*.

Wiley, M.B., Bauer, J., Mehrotra, K., Zessner-Spitzenberg, J., Kolics, Z., Cheng, W., Castellanos, K., Nash, M.G., Gui, X., Kone, L., Maker, A.V., Qiao, G., Reddi, D., Church, D.N., Kerr, R.S., Kerr, D.J., Grippo, P.J., Jung, B., 2023. Non-Canonical Activin A Signaling Stimulates Context-Dependent and Cellular-Specific Outcomes in CRC to Promote Tumor Cell Migration and Immune Tolerance. Cancers (Basel) 15. https://doi.org/10.3390/cancers15113003.

Wilson, V.S., Lambright, C., Furr, J., Ostby, J., Wood, C., Held, G., Gray Jr., L.E., 2004. Phthalate ester-induced gubernacular lesions are associated with reduced insl3 gene expression in the fetal rat testis. Toxicol. Lett. 146, 207–215.