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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

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This study was designed to develop and validate a short-term *in vivo* protocol termed the Fetal Phthalate Screen (FPS) to detect phthalate esters (PEs) and other chemicals that disrupt fetal testosterone synthesis and testis gene expression in rats. We propose that the FPS can be used to screen chemicals that produce adverse developmental outcomes via disruption of the androgen synthesis pathway more rapidly and efficiently, and with fewer animals than a postnatal one-generation study. Pregnant rats were dosed from gestational day (GD) 14 to 18 at one dose level with one of 27 chemicals including PEs, PE alternatives, pesticides known to inhibit steroidogenesis, an estrogen and a potent PPARα agonist and *ex vivo* testis testosterone production (T Prod) was measured on GD 18. We also included some chemicals with "unknown" activity including DMEP, DHeP, DHEH, DPHCH, DAP, TOTM, tetrabromodiethyl hexyl phthalate (BrDEHP), and a relatively potent environmental estrogen BPAF. Dose-response studies also were conducted with this protocol with 11 of the above chemicals to determine their relative potencies. CD-1 mice also were exposed to varying dose levels of DPeP from GD 13 to 17 to determine if DPeP reduced T Prod in this species since there is a discrepancy among the results of *in utero* studies of PEs in mice. Compared to the known male reproductive effects of the PEs in rats the FPS correctly identified all known "positives" and "negatives" tested. Seven of eight "unknowns" tested were "negatives", they did not reduce T Prod, whereas DAP produced an "equivocal" response. Finally, a dose-response study with DPeP in CD-1 mice revealed that fetal T Prod can be inhibited by exposure to a PE *in utero* in this species, but at a higher dose level than required in rats.Key words. Phthalate Syndrome, Fetal endocrine biomarkers, Phthalate adverse outcome pathway, testosterone production, fetal rat testis.

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***Key words*: Phthalate Syndrome; Fetal endocrine biomarkers; Phthalate adverse outcome pathway; testosterone production; fetal rat testis.**

ABBREVIATIONS

BPAF	bisphenol AF; hexafluorobisphenol A
BBP	benzylbutyl phthalate
BrDEHP	di-2-ethylhexyl tetrabromo phthalate (Uniplex FRP-45)
DAP	diallyl phthalate
DBP	di(*n*-butyl) phthalate
DCHP	dicyclohexyl phthalate
DEHP	di(2-ethylhexyl) phthalate
DEP	diethyl phthalate
DHEH	1,2-cyclohexanedicarboxylic acid, bis(2-ethylhexyl) ester
DHeP	di(*n*)heptyl phthalate
DHP	di-*n*-hexyl phthalate
DiBP	diisobutyl phthalate
DIDP	di-isodecyl phthalate
DiHeP	di-isohepthyl phthalate
DINCH	1,2-cyclohexane dicarboxylic acid, di-isononyl ester
DiNP	diisononyl phthalate
DMEP	bis(2-methoxyethyl) phthalate
DMP	dimethyl phthalate
DOTP	dioctyl terephthalate
DPeP	dipentyl phthalate
DPHCH	1,2-cyclohexanedicarboxylic acid, bis(2-propylheptyl) ester
DPHP	bis(2-propylheptyl) phthalate (palatinol 10-P)
DPP	dipropyl phthalate

LIN	linuron
PZ	prochloraz
TOTM	palatinol TOTM (tri octyl trimellitate)
WY-14643	pirinixic acid (PPARα agonist)

Phthalate esters (PEs) are a family of compounds used in a wide array of products including medical tubing, toys for children and adults, pharmaceuticals, personal care products, flooring, and cables, for example. There are concerns about the potential effects of PEs on human health due to widespread indirect and direct exposures (Adibi *et al.*, 2003, 2008; Blount *et al.*, 2000; Silva *et al.*, 2004, 2011) and the adverse developmental and reproductive effects seen in laboratory animal studies (Gray *et al.*, 2000; Mylchreest *et al.*, 1998a; Saillenfait *et al.*, 2009b). Recent trends indicate that while some human PE exposures are declining, others are increasing. For example, from 2001 to 2010 exposures to diethyl phthalate (DEP), dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), and di-(2-ethylhexyl) phthalate (DEHP) have declined whereas DiBP, a reproductive toxicant in rats (Boberg *et al.*, 2008; Hannas *et al.*, 2011b; Saillenfait *et al.*, 2008b), has increased by 260% (Zota *et al.*, 2014). One would hope that as new PEs or alternatives replace older PEs in consumer products that well-studied, relatively nontoxic PEs are not replaced by ones that are less well studied, and more toxic.

At present, several regulatory bodies including the Consumer Product Safety Commission (CPSC) and U.S. Environmental Protection Agency, Office of Research and Development, National Center for Environmental Assessment, and Office of Chemical Safety and Pollution Prevention are developing risk assessments for individual phthalates and mixtures of phthalates. The CPSC Modernization Act of 2008 (Public Law 110–314) specifies that several phthalates, their alternatives, and mixtures of phthalates be regulated and/or evaluated "for endocrine disrupting effects." The law bans the manufacture for sale, offer for sale, distribution in commerce, or import into the United States any children's toy or child care article that contains concentrations of >0.1% of DEHP, DBP, or BBP and the law contains a similar interim prohibition on diisononyl phthalate (DINP), diisodecyl phthalate (DIDP), and di-n-octyl phthalate (DnOP). CPSC was also required to establish a Chronic Hazard Advisory Panel (CHAP) to study the effects on children's health of all phthalates and phthalate alternatives as used in children's toys and child care articles. The CHAP was established to conduct a complete examination of the full range of phthalates that are used in products for children and to (1) examine all of the potential health effects (including endocrine disrupting effects) of the full range of phthalates; (2) consider the potential health effects of each of these phthalates both in isolation and in combination with other phthalates; (3) examine the likely levels of children's, pregnant women's, and others' exposure to phthalates, based on a reasonable estimation of normal and foreseeable use and abuse of such products; (4) consider the cumulative effect of total exposure to phthalates, both from children's products and from other sources, such as personal care products; (5) review all relevant data, including the most recent, best-available, peer-reviewed, scientific studies of these phthalates and phthalate alternatives that employ objective data collection practices or employ other objective methods; (6) consider the health effects of phthalates not only from ingestion but also as a result of dermal, hand-to-mouth, or other exposure; (7) consider the level at which there is a reasonable certainty of no harm to children, pregnant women, or other susceptible individuals and their offspring, considering the best available science, and using sufficient safety factors to account for uncertainties regarding exposure and susceptibility of children, pregnant women, and other potentially susceptible individuals; and (8) consider possible similar health effects of phthalate alternatives used in children's toys and child care articles.

In addition, the USEPA has developed a Chemical Action Plan that includes eight phthalates: DBP, diisobutyl phthalate (DIBP), BBP, di-n-pentyl phthalate (DnPeP), DEHP, DnOP, DINP, and DIDP. EPA intends to initiate rulemaking to add these eight phthalates to the Concern List under TSCA section 5(b) (4) as chemicals that present or may present an unreasonable risk of injury to health or the environment and to add the six phthalates not already on the Toxics Release Inventory (TRI). In addition, EPA plans to consider the results of the cumulative assessment currently being developed and that was due to be completed by CPSC in 2012 pursuant to the Consumer Product Safety Improvement Act of 2008 (CPSIA), as well as the ongoing review of phthalates at FDA and the assessment for USEPA's IRIS program. When complete, these assessments would inform EPA's decision on future action to address these chemicals. EPA's potential control measures may include a ban of all or several of these chemicals, as appropriate. In 2012, EPA announced that it was proposing to regulate DPeP by issuing a significant new use rule (SNUR) under the Toxic Substances Control Act (Fed. Reg. Vol. 77, No. 66, p. 18752). This action is still pending. A SNUR requires persons who intend to manufacture, import, or process this chemical to notify EPA at least 90 days before commencing that activity.

Historically, long-term, resource-intensive multigenerational studies are required to identify the phthalates that disrupt the endocrine system and induce male reproductive tract malformations since there currently are no accepted *in vitro* or short term *in vivo* assays for this purpose. While animal studies have shown that *in utero* treatment with PEs such as diethylhexyl- (DEHP) (Blystone *et al.*, 2010; Gray *et al.*, 2000), benzyl butyl- (BBP) (Gray *et al.*, 2000; Nagao *et al.*, 2000; Tyl *et al.*, 2004), dibutyl- (DBP) (Mylchreest *et al.*, 1998b; Mylchreest and Foster 2000; Mylchreest *et al.*, 1999), diisobutyl- (DiBP) (Saillenfait *et al.*, 2006, 2008b), dicyclohexyl- (DCHP) (Saillenfait *et al.*, 2009a), dipentyl- (DPeP) (Hannas *et al.*, 2011a), dihexyl- (DHP) (Hannas *et al.*, 2011b; Saillenfait *et al.*, 2009a,b), diisoheptyl- (DiHeP) (Hannas *et al.*, 2011b; McKee *et al.*, 2006), and diisononyl- (DINP) (Borch *et al.*, 2004; Gray *et al.*, 2000) ph-

thalate during the critical period of sexual differentiation cause male reproductive malformations known as the Phthalate Syndrome, many phthalates and alternatives have not been studied using such protocols. In contrast to androgen receptor antagonists like vinclozolin (Kelce *et al.*, 1994), procymidone (Hosokawa *et al.*, 1993), prochloraz (Noriega *et al.*, 2005), and flutamide (McIntyre *et al.*, 2001; Miyata *et al.*, 2002; Wong *et al.*, 1995; Yamasaki *et al.*, 2005) which also cause male reproductive tract malformations, the PEs do not bind the androgen receptor, but instead disrupt Leydig cell maturation and gene expression in the fetal rat testis resulting in decreased Leydig cell androgen and insulin-like-3 (insl3) hormone production. The reduction in these hormones during sexual differentiation is causally linked to phthalate-induced malformations of several reproductive tissues in the male offspring (Hannas *et al.*, 2011a; Howdeshell *et al.*, 2008a; Mylchreest *et al.*, 2002; Parks *et al.*, 2000).

The goal of this study was to develop and validate a relatively rapid, medium-throughput *in vivo* screen that detects disruption of fetal testosterone synthesis and uses a minimum number of animals to identify PEs with potential to induce the Phthalate Syndrome (Foster 2006; Skakkebaek 2002). We propose that the FPS can be used to rapidly and efficiently screen phthalates to identify those with the potential to produce adverse developmental outcomes in male offspring by disrupting testosterone synthesis.

In the current study, pregnant Harlan or Charles River (CR) Sprague Dawley (SD) rats were treated by oral gavage with a single, relatively high dose of the chemical from gestational day (GD) 14 to 18, the critical period for sexual differentiation of the reproductive tract, and necropsied on GD 18. On GD 18, testis testosterone production (T Prod) was measured *ex vivo* from three males per litter from three litters per chemical and the remaining testes pooled by litter and used to measure mRNA levels by quantitative RT-PCR (qRT-PRC). These sample sizes were based upon power calculations from earlier work and were found to be adequate to detect reductions in T Prod greater than 50% of control (Hannas *et al.*, 2011a,b; Howdeshell *et al.*, 2008a,b). However, these sample sizes are not adequate to provide the statistical power needed to consistently detect anything but rather large alterations of maternal weight gain and fetal viability or the effects of chemicals that only reduce T Prod by 20–25%.

In addition to measuring fetal testis testosterone production *ex vivo*, we also measured mRNA expression levels in the fetal rat testis (Lambright *et al.*, manuscript in preparation) using targeted, custom-designed qRT-PRC 96 gene arrays (arrays described in detail by (Hannas *et al.*, 2012)) to detect phthalate-induced alterations in gene expression.

Among the PEs expected to be "positive" in the FPS, the "weak" PE used herein was DINP, and the most potent was DPeP. Other known or suspected positives were DBP, DiBP, BBP, DBP, DHP, DEHP, DiHP, DCHP, and pesticides that produce small but significant reductions in T Prod; linuron and prochloraz. The known or suspected negatives studied included dimethyl- (DMP), diethyl- (DEP), dipropyl- (DPP), dioctyl-ter- (DOTP), dipropyl heptyl (DPHP) phthalate, and diisononyl cyclohexane-1,2-dicarboxylate (DINCH) a phthalate alternative. We also included some chemicals with "unknown" activity including diheptyl- (DHeP), dimethoxy ethyl- (DMEP), tetrabromo-diethyl hexyl- (BrDEHP) phthalate, diallyl- (DAP) phthalate, 1,2-cyclohexanedicarboxylic acid, bis(2-ethylhexyl) ester (DHEH) which is a plasticizer similar in structure to DEHP but has a completely hydrogenated ring, 1,2-cyclohexanedicarboxylic acid, bis(2-propylheptyl) ester (DPHCH) which is a plasticizer similar in structure to DPHP but has a completely hydrogenated ring, Palatinol TOTM (tri octyl trimellitate), and hexafluorobisphenol A (bisphenol AF; BPAF) (Bermudez *et al.*, 2010), an environmental estrogen in that is relatively potent *in vivo* with oral administration (Table 1).

As a follow-up to these single-dose screening studies, we also have conducted dose-response studies using the FPS protocol to determine the relative potencies of the chemicals for reducing T Prod and alter gene expression (using the qRT-PCR arrays) and the results of these studies are being used to design mixture studies to determine if the chemicals in the mixture behave in a dose-additive manner. Some of the data from the dose-response studies with DPeP, DiBP, DHP, DHeP, DINP, DIDP, and the PPARα agonist Wyeth 14643 were recently published (Hannas *et al.*, 2011b, 2012) and new dose response data from the FPS are presented herein.

We also examined the effects of DPeP, one of the more potent phthalates, in the CD-1 mouse since there is considerable uncertainty in the literature about the effects of phthalates on testosterone production *in utero* in this rodent species

MATERIALS AND METHODS

Animals—Rats

This project was conducted over about 2–3 years in 66 blocks. Each block consisted of about 15 pregnant rats that were typically divided into four to five different treatment groups with three to four dams per group. Block numbers that are not discussed were used for other projects and have been or will be published separately.

For the first 43 blocks, adult female Harlan SD rats (Harlan Laboratories, Inc., Indianapolis, IN) were mated by the supplier and shipped on GD 1 (Table 2). Mating was confirmed by sperm presence in vaginal smears by the supplier (day of sperm plug positive = GD 0). Following block 43, several blocks were conducted with Charles River SD rats (1) to compare effects in the CR SD rat with those seen with the Harlan SD rat and (2) to provide data on the fetal effects of *in utero* exposure to phthalates and mixtures in the CR SD rat to compare to the treatment-induced reductions in fetal T Prod. The reason for using the CR SD rat for postnatal studies as opposed to the Harlan SD rat is

TABLE 1

Observed and Expected Effects of the 27 Different Chemical Treatments on Fetal Testis Testosterone Production on Gestational Day 18. Chemicals Were Administered to the Dam on Gestational Days 14 to 18. The "Expected Outcome" Was Based Upon the Ability of the Chemical to Induce Some Component of the Phthalate Syndrome in F1 Male Rats After *In Utero* Exposure During Sexual Differentiation, Reduce Fetal or Neonatal AGD in the Absence of an Effect of Body Weight or Induce Reproductive Toxicity in a Transgenerational or Multigenerational Study

Treatment	CAS #	Expected Outcome	Observed Outcome	Reference to a positive or negative
Corn Oil** (Vehicle)	CAS 8001-30-7	Control	-	-
Diethyl Phthalate (DEP)	CAS 84-66-2	Negative	Negative	Gray *et al*. 2000
Dimethyl Phthalate (DMP)	CAS 131-11-3	Negative	Negative	Gray *et al*. 2000
Dipropyl Phthalate (DPP)	CAS 131-16-8	Negative	Negative	Saillenfait *et al*. 2010
Dioctyl Terephthalate (DOTP)	CAS 6422-86-2	Negative	Negative	Gray *et al*. 2000
Dipropyl heptyl phthalate (DPHP)	CAS 53306-54-0	Negative	Negative	Robust summary from BASF
DINCH (1,2-cyclohexane dicarboxylic acid, di-isononyl ester)	CAS 474919-59-0	Negative	Negative	The EFSA Journal (2006)
Di-isodecyl phthalate (DIDP)	CAS 26761-40-0	Negative	Negative	EU RISK ASSESSMENT
Diisononyl Phthalate (DiNP)	CAS 28553-12-0 CAS 68515-48-0	Weak Positive Weak Positive	Weak Positive Weak Positive	Borch *et al*. 2004; Gray *et al*. 2000; Kwack *et al*. 2009
Linuron (LIN; herbicide)	CAS 335-55-2	Weak Positive	Equivocal	Gray *et al* 2000; Mcintyre *et al* 2000; Lambright *et al* 2000
Prochloraz (PZ; fungicide)	CAS 67747-09-5	Weak Positive	Very weak Positive	Noriega *et al* 2005; Blystone *et al*. 2007
Di(n)heptyl Phthalate (DHeP)	CAS 3648-21-3	Weak Positive	Positive	Sallienfait *et al*. 2011
Diisobutyl Phthalate (DiBP)	CAS 84-69-5	Positive	Positive	Saillenfait *et al*. 2006;
Benzylbutyl Phthalate (BBP)	CAS 85-68-7	Positive	Positive	Tyl *et al*. 2004; Nagao *et al*. 2000
Di(n-butyl) Phthalate (DBP)	CAS 84-74-2	Positive	Positive	Mylchreest *et al*. 2008
Dipentyl Phthalate (DPeP)	CAS 131-18-0	Positive	Positive	Hannas *et al*. 2010?
Di-n-hexyl Phthalate (DHP)	CAS 84-75-3	Positive	Positive	Sallienfait *et al*. 2013; 2009
Diethylhexyl phthalate (DEHP)	CAS 117-81-7	Positive	Positive	Gray *et al*. 2009; Blystone *et al*. 2010
Diisoheptyl Phthalate (DiHeP)	CAS 71888-89-6	Positive	Positive	McKee *et al*. 2006
Dicyclohexyl Phthalate (DCHP)	CAS 84-61-7	Positive	Positive	Saillenfait *et al*. 2009
Di-2-ethylhexyl tetrabromo phthalate (BrDEHP; Uniplex FRP-45)	CAS 26040-51-7	Unknown	Negative	
1,2-Cyclohexanedicarboxylic acid, bis(2-ethylhexyl) ester(DHEH)	CAS 84-71-9	Unknown	Negative	
1,2-Cyclohexanedicarboxylic acid, bis(2-propylheptyl) ester (DPHCH)	CAS 228853-15-4	Unknown	Negative	
Dimethoxyethyl phthalate (DMEP)	CAS 117-82-8	Unknown	Negative	
Diallyl Phthalate (DAP)	CAS 131-17-9	Unknown	Equivocal	Saillenfait *et al*. 2008
WY-14643 ** *(PPARα agonist)*	CAS 50892-23-4	Unknown	Negative	
TOTM - (Palatinol TOTM (Tri octyl Trimellitate)	CAS 3319-31-1	Unknown	Negative	
BPAF (Hexafluorobispenol A)	CAS 1478-61-1	Unknown	Negative	Potent estrogen; Bermudez *et al*. 2010; Yamasaki *et al*. 2003

in our hands the CR SD F1 litters are more robust after birth and during lactation than are Harlan SD F1 litters.

Dams were housed individually in clear polycarbonate cages (20 cm × 25 cm × 47 cm) with laboratory grade heat-treated pine shavings (Northeastern Products, Warrensburg, NY) as bedding. Pregnant dams were fed NIH07 Rat Chow and filtered (5 μm filter) municipal drinking water (Durham, NC) *ad libitum*.

Pregnant rats were maintained on a 12:12 h photo period (light/dark cycle, lights off at 7:00 P.M.) and 20–22° C temperature with a 45–55% relative humidity. Water was tested monthly for Pseudomonas and every 4 months for a suite of chemicals, including pesticides and heavy metals. The current study was conducted under protocols approved by the National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee and the Association for Assessment and Accreditation of Laboratory Animal Care.

Dosing and Administration of Chemicals: FPS Protocol for Single Dose Studies with 27 Chemicals in the Rat

One of our objectives was to determine whether 750 mg/kg/day was sufficiently high to detect the endocrine activity of the weaker PEs like DINP, without inducing maternal or fetal toxicity with one of the most potent PEs and DPeP. We also wanted to determine if examining testosterone production from three males per litter from three to four litters per treatment group had sufficient statistical power to discriminate known "positives" from known "negatives"; the known "positives" versus "negatives" being determined by the ability of the PE to induce some aspect of the Phthalate Syndrome in male rat offspring or reduce fetal or neonatal anogenital distance in males without reducing body weight, regardless of the dosage level. PEs that induce testicular effects in pubertal male rats (Creasy *et al.*, 1983; Foster *et al.*, 1981, 1982, 1983; Gray *et al.*, 1988, 1999; Gray and Butterworth 1980; Lake *et al.*, 1984; Mangham *et al.*, 1981; Noriega *et al.*, 2005) were also "expected" to be positive in the FPS and reduce T Prod. This "expectation" was based upon the observation that the PEs that disrupt pubertal male testis function like DBP, DEHP, and DPeP also induce reproductive tract malformations *in utero* whereas PEs like DEP, DPP, and DMP do not induce reproductive effects at either life stage.

In addition, several PEs or PE alternatives with unknown activity were included in the study (Table 1). Effects on T Prod that were reduced significantly from the concurrent control by $p \leq 0.01$ were considered to be "positive" responses, significant effects less than $p \leq 0.05$ but greater than $p > 0.01$ were considered "equivocal" and often repeated, and effects that did not differ from control by $p \leq 0.05$ were considered to be "negatives". Chemicals that reduced T Prod greater than 50% of control, but at relatively high dosage levels were termed as "weak positives." Chemicals that reduced T Prod significantly ($p < 0.01$) were termed "very weak positives" if the reduction in T Prod did not attain a 50% reduction of control because of the chemical's maternal or fetal toxicity. This threshold was selected in order to keep sample sizes small in the "screening" protocol. In addition, the literature indicated that this threshold was attained with phthalates (Hannas *et al.*, 2011a,b, 2012) like DBP (Struve *et al.*, 2009) and DEHP (Parks *et al.*, 2000) without inducing overt maternal or fetal toxicity. In addition, we are currently conducting postnatal studies to determine how much of a reduction in T Prod is necessary to produce permanent alternations later in life.

Pregnant rat dams were randomly assigned to treatment groups on GD 14 in a manner that provided each group with similar means and variances in body weight. Dams were weighed and dosed daily by oral gavage at ~07:30 h. from GD 14 to GD 18 with 0 (vehicle control; laboratory-grade corn oil [CAS no. 8001-30-7] at 2.5 ml/kg) or with the different phthalates at 750 mg/kg/day (unless otherwise noted). Approximately 2 h after dosing on GD 18, dams were euthanized by decapitation and exsanguination, and the fetuses immediately removed and euthanized by decapitation. All fetal necropsies were conducted within a 2-h period to ensure that a similar developmental period was sampled. Generally, a block contained about 15 pregnant rats with 3–4 dams per treatment group. DMEP and DPeP also were administered at 325 mg/kg/day because the fetuses of dams treated with 750 mg DMEP /kg/day displayed anasarca and small testes (although the fetuses were viable and there was no maternal toxicity) and 750 mg DPeP/kg/day induced a high rate of fetal loss. DAP (top dose 200 mg/kg/d), and prochloraz (150 mg/kg/day) were not administered at 750 mg/kg/day because this dosage level would be toxic to the dam and/or fetus (Gray *et al.*, 1999; Noriega *et al.*, 2004; Saillenfait *et al.*, 2008a) and the dose was selected from the literature.

Dosing and Administration of Chemicals: FPS Dose-Response Studies with 11 Chemicals in Rats

FPS dose-response studies were conducted using seven chemicals that we have not reported on previously including DBP (0, 1, 10, 33, 50, 100, and 300 mg/kg/day in the Harlan and CR SD rat), DAP (0, 50, 100, or 200 mg/kg/day in the Harlan SD rat), DCHP (0, 33, 100, 300, 600, or 900 mg/kg/day in the Harlan SD rat), BBP (0, 11, 33, 100, 300, 600, or 900 mg/kg/day in the Harlan SD rat), prochloraz (0, 37.5, 75, or 150 mg/kg/day in the Harlan SD rat), BPAF (0, 200, 300, 400, or 500 mg/kg/day in the CR SD rat), and TOTM (0, 250, 500, and 1000 mg/kg/day in the CR SD rat). In addition, we have new DEHP (0, 100, 300, 600, and 900 mg/kg/day in the Harlan SD rat), DHP (0, 11, 100, and 300 mg/kg/day), DiBP (0, 100, 200, 300, 500, 600, 750, and 900 mg/kg/day) and DPeP data (0, 11, 33, 100, and 300 mg/kg/day in both Harlan and CR SD rats) data that was combined with previously published data and reanalyzed (Hannas *et al.*, 2011a,b, 2012). The sample sizes for these studies are shown in Supplemental File 2.

TABLE 2

Treatment Effects On Fetal Testosterone Production (T Prod) and Survival and Maternal Weight Gain. Means, Standard Errors of the Means and the Numbers of Litters Used to Measure Testosterone Production (TPROD). Also Shown are the Effects of the Chemical Treatments on Maternal Weight Gain During Dosing and Fetal Viability at GD18, and the F and *p* Values for the Effects of the Treatments Within Each Block on T Prod. Shaded Values Were Significant From Control by At Least $p < 0.05$ by a Post Hoc *t*-test Following a Significant *F* Value from the ANOVA for the Entire Block. Values Shaded in Gray Were Significant ($p < 0.05$) from the Control Value by a Post Hoc *t*-test But the Overall ANOVA Was Not Significant

Table 2		TPROD ng/testis			Fetal Viability %			Dam weight gain g		F for T Prod
BLOCK	CHEMICAL	Mean	SE	N	Mean	SE	N	Mean	SE	
1	Control	5.40	0.39	5	98.3	1.7	5	45	4.4	34.8
	DBP	0.58**	0.15	3	100	0	3	36.6	2.3	p<0.0001
	DINP EXXON	4.11	0.44	3	97.8	2.2	3	41.3	2.8	
	DHeP EPA	1.48**	0.43	3	91.7	8.3	3	51.2	8.2	
2	Control	5.67	0.32	5	100	0	5	57.8	5.6	86.1
	BBP	0.61**	0.02	2	100	0	2	40.8	9.3	p<0.0001
	DEP	6.08	0.12	2	92.3	7.7	2	50.4	3.2	
	DiBP	1.06**	0.12	3	100	0	3	58.5	5.5	
3	Control	4.99	0.22	5	100	0	5	46	2	135.5
	Br-DEHP	5.01	0.33	3	100	0	3	54.3	6.1	p<0.0001
	DPeP325	0.63**	0.04	3	97.8	2.2	3	50.6	0.5	
	DPeP750	0.62**	0.03	3	49.4**	5.3	3	10.2**	3.1	
4	Control	5.31	0.34	5	90.9	9.1	5	44.8	2.7	19.8
	DINCH	4.98	0.49	3	97.6	2.4	3	51.9	4.6	p<0.0002
	DOTP	5.17	0.70	3	100	0	3	55.1	4.9	
	DiHeP	0.90**	0.33	3	97.4	2.6	3	42	2.2	
5	Control	5.24	0.45	5	98.5	1.5	5	44.5	2.7	38.3
	DEP	5.89	0.26	3	97.6	2.4	3	48.1	1.8	p<0.0001
	DINP EXXON	3.23**	0.34	3	100	0	3	47.1	1.7	
	DHP	0.44**	0.04	3	88.2	2.6	3	36	7	
6	Control	5.42	0.15	3	100	0	3	40.5	3.7	12.6
a	DPeP 11	4.57	1.15	3	88.9	11.1	3	39.7	6.2	p<0.001
	DPeP 33	3.52*	0.29	3	100	0	3	48.2	0.4	
	DPeP 100	1.24**	0.10	3	97.4	2.6	3	38.6	7	
	DPeP 300	0.38**	0.02	2	100	0	2	35.3	0.2	
7	Control	5.37	0.31	5	98.7	1.3	5	47.4	3.6	52.9
	DCHP	1.13**	0.06	3	95.8	4.2	3	48.6	1	p<0.0001
	DINP BASF	2.66**	0.21	3	97.2	2.8	3	43.3	2.3	
	DHeP NTP	1.48**	0.32	3	100	0	3	50.2	1.7	
14	Control	7.74	0.64	3	100.0	0.0	3.0	50.7	3.0	58.9
	DEP	8.62	0.07	2	100.0	0.0	3.0	37.5	7.7	p<0.0001
	DIBP	2.34**	0.08	4	85.0	9.9	4.0	41.7	4.0	
	DEP+DIBP	2.19**	0.25	3	100.0	0.0	3.0	44.5	2.9	
17	Control	6.82	0.74	3	93.3	6.7	3.0	48.7	2.5	NS
a	DIDP 500	7.00	0.96	3	97.8	2.2	3.0	41.9	1.4	
a	DIDP 750	7.61	0.53	3	100.0	0.0	6.0	37.2	3.9	
a	DIDP 1000	6.88	0.64	3	95.1	2.5	3.0	38.6	2.0	
a	DIDP 1500	6.77	0.61	3	100.0	0.0	3.0	41.5	1.2	
18	Control	9.90	0.39	3	100.0	0.0	3.0	50.1	1.3	18.9
	DBP 33	3.13	0.78	3	100.0	3.0	3.0	41.9	6.5	p<0.0002
	DBP 50	8.47	0.89	2	78.8	21.2	3.0	33.4	10.7	
	DBP 100	6.46*	1.56	3	95.2	7.8	3.0	32.2	10.8	
	DBP 300	2.29**	0.08	3	100.0	0.0	3.0	46.1	1.7	
19	Control	5.18	0.46	3	100.0	0.0	3.0	43.2	3.1	23.8
	DIBP	1.33**	0.17	3	100.0	0.0	3.0	48.6	2.8	p<0.0001
a	WY14643 50	4.37	0.31	3	97.6	2.4	3.0	55.1	2.7	
a	WY14643 100	4.97	0.53	3	97.0	3.0	3.0	46.0	3.7	
a	WY14643 200	5.92	0.94	3	100.0	0.0	3.0	46.0	3.5	
a	WY14643 200	5.92	0.94	3	100.0	0.0	3.0	46.0	3.5	

Table 2 continued		TPROD ng/testis			Fetal Viability %			Dam weight gain g		F for T Prod
BLOCK	CHEMICAL	Mean	SE	N	Mean	SE	N	Mean	SE	
20	Control	5.90	0.39	3	100.0	0.0	3.0	52.7	2	47.8
	Prochloraz 250	2.14**	0.25	3	98.1	1.9	3.0	7.1**	1.2	p<0.0001
	DEHP	0.7**	0.04	3	94.7	2.7	3.0	8.6**	3.5	
	DMP	5.78	0.64	3	89.5	6.7	3.0	43.1*	2.4	
	DPHP	5.01	1.05	3	95.6	4.4	3.0	30.5**	3.4	
22	Control	9.41	0.12	3	100	0.0	3.0	33.5	2.7	3.42
	DBP 1	8.29	0.61	3	97.2	2.8	3.0	46.5	5.4	p<0.07
	DBP 10	7.49	0.70	4	98.1	1.9	4.0	44.6	3.3	
	DBP 100	6.02#	1.03	4	97.9	2.1	4.0	44.7	1.8	
23	Control	9.87	0.58	3	97.6	2.4	3.0	51.7	6.5	pooled
	DCHP 100	3.1**	0.40	3	97.4	5.6	3.0	48.6	4.9	with
	DCHP 300	2.2**	0.43	2	94.4	5.6	3.0	41.1	5.9	block 33
	DCHP 600	2**	0.31	3	100.0	0.0	3.0	43.4	2	for
	DCHP 900	5.39**	0.80	3	100.0	0.0	0.0	45.5	3.5	analysis
24	Control	9.58	0.42	5	98.5	1.5	5.0	62.3	10.9	172.0
	DEHP	0.84**	0.13	5	100.0	0.0	5.0	33.4#	6.3	p<0.0001
	DHEH	8.92	0.83	5	98.3	1.7	5.0	59.6	8.4	
25	Control	9.37	0.22	5	100.0	0.0	5.0	47.5	2.3	13.5
	DMEP 750	7.43**	0.25	5	100.0	0.0	5.0	49.6	10.6	p<0.002
	DPHCH	8.58	0.66	2	100.0	0.0	2.0	41.5	6.1	
26	Control	4.75	0.48	3	77.0	13.4	3.0	64.4	8.2	15.6
	DBP 1	7.60	0.26	4	100.0	0.0	4.0	48.1	3.5	p<0.0004
	DBP 10	5.64	0.29	4	97.9	2.1	4.0	51.0	3.2	
	DBP 100	3.58	0.75	3	98.2	1.9	3.0	52.0	4.1	
27	Control	8.99	0.80	4	98.2	1.8	4.0	53.8	3.8	NS
	DMEP 325	10.50	0.70	3	97.8	2.2	3.0	39.2	11.5	
	DPP	9.30	0.90	4	100.0	0.0	4.0	45.7	6.6	
28	Control	12.50	0.28	3	100.0	0.0	3.0	54.7	0.8	50.5
	DHP 11	10.70	0.54	4	94.9	3.4	4.0	64.6	8.5	p<0.0001
	DHP 100	5.53**	0.97	4	100.0	0.0	4.0	53.4	13.0	
	DHP 300	2.02**	0.02	4	100.0	0.0	4.0	58.0	3.9	
29	Control	11.55	1.30	4	98	2.00	3	48.7	12.7	3.95
	DAP 50	8.95	0.66	4	100	0.00	4	52.6	4.2	p<0.043
	DAP 100	7.97*	0.38	3	100	0.00	3	55.2	3.2	
	DAP 200	7.87*	0.38	3	100	0.00	3	32.9	11.4	
30	Control	9.21	0.57	4	97.7	2.30	4	47.2	2.8	4.78
	DAP 200	7.47	0.62	2	66.7	33.30	3	45.9	13.8	p<0.04
	DiBP 200	4.9**	1.63	2	64.1	32.10	3	49.7	13.3	
	LIN 75	6.77*	0.42	4	97.5	2.50	4	30.5	7.3	
31	Control	11.40	0.72	3	91.7	8.3	3	68.6	10.3	pooled
	DEHP 100	4.24**	0.01	2	100	0.00	2	45.6*	0.25	with
	DEHP 300	2.05**	0.37	3	84.3	12.30	3	43.8	10.4	block 32
	DEHP 600	0.81**	0.16	3	95	2.50	3	33.2*	3	for
	DEHP 900	0.70**	0.17	2	85	15	2	7.4**	3.6	analysis
32	Control	10.70	2.30	2	92.9	7.14	2	39.3	4.8	34.9
	DEHP 100	8.47**	0.62	3	100	0.00	3	53.3	2.2	p<0.0001
	DEHP 300	3.72**	0.85	3	88	7.20	3	37.1	11	
	DEHP 600	1.62**	0.16	3	95.7	2.20	3	39.4	6.40	
	DEHP 900	1.25**	0.21	2	69.6	14.1	3	15.8##	5.5	

Table 2 continued		TPROD ng/testis			Fetal Viability %			Dam weight gain g		F for T Prod
BLOCK	CHEMICAL	Mean	SE	N	Mean	SE	N	Mean	SE	T Prod
33	Control	**13.25**	1.57	**4**	96.8	3.12	4	49.4	3.50	**14.98**
	DCHP 33	9.89	1.15	4	97.2	2.80	4	50.3	3.84	**p<0.0001**
	DCHP 100	5.92**	1.66	4	97.2	2.80	3	51.6	6.6	
	DCHP 300	4.10**	0.46	3	100	0.00	3	41.2	113	
34	Control	**10.85**	**0.91**	**6**	93.6	6.40	6	45.6	1.1	**457.4**
	DBP 750	1.48**	0.23	6	100	0.00	6	37.3	1.4	**p<0.0001**
36	Control	**11.63**	0.13	3	100	0	3	54.7	7.5	**93.2**
	BBP 100	5.43**	0.58	2	97.9	2.10	3	41.5	9.8	**P<0.0001**
	BBP 300	3.81**	0.21	2	97.6	2.40	3	31.7	9.6	
	BBP 600	2.77**	0.66	3	100	0.00	3	53.2	0.9	
	BBP 900	1.73**	0.09	3	100	0	3	35.5	3.2	
37	Control	**10.94**	1.62	4	94.2	5.8	4	69.1	10.2	**0.8**
	BBP 11	12.17	0.31	3	100	0.00	3	58.5	2.4	**p>0.5**
	BBP 33	10.00	1.65	4	98.1	1.90	4	52.6	2.1	
	BBP 100	9.63	1.08	4	100	0	4	45.9	6.5	
38	Control	**6.11**	0.20	4	100	0	4	48.3	4.4	**7.3**
	Prochloraz 37.5	4.8*	0.33	4	100	0	4	43.2	8.4	**p<0.006**
	Prochloraz 62.5	4.71*	0.40	4	98.4	1.6	4	47.5	4.5	
	Prochloraz 150	3.78*	0.11	3	100	0	3	46.1	19.8	
40	Control	8.78	1.2	3	97.6	2.4	3	59.2	6.4	**10.7**
	DPeP 11	6.81	1.36	3	100	0	3	48.7	4.2	**p<0.002**
	DPeP 33	5*	1.46	2	93.8	6.3	2	51.8	10.7	
	DPeP 100	2.38**	0.03	3	97.9	2.1	3	54.2	3.7	
	DPeP 300	1.16**	0.27	3	100	0	3	29.3#	12	
42	Control	8.54	2.9	3	100	0	3	43.6	11.7	**7.8**
	DPeP 11	9.57	1.03	3	100	0	3	46.8	2.5	**p<0.004**
	DPeP 33	3.28*	0.72	3	100	0	3	45.5	3.4	
	DPeP 100	1.96**	0.20	3	97.9	2.1	3	52	2	
	DPeP 300	0.8**	0.34	3	77.3	19.5	3	41.2	3.6	
47	Control	7.36	0.89	3	97.3	2.6	3	37.5	2.9	**18.4**
	DPeP 11	6.34	0.37	3	97.6	2.4	3	41.1	2.7	**p<0.0001**
	DPeP 33	5.29*	0.61	3	46.6	2.7	3	46.6	2.7	
	DPeP 100	3.89**	0.52	3	100	0	3	44.9	2.2	
CRSD	DPeP 300	1.09**	0.18	3	100	0	3	40	6.2	
48	Control	6.85	0.76	2	100	0	3	47.9	0.45	**20.2**
	DPeP 11	6.11	0.54	3	97.4	2.6	3	32.8	1.5	**P<0.0002**
	DPeP 33	6	0.23	3	100	0	3	37.5	2.3	
	DPeP 100	3.45**	0.56	3	97.4	2.6	3	42.2	2.4	
CRSD	DPeP 300	1.87**	0.24	3	66.9*	16.6	3	27.4**	6.1	

Table 2 continued		TPROD ng/testis			Fetal Viability %			Dam weight gain g		F for T Prod
BLOCK	CHEMICAL	Mean	SE	N	Mean	SE	N	Mean	SE	
52	Control	5.37	0.12	2	100	0	2	44.2	0.45	137.1
	DPeP 11	6.18	0.17	2	63.7	31.9	3	20.2	13.1	p<0.0001
	DPeP 33	3.11**	0.21	2	100	0	2	45.5	0.65	
	DPeP 100	1.54**	0.24	3	97.2	2.8	3	42.6	3.2	
Harlan SD	DPeP 300	1.05**	0.13	3	100	0	3	43.3	6.8	
56	Control	6.82	1.87	2	100	0	2	30.5	9	2.87
	Linuron 20	6.89	0.11	3	96.7	3.3	3	25.7	0.6	p>.05
	Linuron 40	5.88	0.16	2	100	0	2	-1	6.9	p<0.10
	Linuron 60	4.27*	0.53	3	100	0	3	2.8	7.8	
CRSD	Linuron 80	5.28	0.02	3	100	0	3	-5.6	18.7	
58	Control	8.2	1.05	3	100	0	3	39.4	6	2.2
	TOTM 250	8.9	0.93	3	100	0	3	31.2	5.9	p>0.15
	T0TM 500	9.57	0.44	2	100	0	2	37.8	12.2	
	TOTM 750	11.28	0.55	3	100	0	3	40.6	2.7	
CRSD	TOTM 1000	10.52	0.52	3	100	0	3	51.5	11.1	
59	Control	5.54	0.13	2	96.7	3.3	3	40.1	3.4	2.58
	TOTM 250	6.88	0.61	3	97.9	2.1	3	36.5	2.9	p>0.10
	T0TM 500	8.05	0.37	3	100	0	3	38.8	5.7	
	TOTM 750	7.51	0.68	3	97.9	2.1	3	35.7	3	
CRSD	TOTM 1000	7.61	0.55	3	95.6	4.4	3	37.3	2.8	
66	Control	7.28	0.27	3	95.2	2.4	3	41	4.6	NS
	BPAF 200	6.85	0.5	3	100	0	3	28.7	4	1.02
	BPAF 300	5.57	0.02	3	100	0	3	29.2	2.4	p>.43
	BPAF 400	5.84	0.21	3	95.2	4.8	3	9.3*	11	
CRSD	BPAF 500	6.31	0.17	2	100	0	2	15.05	19.5	

* $p < 0.05$ for ANOVA and *post hoct*-test.
** $p < 0.01$ for ANOVA and *post hoct*-test.
# $p < 0.05$ by *post hoct*-test by not overall ANOVA.
$p < 0.05$ by overall ANOVA but not *post hoct*-test.
[a]Published by Hannas *et al.*, 2010, 2011a,b,2012.

Dosing and Administration of DPeP: FPS Protocol

Dose-Response Studies in CD-1 Mice

Since several investigators have claimed that PEs do not reduce fetal testosterone in the mouse, we conducted a dose-response study with DPeP in the CD-1 mouse to thoroughly examine this hypothesis over a wide dose range with a PE that is relatively potent in reducing T Prod in the rat. This PE and mouse strain were selected because the literature indicates that chronic dietary administration of DPeP produces adverse testicular effects and reduces fertility in CD-1 mice (Heindel *et al.*, 1989).

In the dose-response study, DPeP was administered by gavage to pregnant CD-1 mice from GD 13 to 17 at 0, 50, 100, 200, 300, 400, 500, or 600 mg/kg/day. This study was conducted in several blocks with T Prod (measured as in the rat) on GD 17 from individual testes from 3 males/litter from 24, 12, 9, 14, 18, 3, 3, and 2 litters per dose group, respectively. Fewer litters were examined in the three higher dose groups because of extensive fetal loss at these dosage levels.

Dosing and Administration of Mixtures of Phthalates Using Rats

A binary mixture of phthalates. In addition to administration of individual phthalates, we also conducted a binary mixture study with DEP and DiBP using the current protocol to determine if DEP (a negative) interacted in an antagonistic or synergistic manner with DiBP (a strong positive at the dose administered in this study). Dams were dosed with the vehicle, a high level of DEP (900 mg/kg/day), an effective dose of DiBP (500

mg/kg/day) or a combination of DEP (900 mg/kg/day) and DiBP (500 mg/kg/day).

Ex Vivo *Testicular Testosterone Production—Methods*

The method for assessing T Prod in this study is identical to that used by Wilson *et al.* (2004) which is a modification of the methods used by Parks *et al.* (2000); methods we derived from those used in studies of fetal T Prod conducted in the 1970s (Warren *et al.*, 1972) and 1980s (Habert and Picon 1986). Fetal testes were removed from fetal male rats and mice using a dissecting scope and three testes (one testis from three different fetuses) per litter were analyzed individually for *ex vivo* testosterone production as described below. Necropsies were conducted in the morning from 8:00 A.M. to 10:00 A.M, similar to previously published fetal necropsies in our laboratory. Testes were immediately transferred to a well (1 testis per well) containing 0.5 ml M199 media without phenol red for *ex vivo* testis hormone production (Wilson *et al.*, 2004) and incubated with gentle rocking for 3 h at 37°C. Following incubation, the media was stored in siliconized microcentrifuge tubes and stored at −80°C until testosterone was measured by radioimmunoassay. Testosterone levels in the incubation media were measured by radioimmunoassay (RIA) using Coat-a-Count kits according to manufacturer's protocols (Siemens Corporation, Los Angeles, CA).

The testosterone intra-assay coefficient of variation was 1.25% based on variability of the standard curve and the inter-assay coefficient of variation was 9.1%. Cross-reactivity with dihydrotestosterone was 3.2%. The limit of detection of the RIA was 0.2 ng/ml testosterone for testosterone production. Data are presented and analyzed using litter mean values.

Statistics

The data from this study were analyzed by block using a one-way analysis of variance (ANOVA) using the general linear measures procedures from the Statistical Analysis Systems (SAS, Inc., Cary, NC). T Prod data were $\log_{10}$ transformed to correct for heterogeneity of variance for statistical analyses and percentage of control values for T Prod were generated using the control values within the same block for graphical representation of the results.

For all analyses, litter means were used as the sample size and differences were considered significant at $p \leq 0.01$ (a positive response), whereas effects falling between $p \leq 0.05$ and $p > 0.01$ were considered "equivocal" effects, and responses with $p > 0.05$ were considered as "negatives." If a treatment produced an "equivocal" response, the treatment was repeated in a subsequent block and the data from the blocks were pooled and the data reanalyzed to determine if the effect was statistically significant ($p \leq 0.01$) or not. *Post hoc* treatment comparisons were made by block using the Least Squares Means procedure on SAS, which is appropriate for *a priori* hypotheses. Data from the dose-response studies were also analyzed using a logistic regression model with GraphPad Prism software, version 5.00 for Windows (GraphPad Software, San Diego CA, www.graphpad.com) to determine the ED_{50} values for each chemical.

Because DEP and DINP at 750 mg/kg/day in blocks 2 and 1, respectively and DAP at 200 mg/kg/day produced "equivocal effects" ($p > 0.01$ but $p \leq 0.05$) on some of the endpoints (T Prod or mRNA expression), each of these was repeated at this dose level and the data pooled to determine if the PE significantly ($p < 0.01$) reduced T Prod or gene expression levels. The T Prod data for DINP and DEP were analyzed as $\log_{10}$ transformed percentage of control data for each block to adjust for heterogeneity of variance and block to block differences in the absolute T levels.

RESULTS

Rat Studies

The results of the different treatments on fetal T Prod, fetal viability and maternal weight gain are shown by block in Table 2. *In utero*, maternal treatment with phthalates and other chemicals during sexual differentiation produced the expected reductions in T Prod: DBP, DiBP, BBP, DPeP, DEHP, DHP, DiHeP, DCHP, DINP, and DHeP, were positive and DEP, DMP, DPP, DoTP, DPHP, DIDP, and DINCH were negative. Prochloraz was a "weak positive" and linuron produced an "equivocal" reduction in T Prod with four litters per group ($p < 0.05$ but $p > 0.01$).

The "unknowns" BPAF, BrDEHP, DPHCH, WY 14643 (Hannas *et al.*, 2012), TOTM, and DHEH did not reduce T Prod. When blocks 29 and 30 were pooled for statistical analysis of the effects of varying doses of DAP on T Prod was considered to be an "equivocal" effect since T Prod at was reduced at 100 ($p < 0.05$) and 200 mg/kg/day ($p < 0.02$). Because the F and p values are greater than $p < 0.01$ for the effects of DAP, we consider this response to be "equivocal" and it is likely that the higher dose reduced fetal body weight (Saillenfait *et al.*, 2008a). In the current study, administration of DAP did not significantly reduce maternal weight gain or induce fetal toxicity but is possible that the higher dose reduced fetal body weight (Saillenfait *et al.*, 2008a); an endpoint we did not collect. In contrast, the effect of DMEP on T Prod at 750 mg/kg/day (block 25) was concurrent with 100% incidence of fetal anasarca, whereas treatment with 325 mg/kg/day (block 27) did not reduce T Prod or induce anasarca.

Because only 2/3 dams were pregnant in the first block that exposed pregnant rats to DEP (block 2), DEP was repeated at 750 mg/kg/day (block 5). The results of the pooled analyses indicate that DEP did not significantly reduce T Prod at 750 mg/kg/day. In addition, T Prod was not reduced by DEP administration at 900 mg/kg/day in the mixture study with DiBP (block 14).

DINP was run first in block 1 and since the effect on T Prod was equivocal (being statistically significant using un-

transformed T Production values ($p < 0.03$) but not with the $\log_{10}$ transformed data ($p > 0.25$). DINP was rerun at 750 mg/kg/day in block 5 (Fig. 1). The pooled results indicated that T Prod was significantly reduced by DINP exposure at 750 mg/kg/day.

The only treatments that significantly ($p \leq 0.0001$) reduced maternal weight gain from GD 14 to 18 were DPeP and DEHP at 750 mg/kg/day (Table 2). DPeP at 750 mg/kg/day also significantly reduced litter sizes so there only were eight viable male fetuses (3 + 3 + 2 males from three litters) for assessment of T Prod. None of the other treatments reduced litter sizes at GD 18.

In the dose-response studies, there were dose-related reductions in T Prod (no statistically significant nonmonotonic alterations of T Prod were noted) (Fig. 2). The potency of the PEs is ranked in Table 2 and the results of the logistic regression and the relative potencies are both shown in Fig. 3).

When the dose-response data for DPeP, DEHP, and DBP were analyzed by strain, rather than with the data from the Harlan and CR SD rats pooled, we found that T Prod in the Harlan SD was significantly more sensitive to disruption than in the CR SD rat for each PE (Fig. 4)

In the binary mixture study with DEP and DiBP, DiBP alone significantly reduced T Prod from 7.74 (±0.64) ng/testis in controls to 2.34 (±0.37) whereas DEP was without effect (8.62 ± 0.07). DEP did not interact with DiBP; the combination of DEP plus DiBP (2.19 ± 0.24) did not differ from the effect of DiBP alone (Table 2, block 14).

Mice

In the dose-response study, oral DPeP administration to pregnant CD-1 mice from GD 13 to 17 significantly reduced fetal testis T Prod at 100 mg/kg/day and above in a dose-related manner ($F(7, 77) = 5.9$; $p < 0.0001$). However, the ED_{50} was about four fold higher than in the rat ($ED_{50} = 193$ mg/kg/day vs. 48 mg/kg/day) and the dose-related decline in T Prod reached a plateau at about 50% of control, whereas T Prod in the rat reached a plateau at about 10–15% of control. In contrast to T Prod, DPeP induced fetal loss and reduced maternal weight gain during dosing at lower doses in the mouse than in the rat (Fig. 5).

DISCUSSION

Concordance of Fetal Phthalate Screening Results on Individual Chemicals with "Expected" Outcomes

A major objective of this study was to develop a screening protocol to identify and characterize PEs that disrupt fetal rat testis endocrine function and potential disruptions of sexual differentiation. Although there are literally thousands of publications on the reproductive toxicity of the PEs, the vast majority of these have studied two active PEs, DBP, and DEHP. Beyond these two well-studied PEs only a handful of other PEs or alternatives have been studied *in utero* for reproductive toxicity out of the several hundred extant PEs and alternatives. The data from the published studies (listed in Table 1) were used to predict expected "positives" and "negatives." Chemicals reported to produce the Phthalate Syndrome in F1 male rats after *in utero* exposure, or reduce AGD in male rats during neonatal or late fetal life without affecting body weight were expected to be "positive" in the FPS and reduce T Prod. PEs that induce testicular effects in pubertal male rats (Creasy *et al.*, 1983; Foster *et al.*, 1981, 1982, 1988; Gray *et al.*, 1988, 1999; Gray and Butterworth, 1980; Lake *et al.*, 1984; Mangham *et al.*, 1981; Noriega *et al.*, 2005) were also expected to be positive in the FPS and reduce T Prod. We also expected the pesticides prochloraz (Blystone *et al.*, 2007) and linuron (Wilson *et al.*, 2009) to reduce T Prod, as reported in the literature. However, we would not necessarily expect chemicals like vinclozolin (Kelce *et al.*, 1994) or procymidone (Hosokawa *et al.*, 1993; Ostby *et al.*, 1999) that disrupt male rat testis function and pubertal development via other adverse outcome pathways (e.g., androgen receptor antagonism) to reduce T Prod in the FPS.

In the screening protocol termed the FPS, pregnant rats and rat fetuses were exposed to one of 27 chemicals, including PEs, several PE alternatives, two pesticides and a potent PPARα agonist (Hannas *et al.*, 2011b, 2012) to determine which chemicals suppressed fetal rat testis testosterone production during the "masculinizing window" of fetal development (Carruthers and Foster 2005; Scott *et al.*, 2008; Wolf *et al.*, 2000) (Tables 1 and 2). A companion paper (in preparation) describes a highly reproducible genomic "signature" (mRNA expression levels) for the effects of these chemicals on testis gene expression using a custom-designed 96 gene qRT-PCR array containing mRNA for key genes involved in androgen synthesis, gonadal and sexual differentiation and PPAR function; a genomic signature identical that reported by Hannas *et al.* (2012) and it compares the ED_{50} values for the reductions in mRNA levels with the ED_{50} values for T Prod.

We found that "expected positive" PEs could be correctly identified with the screen and in most cases evaluating T Prod from only 3 litters per chemical (one testis each from 3 males per litter) provided an adequate sample size. However, a sample size of four litters per dose group was not sufficient to label the 26% reduction in T Prod as statistically significant at a $p < 0.01$ level by 75 mg linuron/kg/day.

We also determined that administering a PE at a dose of 750 mg/kg/day for 5 days during sexual differentiation was appropriate for most, but not all, of the PEs. This dose was high enough to detect the reduction in T Prod induced by the weaker PEs like DINP, without inducing maternal or fetal toxicity, whereas PEs like DAP, DMEP ,and DPeP had to be administered at lower dosage levels. Our use of a single, relative high dosage level to screen for reductions in T Prod is based on the assumption that few if any chemicals reduce fetal testis T Prod at a low dose level but have no effect at high dosage levels. This assumption is supported by the PE dose response data on fetal rat Prod from our laboratory and other laborato-

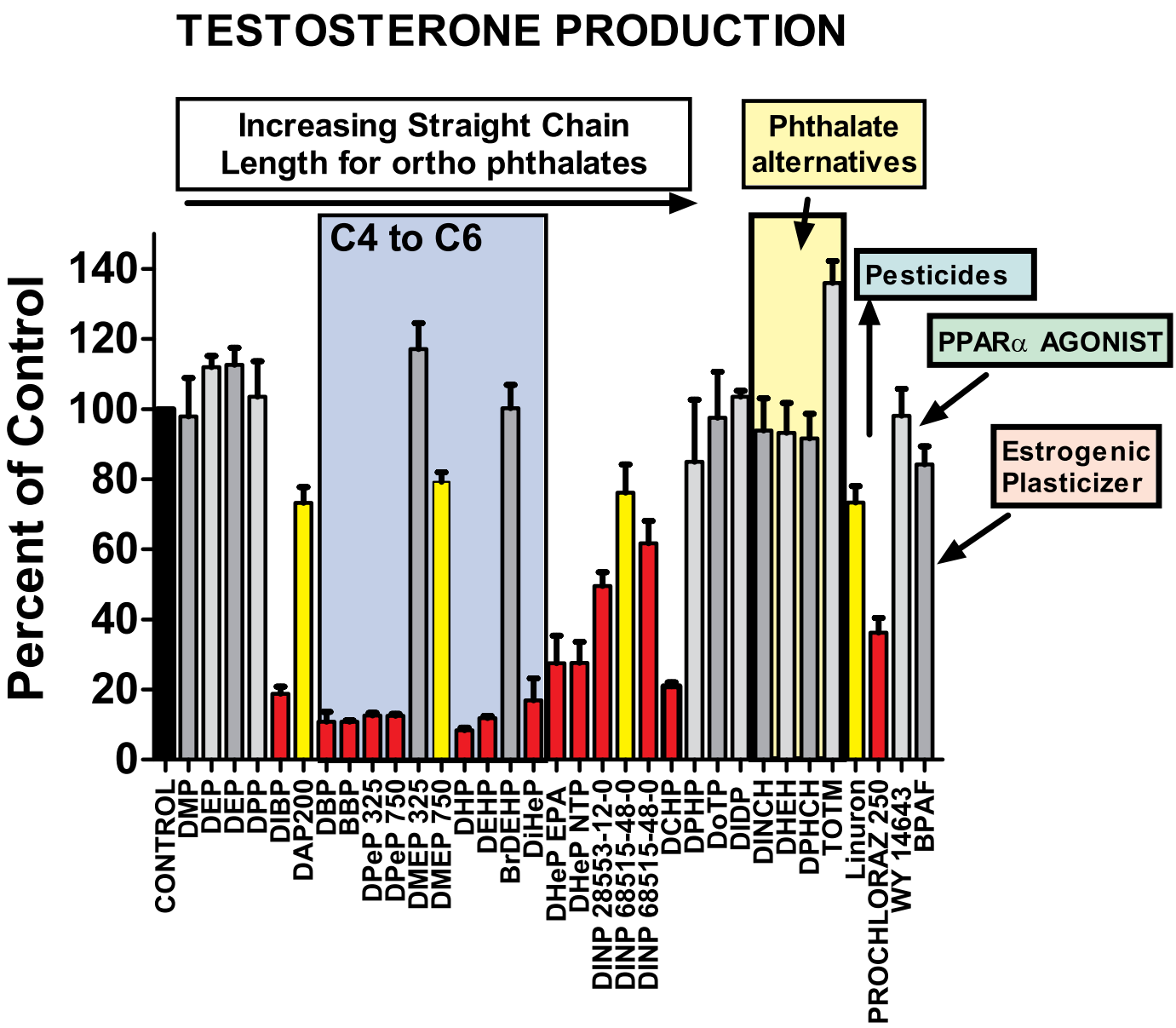


FIG. 1. Effects of the different *in utero* maternal treatments on fetal testis testosterone production, collected *ex vivo* for 3 h incubation (one testis for each of three males per litter, with 3–4 litters per dose group in most cases). Data are expressed as percentage of control from the respective block in which the PE was tested; T Prod data were $\log_{10}$ transformed to correct for heterogeneity of variance. Phthalates are listed from left to right by increasing ester straight side chain length from C2 to C9. Several phthalates which do not have straight side chains from C4 to C6 disrupt fetal testis testosterone production including DIBP, DHeP, DINP, and DCHP. Gray histograms are not significantly different from control ($p > 0.10$), yellow were equivocal ($p \leq 0.05$ to $p > 0.01$) and red differed significantly ($p \leq 0.01$) from the concurrent control value.

ries including one study that administered DBP *in utero* with a low dose of 0.1 mg/kg/day which they reported as equivalent to high dose human exposures (Lehmann *et al.*, 2004). The use of dosage levels lower than 0.1 mg/kg/day is problematic because PEs are found in rodent diets and beddings within this dose range (Kondo *et al.*, 2010).

FPS Dose-Response Studies

In addition to executing single dose level studies to identify "positives" and "negatives" we also conducted dose-response studies on 11 of the chemicals in order to determine the ED_{50} values for reduced T Prod (Fig. 3 and Table 3). Some of these data were presented previously by Hannas *et al.* (2011a,b, 2012) whereas others are presented here for the first time. We found that the ED_{50} dose of the chemicals that significantly reduced

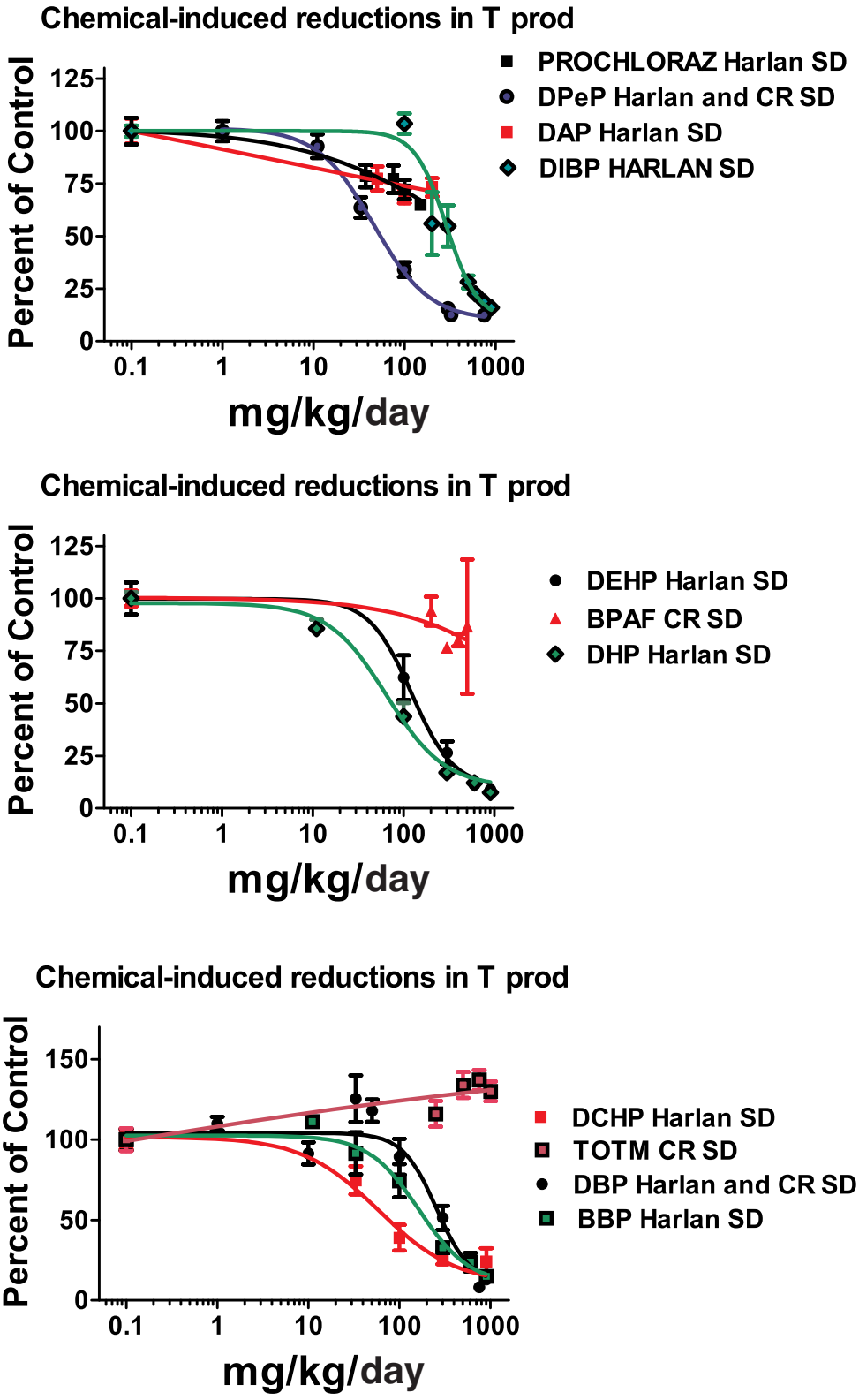


FIG. 2. Dose-related reductions in male rat testis testosterone production on gestational day 18, expressed as percentage of control values. The graph was generated using GraphPad Prism software using the nonlinear, four parameter logistic regression model, with the bottom constrained to 10% of control testosterone production from the same block as the treatment.

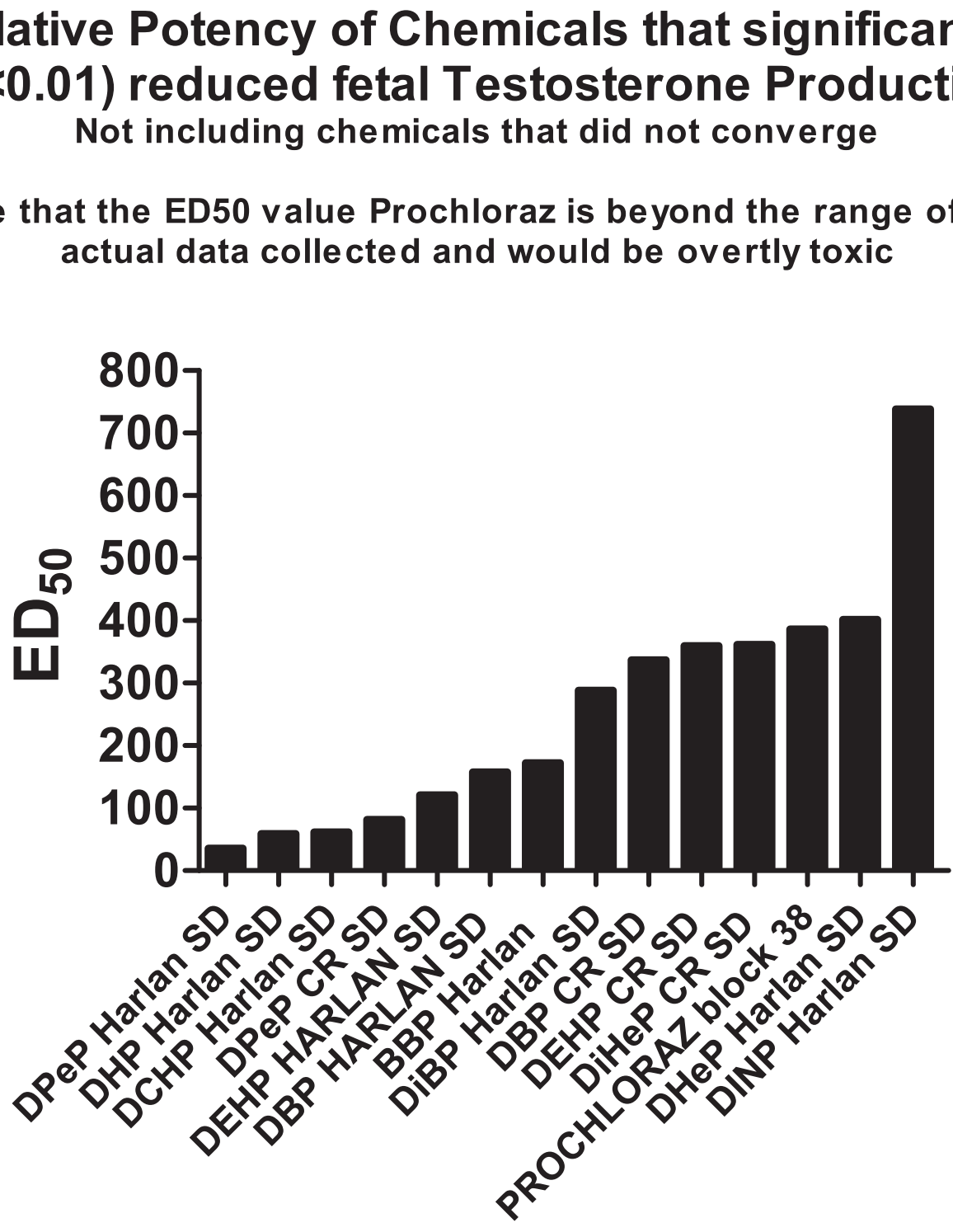


FIG. 3. The ED_{50} values from the logistic regression analyses of the dose-response data were ranked from left to right by decreasing potency to reduce testosterone production with the most potent chemical with the lowest ED_{50} value on the left and the weakest chemical. Chemicals that did not significantly reduce testosterone production are not included in the figure.

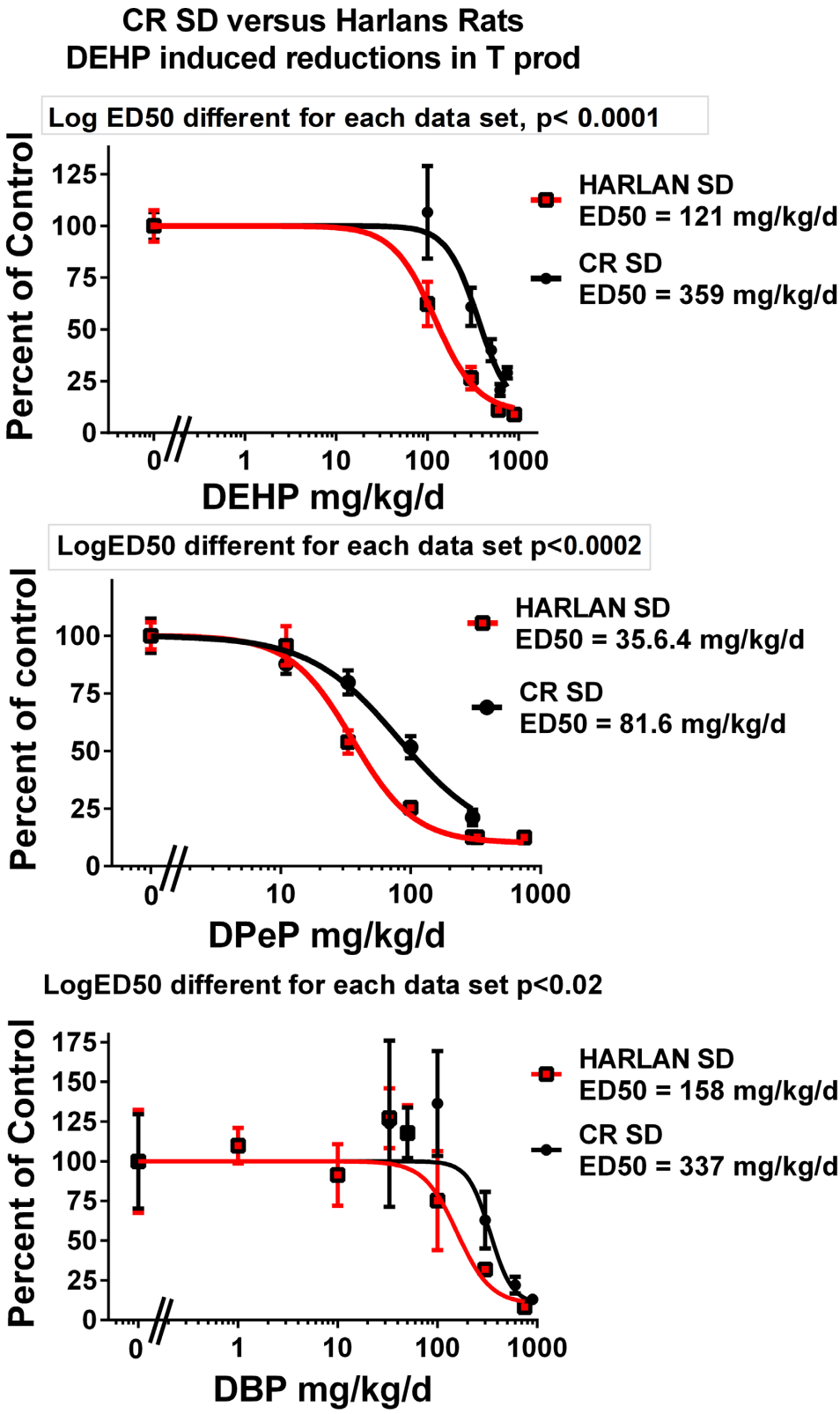
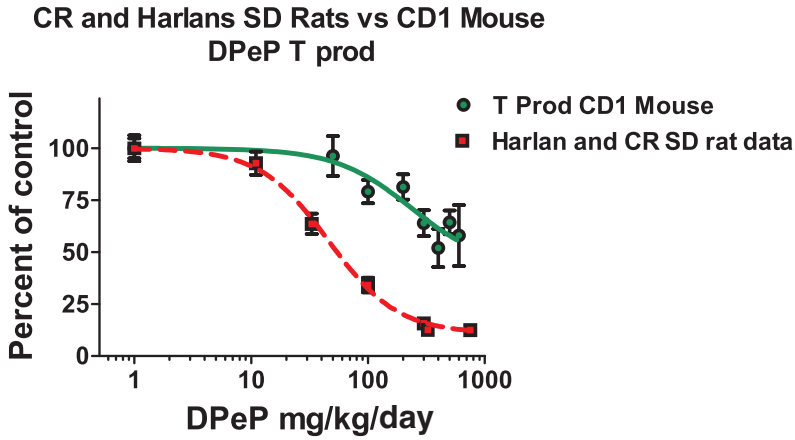
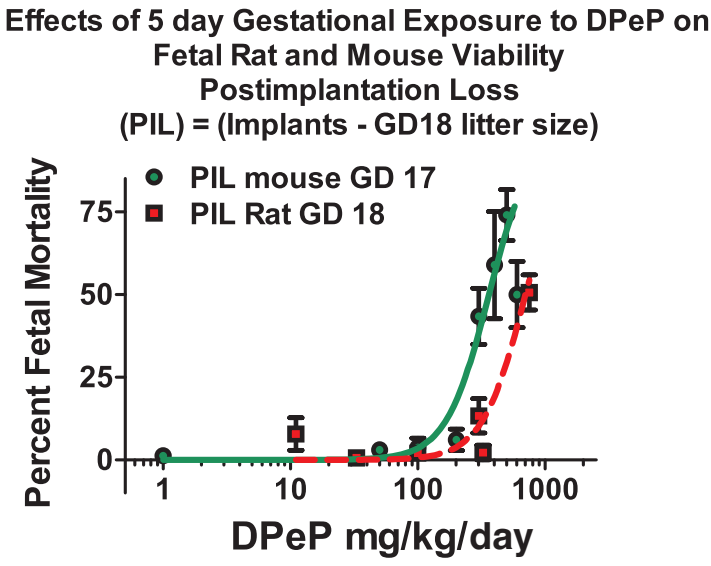


FIG. 4. Dipentyl, dibutyl, and diethylhexyl phthalate were run in several blocks in both Harlan SD and Charles Rivers SD (CR SD) rats in order to compare the sensitivity of these SD rats from different suppliers to phthalate-induced reduction of fetal testosterone production on GD 18. The results of the statistical comparison of the two logistic regression models with GraphPad Prism software indicate that the Harlan SD was slightly more sensitive than is the CR SD.



Sigmoidal Dose-Response Results		
	Harlan and CRSD rat data	CD-1 Mouse
Best-fit values		
Bottom	10.76	42.45
Top	= 100.0	= 100.0
LogED50	1.663	2.371
LogED50 SE	0.07518	0.6114
EC50	46.01	235.1
HillSlope	-1.47	-1.332
R square	0.7917	0.3371
Number of data points	95	64



LogEC50 different for each data set
< 0.0001
Reject null hypothesis
LogEC50 different for each data set
35.17 (1,155)

FIG. 5. Comparison of the dose-related effects of dipentyl phthalate (DPeP) on testosterone production (T Prod) on gestational day 18 and fetal mortality as measured by postimplantation loss (PIL = 100 × (number of implantation sites – number of live fetuses)) in the fetal male rat and mouse. The results of the statistical comparison of the two logistic regression models with GraphPad Prism software indicate that T Prod was more sensitive to DPeP in the rat versus the mouse, whereas, fetal mortality was more affected in the mouse than the rat.

T Prod varied by about 25-fold from 45 to 1100 mg/kg/day. These potency values are currently being used to design a fixed-ratio mixture study with nine active phthalates to determine if the mixture reduces T Prod and testis mRNA expression in a dose-additive manner in the CR SD rat as was seem in the Harlan SD rat (Hannas *et al.*, 2011b) and to determine how much of a reduction in each fetal endocrine endpoint is required to induce permanent effects later in life. For the three PEs (DPeP, DEHP, and DBP) administered in the FPS to both Harlan and CR SD rats, we found that T Prod in Harlan SD was reduced at a significantly lower ED_{50} values than in the CR SD (Fig. 4).

As discussed by Hannas *et al.* (2011a), the relative potencies of the different PEs in the FPS also are well correlated with the relative potencies for induction of the postnatal Phthalate Syndrome and other reproductive effects as well. For example, among the positive chemicals DPeP was one of the most potent PEs in reducing fetal T Prod in the FPS and also in producing Phthalate Syndrome malformations, while DINP is one of the weakest PEs examined in the FPS and it produced a low incidence of Phthalate Syndrome malformations and only at very high dosage levels. With DINP, we found that 7.7% of the F1 males were affected at 750 mg/kg/day whereas 100% of the F1 males were severely malformed following exposure to DPeP at 300 mg/kg/day. Although DPeP and DINP represent the extremes among the active PEs in regards to their potencies, others like DiBP, DBP, and BBP for example, are intermediary in their potencies between these two in the FPS and in postnatal assessments. Furthermore, we have shown (Hannas *et al.*, 2011b) that the relative potencies obtained in the FPS can be used to accurately predict the effects of mixtures of PEs on fetal T Prod and testis gene expression.

The potency estimates from the FPS are likely to be less useful in predicting the postnatal effects of *in utero* exposure to chemicals like prochloraz ($p < 0.01$ reduction in T Prod) (Blystone *et al.*, 2007b; Noriega *et al.*, 2005; Vinggaard *et al.*, 2006) and linuron (Lambright *et al.*, 2000; Wilson *et al.*, 2009) because these chemicals disrupt fetal endocrine pathways by at least two mechanisms of toxicity; by weakly inhibiting testis T Prod, and by acting as an androgen receptor antagonist, and for chemicals like vinclozolin and procymidone that disrupt male reproductive tract differentiation as androgen receptor antagonists.

PE SAR and In Utero *Effects on T Prod*

In the current project, we examined the ability of ortho PEs and PE alternatives with ortho ester groups varying in from C1 (one carbon side chain; DMP) to as many as 10 carbons (DIDP). As noted by other authors, although most if not all alkyl PEs with side-chain lengths of C4 to C6 are reproductive toxicants (Fabjan *et al.*, 2006), some C3 and C7 PEs reduce fetal T Prod and alter male rat reproductive development. It would seem that the claim "…that molecules with linear alkyl chains of 4–6 carbons profoundly affect fertility in rodents, with DEHP being the most active. Molecules with longer or shorter side chains are essentially inactive in these assays." (report from Exxon/Mobil to USEPA, 2001) is not consistent with more recent observations. DPeP is clearly more potent than DEHP as a reproductive toxicant and PEs like DIBP (straight chain length of C3) (Saillenfait *et al.*, 2006, 2008b) and DnHeP (C7 straight chain) (Saillenfait *et al.*, 2011) also can reduce fetal T Prod in the FPS and induce reproductive toxicity.

*Screening PEs I*n Vivo *Versus* In Vitro

Although our protocol does not eliminate animal use, it reduces the numbers of animals needed to detect PEs that induce the Phthalate Syndrome in rats by altering testis endocrine function during sexual differentiation and also allows us to characterize the relative potency of the "positive" PEs. An *in vivo* screening protocol is necessary for chemical classes like the phthalates since tests conducted *in vitro* do not accurately reflect the effects that phthalates have in the more complex and complete environment of the developing whole animal (*in vitro* limitations discussed by McPartland, 2011; http://blogs.edf.org/nanotechnology/2011/06/14/chemical-safety-evaluation-limitations-of-emerging-test-methods/). Some of the major limitations of the current batteries of *in vitro* assays that are relevant to the phthalates are the lack of metabolic activity (activation and detoxification), the absence of important biological pathways and key molecular events, the inability to integrate all of the *in vitro* effects across all the biological systems in a whole animal and the absence of assay validation. For example, the reported reproductive toxicity "signature" for phthalates, focused on PPAR activation (Knudsen *et al.*, 2013; Martin *et al.*, 2011), is incongruous with the endocrine pathways actually disrupted in the developing testis *in utero* by PEs (Hannas *et al.*, 2011b, 2012). In fact, in the FPS protocol the potent PPARα agonist WY 14643 (Pirinixic Acid), had no effect on testis T Prod or expression of the mRNA for any of the genes disrupted by PEs (Hannas *et al.*, 2011b, 2012). Additionally, the PPARγ agonist rosiglitazone also did not affect these fetal endocrine measures or induce any aspect of the Phthalate Syndrome in F1 animals (Boberg *et al.*, 2008). The lack of effect of a potent PPARα or PPARγ agonist on testis endocrine function indicates that activation of either PPAR pathway is unlikely to be a key event in the adverse outcome pathway for the effects of PEs on fetal testis endocrine function (Boberg *et al.*, 2008; Hannas *et al.*, 2012). Furthermore, the structure activity relationship (SAR) for the reproductive toxicity of the PEs is very different than the SAR for activation of PPARα pathways (Bility *et al.*, 2004). In the current study, we stated that we "expected" PEs that induce testicular effects in pubertal male rats (Creasy *et al.*, 1983; Foster *et al.*, 1981, 1982, 1983; Gray *et al.*, 1988, 1999; Gray and Butterworth 1980; Lake *et al.*, 1984; Mangham *et al.*, 1981; Noriega *et al.*, 2005) would be positive in the FPS and reduce T Prod. This hypothesis was based upon the observation that the PEs that induce testicular lesions in the pubertal male testis also induce reproductive tract malformations *in utero* whereas PEs

TABLE 3
Logistic Regression Analyses of the Effects of Chemicals Testosterone Production

Chemical and strain	ED_{50}	ED_{50} 95% CI	log ED_{50}	log ED_{50} SE	Hill slope	No. of litters	R^2	Rank
DPeP Harlan SD	35.59	28.26 to 44.82	1.551	0.05011	−1.816	66	0.819	1
DHP Harlan SD	59.21	42.85 to 81.83	1.772	0.06845	−1.255	29	0.93	2
DCHP Harlan SD	61.62	39.56 to 95.97	1.79	0.09279	−1.035	24	0.7683	3
DPeP CR SD	81.62	62.96 to 105.8	1.912	0.05494	−1.259	29	0.8534	4
DEHP HARLAN SD	121.2	92.02 to 159.5	2.083	0.05789	−1.87	26	0.8675	5
DBP HARLAN SD	157.9	100.5 to 248.0	2.198	0.09746	−2.576	50	0.5364	6
BBP Harlan	172.4	115.7 to 256.6	2.236	0.0841	−1.63	28	0.7949	7
DiBP Harlan SD	288.2	247.9 to 335.2	2.46	0.03273	−2.508	60	0.8778	8
DBP CR SD	337.1	250.2 to 454.2	2.528	0.06286	−3.906	27	0.6911	9
DEHP CR SD	359.8	281.3 to 460.3	2.556	0.05183	−2.517	26	0.8059	10
DiHeP CR SD	361.6	290.0 to 450.8	2.558	0.0454	−2.396	19	0.8639	11
DHeP Harlan SD	401.7	310.3 to 520.0	2.604	0.05146	−2.539	14	0.8383	12
DINP Harlan SD	738.3	616.7 to 883.9	2.868	0.03853	−1.681	38	0.7691	13
PROCHLORAZ	386.5[a]	50.32 to 2968	2.587	0.4257	−0.565	23	0.5336	14
DAP CR SD	BAD FIT	None	None	None	None	20	None	
BPAF CR SD	BAD FIT	None	None	None	None	14	None	
TOTM CR SD	BAD FIT	None	None	None	None	28	None	
DIDP Harlan SD	Not converged	None	None	None	None	15	None	
WYTHE 14643 Harlan SD	Not converged	None	None	None	None	12	None	

***Note*. Chemicals in the table are ranked from the lowest to highest ED_{50} value in mg/kg/day.**
[a]Indicates that the ED_{50} value would be toxic to the dam and was above the dose range tested in the current study.

like DEP, DPP and DMP do not alter testis function at either life stage. Since the phenotypic effects of PEs on testis function during these two stages of development are so different, one would not necessarily expect this to be the case. The fact that the SAR for testicular toxicity appears to be similar during these two stages of development may indicate that the same molecular initiating event (MIE) is disrupted by PEs *in utero* and during puberty but the downstream events regulated by this MIE differ considerably; plausible outcomes given the major differences between the signaling pathways in the fetal and pubertal rat testis (Scott *et al.*, 2008).

In Utero *Dipentyl Phthalate Exposure Reduces Fetal Testosterone in the CD-1 Mouse*

In the current study, we also conducted a dose-response study of DPeP in the pregnant CD-1 mouse and found that this relatively potent PE did significantly reduced T Prod (Fig. 5). This mouse strain and PE were selected because DPeP has been shown to produce testicular effects in this strain in a high dose Reproductive Assessment by Continuous Breeding (RACB) study with effects sufficient to render the F1 males and females infertile (Heindel *et al.*, 1989). In addition, DEHP induces testicular lesions in this mouse strain, but not in the ICR mouse strain (Oishi 1993). This study was conducted to begin to clarify some of the discrepancies on effects of PEs *in utero* on fetal mouse T Prod.

Although some authors have stated emphatically that, unlike the rat, T levels in the mouse do not respond to PEs at this stage of development (Gaido *et al.*, 2007; Johnson *et al.*, 2012), other authors have reported reductions in fetal and neonatal (Moody *et al.*, 2013) testosterone and related gene expression levels and increases in malformations of androgen- and insl3-dependent tissues in the male mouse reproductive tract. For example, Johnson *et al.* (2012) stated in their review article "Of mice and men (and rats): phthalate-induced fetal testis endocrine disruption is species-dependent" that inhibition of fetal Leydig cell hormone synthesis is not observed in the mouse following *in utero* PE administration. These authors conclude that T Prod in mice is not affected by PE exposures *in utero* and, for this reason; mice are a better animal model for humans than is the fetal rat. However, our results demonstrate that T prod in fetal mice is significantly reduced at 100 mg DPeP/kg/day and above. In the current study with CD-1 mice, we found that DPeP significantly reduced T Prod by 25–30% at a dose level that did not induce any maternal or fetal toxicity. The ED_{50} for this effect was about fourfold higher than in the rat and the effect reached a plateau at about 50% of control, whereas the T Prod in the rat can be reduced to ~10–15% of control before reaching a plateau. Although the biological basis for the species difference in the level of T Prod at the plateau is not known, this could result if the MIE disrupted by the PEs only inhibited one of multiple potential pathways regulating T Prod and the affected pathway was more important in the rat than in the mouse.

In contrast to being less sensitive to the effects of DPeP on T Prod, pregnancy maintenance and fetal viability in the mouse were affected at lower dosage levels than in the rat (Fig. 5). In addition to the current investigation, several other studies have reported that PEs reduce fetal mouse T Prod, testis genes related

to T Prod and insl3 synthesis, and increase the incidence of malformations in androgen- and insl3-dependent tissues in postnatal life. For example, Song *et al.* (2006, 2008) and Wang *et al.* (2004) reported that *in utero* DEHP reduced fetal testis insl3 levels, induced abnormal development of the gubernaculum, induced cryptorchidism, and caused testis histopathology, dysplasia and dysfunction of Sertoli cells, Leydig cells, and spermatogenic cells in fetal KM mice. Wu *et al.* (2010) reported that *in utero* DEHP reduced fetal and postnatal testosterone and fetal insl3 levels and Liu *et al.* (2009) found that DEHP induced hypospadias and altered TGFβ1 levels in the genital tubercle. One study even reported a nonmonotonic effect on fetal mouse T Prod, which increased and then decreased with increasing maternal dosages of DEHP (Do *et al.*, 2012)). However, the latter study (Do *et al.*, 2012) reported nonmonotonic effects at several dose levels that are well below those that have been reported in rodent diets and beddings (Kondo *et al.*, 2010).

It is possible that the discrepancies in the literature on the effects of PEs in the mouse can be attributed to strain differences, as it is known that the pubertal effects of PEs on the mouse testis vary greatly from strain to strain (Oishi 1993).

CONCLUSIONS

In summary, in the current project we developed and validated a short-term *in vivo* protocol, termed the FPS, to screen phthalates, phthalate alternatives and other chemicals for their ability to disrupt testis endocrine function *in utero*; an effect causally related to the development of male rat reproductive tract lesions and reproductive problems after birth in adulthood. The FPS protocol also can be used to determine the relative potency of the PEs that reduce fetal T Prod and a comparison of these results with those seen in multigenerational or one-generation studies reveals that the FPS accurately predicts PEs that do, or do not, induce the Phthalate Syndrome in F1 male rats after *in utero* exposure.

Although the current screening protocol for PE-induced reproductive toxicity does not eliminate animal use, as would an *in vitro* study, this protocol significantly reduces the numbers of animals, as well as the amount of other resources (labor, time, etc.) required to predict whether PEs will or will not induce the Phthalate Syndrome and other reproductive effects. Until all the key events in this adverse outcome pathway can be identified and quantified, validated *in vitro* assays developed and the results integrated, it will remain necessary to conduct animal studies for human health assessments with this class of chemicals.

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