Dose Addition Models Based on Biologically Relevant Reductions in Fetal Testosterone Accurately Predict Postnatal Reproductive Tract Alterations by a Phthalate Mixture in Rats

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ABSTRACT

Challenges in cumulative risk assessment of anti-androgenic phthalate mixtures include a lack of data on all the individual phthalates and difficulty determining the biological relevance of reduction in fetal testosterone (T) on postnatal development. The objectives of the current study were 2-fold: (1) to test whether a mixture model of dose addition based on the fetal T production data of individual phthalates would predict the effects of a 5 phthalate mixture on androgen-sensitive postnatal male reproductive tract development, and (2) to determine the biological relevance of the reductions in fetal T to induce abnormal postnatal reproductive tract development using data from the mixture study. We administered a dose range of the mixture (60, 40, 20, 10, and 5% of the top dose used in the previous fetal T production study consisting of 300 mg/kg per chemical of benzyl butyl (BBP), di(n)butyl (DBP), diethyl hexyl phthalate (DEHP), di-isobutyl phthalate (DiBP), and 100 mg dipentyl (DPP) phthalate/kg; the individual phthalates were present in equipotent doses based on their ability to reduce fetal T production) via gavage to Sprague Dawley rat dams on GD8-postnatal day 3. We compared observed mixture responses to predictions of dose addition based on the previously published potencies of the individual phthalates to reduce fetal T production relative to a reference chemical and published postnatal data for the reference chemical (called DA_{ref}). In addition, we predicted DA (called DA_{all}) and response addition (RA) based on logistic regression analysis of all 5 individual phthalates when complete data were available. DA_{ref} and DA_{all} accurately predicted the observed mixture effect for 11 of 14 endpoints. Furthermore, reproductive tract malformations were seen in 17–100% of F1 males when fetal T production was reduced by about 25–72%, respectively.

Key words: dose addition; phthalates; postnatal developmental toxicity; male reproductive tract; endocrine disruptors; mixture models

Phthalate esters are high production volume chemicals, utilized as plasticizers and fragrance stabilizers, to which humans are ubiquitously exposed (Blount *et al.*, 2000; Silva *et al.*, 2004; Swan *et al.*, 2005; Wolff *et al.*, 2008). Exposure to certain individual phthalate esters during the period of sexual differentiation induces reproductive malformations in rats. These malformations, known as the 'phthalate syndrome', include agenesis and hypoplasia of the epididymis, testicular malformations, agenesis of accessory reproductive organs, and abnormal penile development (ie, hypospadias) (Foster, 2006; Gray *et al.*, 2006b). Phthalate treatment also reduces the anogenital distance (AGD) and causes nipple retention in male rats, which are present in infancy and persist through adulthood (Gray *et al.*, 2000). Phthalates disrupt reproductive development in males by acting on Leydig cells in the fetal testes to inhibit testosterone (T) production, suppress associated steroidogenic pathway genes (Lehmann *et al.*, 2004; Liu *et al.*, 2005; Parks *et al.*, 2000; Wilson *et al.*, 2004), and inhibit expression of insulin-like hormone 3 (*Insl3*), a peptide hormone responsible for the maturation of the gubernacular cords which causes the first phase of testicular descent (Ivell and Bathgate, 2002; Zimmermann *et al.*, 1999) (Fig. 1). Phthalates also impact the Sertoli cells and germ cells of the developing testes; however, these effects are thought to occur via a primarily androgen-independent mechanism (Sharpe and Skakkebaek, 2008).

Cumulative risk assessments of the phthalates are ongoing and are dependent on individual phthalate data. In 2008, a National Academy of Sciences panel advised the U.S. Environmental Protection Agency (U.S. EPA) to conduct cumulative risk assessments on the phthalates, given their common mode of action to suppress androgen-dependent reproductive tract development in male rats (NAS, 2008). The Consumer Products Safety Commission recently convened a Chronic Exposure Advisory Panel to evaluate the cumulative risk for children's health from exposure to multiple phthalates (Lioy *et al.*, 2015). However, assessment of the cumulative risk to the phthalate esters has been challenging due to incomplete data on the individual reproductive toxicant phthalates.

To address this data gap, we previously characterized the ability of 5 reproductive toxicant phthalates to reduce fetal testicular T production in the rat, including: benzyl butyl (BBP), di(n)butyl (DBP), diethylhexyl (DEHP), di-isobutyl (DiBP) and dipentyl phthalate (DPP) (Howdeshell *et al.*, 2008b). Based on the individual phthalate dose response data, we designed and tested a mixture of 5 reproductive toxicant phthalates to determine if they would act in a dose additive manner to reduce fetal testicular T. The ratio of the 5 phthalates (BBP, DBP, DEHP, DiBP, and DPP) in the mixture was based on their relative potencies, such that each phthalate would contribute equally to reducing fetal T production; the mixture dose levels were designed to range from a no effect dose to maximally tolerated dose in order to define the dose response range as fully as possible (Table 1). As hypothesized, the 5 phthalate mixture inhibited fetal T production in a dose-additive manner (Howdeshell *et al.*, 2008b). These data on fetal testicular T production in the rat have been corroborated by subsequent experiments from our laboratory testing a 9 phthalate mixture (Hannas *et al.*, 2012).

This experiment was designed to evaluate how predictive decreases in fetal T are as an upstream event to downstream induction of reproductive malformations. The two major objectives of the current study were to (1) test whether a dose additive model based on the fetal T production data of individual phthalates would accurately predict the effects of a 5 phthalate mixture on postnatal reproductive development in male rats, and (2) to define the biological relevance of the reduction in fetal T production (ie, how much of a reduction in fetal T production was required to induce permanent postnatal reproductive abnormalities in the male offspring?). First, we dosed pregnant Sprague Dawley rats with a dose range of the 5 phthalate mixture (60, 40, 20, 10, and 5% of the top dose used in previous fetal T studies consisting of 300 mg/kg per chemical of BBP, DBP, DEHP, DiBP, and 100 mg DPP phthalate/kg) via gavage on GD8-postnatal day 3 and measured the postnatal reproductive tract development of their offspring. As mentioned earlier, the mixture dose was formulated such that each individual phthalate contributed equally to reduce fetal T production

FIG. 1. Adverse outcome pathway for disrupted androgen- and insulin-like hormone 3 (INSL3)-dependent development due to phthalate ester exposure during sexual differentiation in rats. AR, androgen receptor; PPAR, peroxisome proliferator activated receptor; and T, testosterone. *Suppressed maturation of the gubernacular cords contributes to incidence of cryptorchidism (undescended testes) due to role of gubernacular cords in transabdominal descent of the testes. **Undescended testes may contribute to incidence of testicular cancer.

(Howdeshell *et al.*, 2008b). Second, we evaluated whether the observed mixture data conformed to dose addition model predictions based on published individual phthalate data. When data were available on all 5 individual phthalates in the mixture, we calculated mixture model predictions for each endpoint using dose addition (called DA_{all}) and response addition (RA). Because individual phthalate data were not available for all endpoints measured in the current study, we also evaluated the dose additivity of the phthalate mixture based on the published potency of the each individual phthalates to reduce fetal T production relative to a reference chemical (called DA_{ref}) (Fig. 2). Finally, we determined the level of fetal T production that induced postnatal reproductive abnormalities in male rats following *in utero* exposure to the phthalate mixture: we used logistic regression analyses to define biologically significant reduction in fetal T. Our *a priori* hypothesis was that a DA model based on fetal T production (DA_{ref}) would accurately predict the effects of developmental exposure to the 5 phthalate mixture to alter reproductive development in male rats. We employed a mixture dose with equipotent contribution from each individual phthalate, as opposed to using an environmentally-relevant ratio, so that we would be more likely to observe unexpected interactions of the phthalate mixture on postnatal reproductive endpoints.

MATERIALS AND METHODS

General Methods

Animals. Adult female Sprague Dawley (CR:CD(SD)IGSBR) rats (Charles River, Raleigh, North Carolina) were mated by the supplier and shipped on gestation day 2 (GD2). Mating was confirmed by sperm presence in vaginal smears (day of sperm plug positive = GD1). Animals were housed individually in 20 × 25 × 47 cm clear polycarbonate cages with laboratory-grade heat-treated pine shavings (Northeastern Products, Warrensburg,

New York). Pregnant and lactating females were fed Purina Rat Chow 5008, and weanling and adult rats were fed Purina Rat Chow 5001 *ad libitum*. Animals were provided access to filtered (5 micron filter) municipal drinking water (Durham, North Carolina) *ad libitum*. Water was tested monthly for *Pseudomonas* and every 4 months for a suite of chemicals including pesticides and heavy metals. Animals were maintained on a 12:12 h light/dark photoperiod (lights off at 1900) at 20–24°C. This study was conducted under protocols approved by the National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care-approved animal facility.

Source and Administration of Chemicals. The vehicle control, laboratory-grade corn oil (CAS no. 8001-30-7, Catalog no. C-8627), and the phthalates used in this study were purchased from Sigma-Aldrich (St Louis, Missouri): BBP (CAS no. 85-68-7, Catalog no. 308501, Lot no. 08523JQ, purity 98%); DBP (CAS no. 84-74-2, Catalog no. D-2270, Lot no. 109F0386, purity = 99%); DEHP (CAS no. 117-81-7, Catalog no. P-6699, Lot no. 106H3487 for individual dose response and Lot no. 106H3487 for the mixture dose, purity = 99%); diethyl phthalate (DEP) (CAS no. 84-66-2, Catalog no. P-5787, Lot no. 48H3537, purity 99%); DiBP (CAS no. 84-69-5, Catalog no. 152641, Lot no. 103141C, purity = 99%), and DPP (CAS no. 131-18-0, Catalog no. 80154, Lot no. 1151652, purity = 99%). The purity of each chemical was provided by the vendor. Phthalate doses were dissolved in corn oil and delivered in 2.5 µl corn oil per gram body weight. The rat dams were weighed daily during the dosing period to administer the dose per kg body weight and to observe the health of the dams.

Developmental Reproductive Toxicity Study

Treatment Groups. Pregnant rat dams were randomly assigned to treatment groups on GD8 in a manner that provided similar

FIG. 2. DA models used to predict the effects of the 5 phthalate (PE) mixture on postnatal reproductive development. (A) DA_{all} was used when postnatal data were available for each of the 5 PEs in the mixture (eg, AGD). When postnatal data were not available for all 5 PEs, DA was predicted using reference chemical (DA_{ref}), which relied on the fetal T production data published for each individual PEs (Howdeshell *et al.*, 2008b) to calculate potency of each PE relative to a reference PE (eg, dibutyl phthalate [DBP]) and postnatal data for the reference PE (eg, DBP, Mylchreest *et al.*, 1998, 1999, 2000). R, response to the chemical (or mixture); D, concentration of chemical; ED_{50} , concentration of chemical that causes a 50% response; and ρ , power (Hill slope) of the chemical; i, refers to individual chemical data (eg, D_i is the concentration of chemical i); n, number of individual chemical in the mixture; and ρ^i is the average power (Hill slope) associated with the individual chemicals in the mixture.

mean ($\pm$ SE) body weight per treatment group prior to dosing. Dams were dosed via gavage on GD8 to PND3 with 0, 5, 10, 20, 40, and 60% of the top dose of 1300 mg/kg/d total phthalates, including BBP, DBP, DEHP, and DiBP each at 300 mg/kg/d, plus 100 mg/kg DPP/d for a dose ratio of 3:3:3:3:1. This is the same 5 phthalate mixture used in the published fetal T production study (Howdeshell et al., 2008b), and the ratio of the mixture allow each phthalate to contribute equally to the reduction in fetal T production (Supplementary Table S1). Mixture doses >60% of top dose were not used in the developmental reproductive toxicity study due to high rates of fetal mortality observed in the fetal T production study (Howdeshell et al., 2008b). The 60% of top dose (780 mg/kg/d total phthalates) was predicted to induce malformations in the epididymides and seminal vesicles of the male offspring without causing maternal toxicity, if the combination of 5 phthalates acted in a dose additive manner.

Neonatal and Pubertal Data. Control and treated dams ($n=6$ litters per treatment; total of 36 dams) were allowed to deliver naturally. On PND2 (day of birth = PND1), the body weight and AGD of individual pups were recorded. AGD was measured using a dissecting scope with an ocular micrometer as per Hotchkiss et al. (2004). Then, the male AGD data were converted to the percent reduction from control male AGD. Finally, because the maximum reduction in male AGD would be to a control female AGD level (which is 50% of a normal male), the data were multiplied by 2 to express the reduction in male AGD on a 0–100% scale. On PND14, male and female offspring were reweighed and examined for presence or absence of areolae or nipples. Data on areolae/nipple retention were analyzed in 2 different ways: (1) % of males with areolae/nipples to determine incidence of nipple retention and (2) % of total possible areolae/nipples (up to 12) to measure severity of the treatment to induce nipple retention. At PND22, pups were weighed and weaned. Male offspring were housed two siblings per cage. Female offspring were housed 2–3 per cage. The rat dams were euthanized after weaning the pups, their uteri were removed, and the number of uterine implants was recorded. Fetal/neonatal mortality was calculated by subtracting the number of live pups on PND2 from the number of implantations, then dividing by the number of implantations. Maternal and litter characteristics, and adult female necropsy data were published in Hannas et al. (2013).

Adult Male Necropsy Data. At 40–46 weeks of age, males were necropsied following CO₂ anesthesia and decapitation. The ventral surface of each male was shaved and examined for the presence of nipples. The males were then examined for reproductive malformations, including hypospadias, cleft phallus, vaginal pouch, epididymal malformations (eg, epididymal agenesis and hypoplasia), gubernacular cord malformations (eg, gubernacular cord agenesis and hypoplasia), testicular malformations (eg, testicular atrophy, cryptorchid testes, and fluid-filled testes), and agenesis of the vas deferens, ventral prostate and seminal vesicles. Gubernacular hypoplasia was characterized by thread-like gubernacular cords measuring longer than 20 mm length. The percent of males displaying the ‘phthalate syndrome’ (also called total malformed males) was calculated by adding together the number of males that had any occurrence of the following malformations at necropsy: permanent nipple retention, hypospadias, epididymal malformations, testes malformations, vas deferens agenesis, ventral prostate agenesis, seminal vesicle agenesis, and gubernacular malformations. Body and select organ weights (ie, glans penis, ventral prostate, seminal

vesicles, testes, epididymides, levator ani/bulbocavernosus (LABC) muscle, Cowper’s glands, kidneys, and liver) were recorded at time of necropsy. Organ weight reductions were not included in the calculation of total malformed males.

Individual Chemical Assessment for Postnatal Reproductive Endpoints. Mixture model predictions required data on postnatal reproductive endpoints for the individual phthalates. These studies from which individual phthalate data were collected were selected because the individual phthalates were administered during a similar period of *in utero* development as this study (eg, early to mid-gestation through gestation; some studies also dosed during lactation), rat dams were exposed to range of doses via gavage or the diet, and they reported postnatal reproductive development in male offspring through adulthood. Data were retrieved from the following studies: BBP (Aso et al., 2005; Nagao et al., 2000; Tyl et al., 2004), DBP (Aso et al., 2005; Mylchreest et al., 1998, 1999, 2000; Nagao et al., 2000; Wine et al., 1997), DEHP (Blystone et al., 2010; Gray et al., 2009), and DIBP (Saillenfait et al., 2008), and DPP (Earl Gray, Jr., Johnathan Furr, Katoria, Tatum-Gibbs, Christy Lambright, Hunter Sampson, Vickie Wilson, Andrew Hotchkiss, and Paul Foster, in preparation). We graphed the data on a log-linear scale and fit the data with a sigmoidal dose response, 4-parameter logistic regression equation in GraphPad Prism (La Jolla, California)

$$R = \frac{1}{1 + (ED50/D)^{\rho}} \quad (1)$$

where R is the response, D is the daily dose, ρ is the slope of the curve and ED50 is the dose resulting in a 50% effect. We constrained the Bottom to 0% and the Top to 100% for the incidence of reproductive malformations, % reduction in AGD at PND2 (doubled), and % reduction in adult organ weights. The ED50s and Hill slopes of the individual phthalates were used in equations to predict the phthalate mixture response.

In order to more directly model the endpoints studied in the current phthalate mixture study, we reanalyzed the raw data from Mylchreest et al. (1998) for the reduction in organ weights (ie, seminal vesicles, ventral prostate, paired epididymides, and paired testes) following DBP exposure during pregnancy and lactation. This was necessary because Mylchreest et al. (1998) excluded absent, partial, or missing organs from their analysis of male organ weights; whereas, we included the actual weight of the partial or small organs in the 5 phthalate study and assigned 0 mg (or g) to any absent organs. We also used the raw data from Mylchreest et al. (1998) to calculate the incidence of testicular malformations (number of males with either undescended testes, or absent, partial, or small testes) because they only reported the incidence of undescended testes; whereas our calculation of testicular malformations includes undescended testes, or ectopic, fluid-filled, or atrophic testes (ectopic, fluid-filled or atrophic testes are smaller than normal, descended testes). For the organ weight data, we applied the following values for individual data points not included in the previously published analysis: agenesis of any organ (0 g), small testis (0.873 ± 0.254 g), small epididymis (0.464 ± 0.093 g), and partial epididymis (0.296 ± 0.067 g); these averages were calculated from the historical published data of small testes, or small or partial epididymides, respectively, in the laboratory of Earl Gray.

Comparison of Observed Mixture Effects to Mixture Model Predictions. We evaluated whether the phthalate mixture acted in a dose

additive manner on androgen-responsive reproductive development by comparing the observed data of the 5 phthalate mixture to mixture effects predicted from mathematical models. The mixture models were based on dose response data for the individual phthalates from previously published studies (see *Individual Phthalates* section above). Dose addition (DA_{all}) and RA models were calculated when a complete set of individual phthalate data were available for an endpoint. For several endpoints, there was incomplete data available in the published literature for all 5 individual phthalates of the mixture. Thus, a dose addition model based on the inhibition of fetal testicular T by the individual phthalates relative to a reference chemical was also used (called DA_{ref}).

DA has been used to predict the effects of mixtures comprised of individual chemicals that work via a similar mode or mechanism of action. Traditionally, the dose additive model is calculated based on data from all individual phthalates in the mixture (DA_{all}),

$$R = \frac{1}{1 + \frac{1}{\left(\sum_{i=1}^n \frac{D_i}{ED50_i}\right)^{p_i}}} \quad (2)$$

where R is the response to the mixture, D_i is the concentration of chemical i in the mixture, ED50_i is the concentration of chemical i that causes a 50% response, and p_i is the average power (Hill slope) associated with the chemicals. RA (also called independent action) has been used to estimate the effects of mixtures which include individual chemicals that act through different mechanisms of action. The RA model was calculated based on data from all individual phthalates in the mixture,

$$R = 1 - \prod_{i=1}^n (1 - R_i) \quad (3)$$

where R represents the response to the mixture and R_i is the response to individual chemical i in the mixture. The methods for predicting the DA and RA effects of mixtures have been described in detail elsewhere (Howdeshell et al., 2007; Howdeshell et al., 2008b; Rider and LeBlanc, 2005).

Dose addition based on fetal T production relative to a reference chemical (DA_{ref}) was used to predict dose additive effects of the phthalate mixture when a complete set of individual phthalate data were not available for a given endpoint. Specifically, the DA_{ref} model was based on the published potency of the individual phthalates to inhibit fetal testicular T production as reported in Howdeshell et al. (2008a), and the phthalate mixture dose was expressed in units of a reference chemical (eg, DBP mg/kg/d) as per Equation (4) below:

$$D_{ref} = D_i \left(\frac{ED50_{ref}}{ED50_i} \right) \quad (4)$$

where the D_{ref} is the calculated dose in reference chemical units, D_i is the dose of chemical i in the mixture, ED50_{ref} is the dose of the reference chemical that causes a 50% response, and ED50_i is the dose of chemical i that causes a 50% response. The DA_{ref} model then estimated the phthalate mixture effect based on the log ED50 and Hill slope of the reference chemical for each endpoint using a logistic regression equation (Equation 1). We selected DBP as the reference chemical for the DA_{ref} predictions because it is a well-characterized reproductive toxicant with data for a majority of the postnatal reproductive endpoints measured in the current phthalate mixture study (Mylchreest

et al., 1998, 1999, 2000). DEHP was used as the reference chemical with data from Gray et al. (2009) for the following endpoints because DBP data were not available: number of adult males with areolae/nipples and % of total possible areolae/nipples in adult males. The logistic regression parameters of the inhibition of fetal T production by the 5 individual phthalates and their potencies relative to the reference chemical (DBP or DEHP) are included in Supplementary Table S1.

We determined whether the observed data were predicted by mixture models of DA in 2 ways. First, we compared the logistic regression models for the observed data to the mixture models by evaluating whether the ED50 values predicted by the mixture models fell within the ED50 ± 95% confidence limits of the observed data.

Although this approach does not incorporate variability into the model predictions through statistical techniques (eg, bootstrapping), it does reflect the variability in observed data by using the 95% CI as a rational cut-off for determining whether or not the modeled predictions were similar to observed responses. Second, we force fit the observed data to each mixture model by constraining the observed data to the four parameters of each mixture model (DA_{ref}, DA_{all}, or RA). If the mixture model perfectly predicted the observed data, the R² value of the force-fit model would be equal to the R² value obtained with the 'best fit' of the observed data. The greater the mixture model deviated from the observed effects, the greater the decline in the R² of the force-fit model from the 'best fit' of the observed data. We defined a 'best fit' in the force fit analysis to be a forcefit to model R² value that was ≥70% of the observed best fit R²; best fit models were considered to be consistent with observed data.

We also evaluated additional response levels (eg, ED5, ED10, ED20, ED25, ED75, and ED90), so that if clear deviation of observed data from modeled data were detected using the forcefit approach, the dose dependency of the deviation could be evaluated to observe whether there were dose-dependent interactions of the observed data to the mixture models. Furthermore, for the postnatal endpoints not accurately predicted by DA (DA_{ref} or DA_{all}) or RA in the forcefit analysis, we calculated the magnitude of interaction for the observed data relative to the mixture model predictions (Boobis et al., 2011). For example, a magnitude of interaction of 2.0 would be calculated if the observed ED20 of the mixture is 20 and the mixture model predicted ED20 based on additivity is 40.

Biologically Significant Reductions in Fetal T Production that Induce Reproductive Malformations in Males. We evaluated the relationship of the reduction in fetal T production to the induction of postnatal reproductive malformations in male rats following developmental exposure to the phthalate mixture. This analysis was based on the assumption that phthalate-induced decreases in fetal T availability during in utero sexual differentiation lead to reproductive malformations observed in the adult male rat. We fit the previously published data for fetal T production for the 5 phthalate mixture to a 4 parameter logistic regression equation (GraphPad Prism, La Jolla, California) without constraints; fetal T production data were previously published in Howdeshell et al. (2008b). The postnatal malformations were fit to a 4-parameter logistic regression constraining the bottom to 0% and the top to 100%. Next, we used a logistic regression analysis of fetal T production versus postnatal effects to estimate effective dose inducing a 10% (ED10), 50% (ED50) or 90% (ED90) effect (as % affected males for malformation rates, % reduction in AGD, or % reduction in organ weights or AGD); this was determined by interpolation of the data using GraphPad Prism.

Statistics. Data from the developmental study were analyzed by one-way ANOVA using the general linear measures procedures Statistical Analysis Systems (SAS, Inc., Cary, North Carolina). Post hoc comparisons for endpoints in the developmental study were made using the Least Squared Means procedure in SAS, which is appropriate for *a priori* hypotheses. For the analysis of treatment effects, data were analyzed by litters with the exception of the malformation data, which was analyzed as individual means by Fishers exact test (Sigma Stat, Systat Software, San Jose, California). Differences were considered significant at $P < 0.05$.

RESULTS

Developmental Reproductive Toxicity Study

Infant and Pubertal Data. The phthalate mixture significantly influenced androgen-sensitive endpoints in the developing

male offspring (Table 2). The phthalate mixture reduced AGD on PND2 in male pups with significant decreases observed at doses of 20% of top dose and above (Fig. 3). The phthalate mixture significantly increased the percentage of males with areolae and/or nipples on PND13 at doses of 10% of top dose and above, as well as the percent of total areolae/nipples out of 12 possible nipples on PND13 at doses of 20% of the top dose and above (Fig. 3, Table 2). There were no significant treatment effects on body weight of the male pups at PND2, PND13 (data not shown), or at weaning (PND22) (Supplementary Table S2).

Adult Male Offspring Necropsy Data. In utero exposure to the phthalate mixture induced both external and internal reproductive malformations in the male rat offspring (Table 3). The percent of adult males with nipples (Fig. 4A) and the percent of total possible nipples in adulthood were significantly increased at 20% of the top dose and above. Epididymal and total testicular malformations were significantly induced by phthalate

TABLE 1. Individual phthalate composition of the 5 phthalate mixture used in the developmental reproductive toxicity study

	5% of top dose	10% of top dose	20% of top dose	40% of top dose	60% of top dose
Phthalates in mixture:	(mg/kg/d)	(mg/kg/d)	(mg/kg/d)	(mg/kg/d)	(mg/kg/d)
BBP	15	30	60	120	180
DBP	15	30	60	120	180
DEHP	15	30	60	120	180
DiBP	15	30	60	120	180
DPP	5	10	20	40	60
Total phthalates	65	130	260	520	780

The same was used in the previously published fetal T production study, which included a top dose of 300 mg/kg/d each of DBP, BBP, DEHP, and DiBP, and 100 mg DPP/kg/d (Howdeshell et al., 2008b).

TABLE 2. Male reproductive tract malformations in SD rats prenatally exposed to a 5 phthalate mixture from GD8 to PND3 and necropsied on PND280-324

	0% of top dose	5% of top dose	10% of top dose	20% of top dose	40% of top dose	60% of top dose
Number of males at necropsy (individuals, litters)	36, 6	31, 5	44, 6	35, 6	24, 5	9, 3
AGD of male pups on PND2 (mm)	3.46 ± 0.06 ^a (42, 6) ^b	3.35 ± 0.15 (33, 5)	3.32 ± 0.09 (49, 6)	3.04 ± 0.05 ^c (42, 6)	2.99 ± 0.09 ^c (26, 5)	2.54 ± 0.19 ^c (14, 3)
Areolae/nipples on PND13 (%)	0 ± 0 (38, 6)	15.6 ± 6.5 (32, 5)	39.1 ± 7.3 ^c (46, 6)	71.4 ± 7.7 ^c (35, 6)	88.5 ± 6.4 ^c (26, 5)	100.0 ± 0.0 ^c (11, 3)
% of total possible areolae/nipples (out of 12) on PND13	0 ± 0 (38, 6)	3.7 ± 1.4 (32, 5)	9.5 ± 2.5 (46, 6)	21.9 ± 4.8 ^c (35, 6)	48.1 ± 7.4 ^c (26, 5)	92.1 ± 7.9 ^c (11, 3)
Nipple retention in adulthood (%)	0 ± 0	0 ± 0	11.4 ± 4.8	42.9 ± 8.5 ^c	62.5 ± 10.1 ^c	88.9 ± 11.1 ^c
% of total possible nipples in adulthood	0 ± 0	0 ± 0	1.7 ± 0.9	8.1 ± 1.9 ^c	16.7 ± 3.6 ^c	50.0 ± 7.5 ^c
Hypospadias (%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	11.1 ± 11.1 ^c
Epididymal malformations (%)	0 ± 0	0 ± 0	0 ± 0	14.3 ± 6.0 ^c	70.8 ± 9.5 ^c	100.0 ± 0.0 ^c
Testicular malformations (%)	2.78 ± 2.78	0 ± 0	0 ± 0	17.1 ± 6.5 ^c	79.2 ± 8.5 ^c	100.0 ± 0.0 ^c
Undescended testes (%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	8.3 ± 5.8	66.7 ± 16.7 ^c
Seminal vesicle malformations (%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	16.7 ± 7.8 ^c	77.8 ± 14.7 ^c
Seminal vesicle agenesis (%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4.2 ± 4.2	55.6 ± 17.6 ^c
Ventral prostate agenesis (%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	33.3 ± 16.7 ^c
Gubernacular malformations (%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	16.7 ± 7.8 ^c	88.9 ± 11.1 ^c
Total malformed in adulthood (%)	0 ± 0	0 ± 0	11.4 ± 4.8	51.4 ± 8.6 ^c	91.7 ± 5.8 ^c	100 ± 0 ^c

Data are the percent (%) of affected males unless otherwise noted.

^aData are individual rat means (± SE) with the exception of AGD, nipple retention and % total possible nipples, which are litter means (± SE); ^bNumbers in parentheses indicate the number of individuals and litters per treatment group when different than row 1; ^cValues in bold are significantly different than controls ($P < 0.05$).

mixture doses of 20–60% of top dose (Fig. 4B and C). The phthalate mixture significantly increased the incidence of seminal vesicle malformations (defined as agenesis of the seminal vesicles and/or the coagulating glands) at doses of 40 and 60% of the top dose, while SV agenesis alone was significantly increased only at 60% of top dose (Fig. 4D). Seminal vesicle asymmetry and agenesis of the vas deferens were significantly increased at doses of 40 and 60% of the top dose (Supplementary Table S2). The incidence of gubernacular malformations was significantly increased by the phthalate mixture at doses of 40% (4/24 males; 2/5 litters) and 60% of the top dose (8/9 males; 3/3 litters). Gubernacular malformations can lead to

undescended testes as was evidenced by the co-occurrence of these two malformations in 3 of 24 males (12.5%) and 6 of 9 males (66.7%) in the 40 and 60% of top dose groups, respectively. One male with gubernacular malformations in the 40% of top dose group had an undescended right testis and a left testis descended into ipsilateral scrotal sac. Hypospadias and ventral prostate agenesis were observed only at 60% of top dose (Table 3). Finally, the percent of males displaying one or more reproductive malformations was significantly increased by the phthalate mixture at doses of 20% of top dose and above (Table 3). In addition, one male in the 20% of top dose group had a developmentally-stunted tail that was approximately one-half its normal size. There was no effect of treatment on the frequency of kidney malformations in male offspring (data not shown).

Treatment with the phthalate mixture during *in utero* sexual differentiation significantly reduced male reproductive organ weights in adulthood (Table 3). The phthalate mixture at $\geq 40\%$ of the top dose significantly reduced the weights of the paired epididymides, paired testes, seminal vesicles, and ventral prostate (Fig. 5), and the LABC muscle (Table 3). Weight of the paired epididymides was the most dramatically affected organ weight measured in this study with reductions of 37.5 and 5.4% of control values observed in the 40 and 60% of top dose groups, respectively (Table 3). The weight of the paired testes from one male (7.60 g) in the 60% of top dose group was not included in the analysis because this weight was over double the weights of other paired testes at this dose; both testes from this male were undescended and fluid-filled. There was no treatment effect on the weights of the glans penis, Cowper's glands (Supplementary Table S2) or the liver (data not shown). Consistent with body weight measurements at neonatal and pubertal ages, there was no effect of treatment on the body weights of the adult male offspring at necropsy (Table 3).

Comparison of Observed Data to Mixture Model Predictions. The logistic regression parameters of the published postnatal endpoints for the individual phthalates were input into mixture models to determine whether the observed data were accurately predicted by the dose additive models, DA_{all} and DA_{ref} . We report the logistic regression parameters of the individual phthalates in Supplementary Table S3 and the logistic regression parameters of the observed mixture data and mixture model predictions in Supplementary Table S3.

The DA_{all} model was an accurate predictor of the observed effects of the phthalate mixture for 6 of the 6 endpoints with

FIG. 3. AGD on PND2 and areolae/nipple retention on PND13 in SD male rats prenatally exposed to a dose range of a 5 phthalate mixture on GD8 to PND3. Data are observed data (litter means $\pm$ 95% CI) and mixture model predictions for dose addition (DA_{all}) and RA, and DA_{ref} based on fetal T production with DBP as a reference chemical. Paired dotted lines are the 95% prediction interval of the observed data.

TABLE 3. Body and reproductive tissue weights (litter means $\pm$ SE) of adult male SD rats prenatally exposed to a dose range of a 5 phthalate mixture from GD8 to PND3

	0% of top dose	5% of top dose	10% of top dose	20% of top dose	40% of top dose	60% of top dose
Number of males (individuals, litters)	36, 6	31, 5	44, 6	35, 6	24, 5	9, 3
Body weight (g)	789.9 $\pm$ 7.0	810.0 $\pm$ 11.7	794.5 $\pm$ 14.3	743.6 $\pm$ 2.3	827.6 $\pm$ 17.1	817.4 $\pm$ 66.9
Ventral prostate (mg)	800.7 $\pm$ 53.9	764.8 $\pm$ 35.2	850.4 $\pm$ 31.6	749.2 $\pm$ 67.9	443.8 $\pm$ 42.9^a	145.7 $\pm$ 77.9^a
Seminal vesicles (mg)	2209.1 $\pm$ 108.0 (34, 6)	2144.3 $\pm$ 89.6	2123.5 $\pm$ 91.1 (43, 6)	1974.0 $\pm$ 136.7	1570.0 $\pm$ 176.0^a (22, 5)	474.3 $\pm$ 242.2^a (8, 3)
Paired testes (mg)	3961.7 $\pm$ 121.7	4071.1 $\pm$ 180.7	3948.9 $\pm$ 77.6	3804.2 $\pm$ 105.7	2869.5 $\pm$ 138.0^a	2358.3 $\pm$ 419.8^{a,b} (8, 2)
Paired epididymides (mg)	1397.6 $\pm$ 21.0	1354.2 $\pm$ 42.1	1324.6 $\pm$ 33.8	1226.6 $\pm$ 78.8 ^c	524.6 $\pm$ 123.0^a	75.0 $\pm$ 75.0^a
LABC (mg)	1507.0 $\pm$ 29.3	1496.8 $\pm$ 49.6	1469.2 $\pm$ 46.3	1477.3 $\pm$ 69.4	1222.9 $\pm$ 59.3^a	847.3 $\pm$ 206.8^a

^aValues in bold are significantly different than controls ($P < 0.05$); ^bMean and SE values without one male whose testes weights were nearly double the average for the treatment group (right testes = 3.79 g and left testes = 3.813 g); ^c $P = 0.07$ versus control.

FIG. 4. Reproductive malformations observed in adult SD male rats prenatally exposed to a dose range of a 5 phthalate mixture on GD8 to PND3: (A) % of males with nipple retention, (B) epididymal malformations, (C) testicular malformations, and (D) seminal vesicles (SV) agenesis. Data are observed data (individual means $\pm$ 95% CI) and mixture models of DA_{all} and RA, and DA_{ref} based on fetal T production and a reference chemical. DBP was the reference chemical for all malformations except nipple retention, which employed DEHP as the reference chemical. Paired dotted lines are the 95% prediction interval of the observed data.

FIG. 5. Reproductive tissue weights in adult male SD rats prenatally exposed to a dose range of a 5 phthalate mixture on GD8 to PND3: (A) paired epididymides, (B) paired testes, (C) seminal vesicles, and (D) ventral prostate. Data are observed data (individual means $\pm$ 95% CI) and mixture models of DA_{all} , RA, and DA_{ref} based on fetal T production and DBP as a reference chemical. Paired dotted lines are the 95% prediction interval of the observed data.

data for each individual phthalate in the mixture (Figs. 3–5, Table 4), including 5 endpoints whose DA_{all} ED50 predictions fell just outside of the 95% confidence interval (CI) around the observed data ED50. The RA model fell within the observed 95% CI for only one endpoint (AGD at PND2), which was also

predicted by DA_{all} . The force fit analysis indicated that DA_{all} provided a good fit for the observed data for epididymal malformations, testicular malformations, paired epididymal weight and paired testes weight; whereas RA provided a very poor fit (Table 5). AGD on PND2 was accurately predicted by both DA_{all}

TABLE 4. Summary of observed effects in SD rats prenatally exposed to a 5 phthalate mixture versus predictions of dose addition based the potency of each phthalate to reduce fetal T production data relative to a reference chemical and postnatal data for the reference chemical (DA_{ref}), or based on postnatal data for all individual phthalates (DA_{all}) as well as RA

Postnatal endpoints	Observed ED50 (% of top dose ± 95% CI ^a)	Mixture model ED50 (% of top dose)		
		DA _{ref} ^b	DA _{all}	RA
Fetal/neonatal mortality	53.3 (48.4–58.72)	64.9	— ^c	—
AGD on PND2 (% reduction from control doubled)	68.4 (43.7–106.8)	58.0	52.7	66.13
% of males with areolae/nipples on PND13	12.7 (10.4–15.5)	6.5	26.4	40.7
% of males with nipples in adulthood	26.1 (21.6–31.5)	32.2 ^d	—	—
% total possible nipples, adult males	59.2 (52.1–67.3)	64.7^d	—	—
Hypospadias	~62.4 (very wide)	56.7	—	—
Epididymal malformations	30.9 (28.0–34.2)	38.2	40.3	159.2
Testicular malformations	29.0 (26.4–31.9)	33.8	26.0	91.47
Seminal vesicle agenesis	58.4 (56.0–60.9)	52.4	—	—
Ventral prostate agenesis	60.7 (very wide)	119.2	—	—
Paired epididymides weight	34.2 (31.0–37.8)	40.7	52.2	203.2
Paired testes weight	66.2 (53.9–81.4)	79.9	50.1	157.3
Seminal vesicle weight	47.2 (42.6–52.4)	48.1	—	—
Ventral Prostate weight	42.0 (37.4–47.1)	108.9	—	—

AGD and organ weights are % reduction from controls. Fetal/neonatal mortality and reproductive malformations are % of males affected. Best fit model is bolded, italicized and highlighted in gray.

^aCI = confidence interval; ^bDA_{ref} was calculated with DBP as reference chemical for all endpoints with exception of adult nipple retention; ^cModel prediction was not calculated due to a lack of data on the endpoint for all individual phthalates in the mixture; ^dFor adult nipple retention data, DA_{ref} was calculated with DEHP as a reference chemical due to a lack of DBP data.

TABLE 5. Force fit of observed data from SD rats prenatally exposed to a 5 phthalate mixture to logistic regression parameters of mixture models for dose addition based the potency of each phthalate to reduce fetal T production data relative to a reference chemical and postnatal data for the reference chemical (DA_{ref}), or based on postnatal data for all individual phthalates (DA_{all}) as well as RA

Postnatal endpoint	Observed data (R ²)	Force fit of observed data to mixture models (R ²)		
		DA _{ref} ^a	DA _{all}	RA
Fetal mortality	0.62	0.54	— ^b	—
AGD on PND2	0.59	0.54	0.56	0.56
% Males with areolae/nipples on PND13	0.84	0.64	0.50	0.23
% Males with nipples in adulthood	0.82	0.75^c	—	—
% Total possible nipples in adulthood	0.74	0.70^c	—	—
Hypospadias	0.11	-2.7	—	—
Epididymal malformations	0.59	0.55	0.51	-0.20
Testicular malformations	0.64	0.61	0.63	-0.08
Seminal vesicle agenesis	0.45	0.37	—	—
Ventral prostate agenesis	0.32	0.15	—	—
Paired epididymal wt	0.92	0.85	0.64	-0.06
Paired testis wt	0.76	0.71	0.57	-0.08
Seminal vesicle wt	0.74	0.74	—	—
Ventral prostate wt	0.79	0.39	—	—

AGD and organ weights are % reduction from controls. Fetal/neonatal mortality and reproductive malformations are % of males affected. Best fit model (defined as the model R² fitting within 70–100% of the observed data R² value) is bolded, italicized, and highlighted in gray.

^aDA_{ref} was calculated with DBP as reference chemical for all endpoints with exception of adult nipple retention; ^bModel prediction was not calculated due to a lack of data on the endpoint for all individual phthalates in the mixture; ^cFor adult nipple retention data, DA_{ref} was calculated with DEHP as a reference chemical due to a lack of DBP data.

and RA models using both methods of comparing the mixture model predictions to the observed data (Fig. 3). While the observed data were poorly fit in the DA model for the percent of males with areolae/nipples at PND13, the data fit better to the DA model than the RA model (Table 5). For this endpoint (percent of males with areolae/nipples at PND13), the magnitude of the greater-than-dose-additive interaction did not exceed 2.9, respectively and was more pronounced in the low dose region than the high dose region.

Next, we tested whether the observed mixture data would be predicted by DA_{ref}, which is the dose addition model based on the potency of the individual phthalates in the mixture to reduce

fetal testicular T production relative to a reference chemical and postnatal data for the reference chemical. The DA_{ref} model allowed us to test of our hypothesis that fetal T production would be predictive of androgen-sensitive reproductive tract development. This analysis was necessary because a complete set of data on the 5 individual phthalates from the published literature were not available for mixture modeling on 8 of the 14 postnatal endpoints evaluated in the mixture study. DA_{ref} accurately predicted mixture responses for 11 of 14 endpoints (Figs. 3–5, Tables 4 and 5). The DA_{ref} ED50 was within the 95% CI of the observed ED50 for 4 of 14 endpoints: AGD at PND2, % of total possible nipples in adulthood, paired testes weight, and seminal

vesicle weight (Table 4). For an additional 7 endpoints, the DA_{ref} ED50s were only slightly beyond the 95% CI of the observed mixture ED50: fetal mortality, % of males with areolae/nipples at PND13 and in adulthood, malformations of the epididymides and testes, seminal vesicle agenesis, and weights of the paired epididymides (Table 4). The DA_{ref} model was also identified as a good fit for the same 11 endpoints when evaluated by force fit analysis (Table 5). DA_{ref} also accurately predicted the effects of the phthalate mixture for many of these 11 endpoints at lower response levels. For example, the prediction for DA_{ref} fell within the observed 95% CI for: paired testes weight at ED10, ED20, and ED25; epididymal malformations and paired epididymal weight at ED5, ED10, and ED20; and fetal/neonatal mortality, AGD on PND2, testicular malformations, seminal vesicle malformations, and seminal vesicle weight (% reduction) on ED5, ED10, ED20, and ED25 (Supplementary Table S5).

In addition to the postnatal reproductive tract endpoints, fetal testicular mRNA expression of *Star*, *Cyp11a*, and *Insl3* in GD18 SD rat fetuses were also predicted by DA_{ref} (Supplementary Figs. S1 and S2). In brief, fetal testicular gene expression was measured in rat fetuses exposed to a dose range of the same 5 phthalate mixture on GD8–18, identical to the published fetal T production study. The fetal gene study methods and results are located in Supplementary Materials (Supplementary Tables S6–S9). The DA_{ref} model ED50 fell within the observed 95% CI and the force fit analysis yielded a similar R² value to the observed data for the 3 genes (Supplementary Tables S10–S11).

It was not possible to predict the mixture effect for any of the mixture models for 3 endpoints: hypospadias, ventral prostate agenesis, and ventral prostate weight (Tables 4 and 5). While the DA_{ref} ED50 for hypospadias was similar to the observed data ED50 (magnitude of interaction was 0.91), it was not possible to accurately calculate the 95% CI of the observed ED50 because the variation was too great, and DA_{ref} was a very poor fit in the force fit analysis. In addition, ventral prostate agenesis and ventral prostate weight were not accurately predicted by the DA_{ref} models, when evaluated by either the 95% CI around the observed ED50 or the force fit analysis. For both ventral prostate agenesis and ventral prostate weight, greater-than-additive interactions were observed at the higher effect levels with maximum magnitudes of 3.8 (ED90) and 4.3 (ED75), respectively.

Biological Relevance of the Reductions in Fetal T Production with Postnatal Abnormalities in the F1 Males. The relationship between the reduction in fetal testicular T production and the percent of

males with reproductive malformations was fitted to a 4-parameter logistic regression (Table 6). In order to interpret the relationship between fetal T production (graphed as % of control) and the incidence of postnatal abnormalities, we fit the fetal T production without constraining the Top and Bottom (Fig. 6). The ED50 values of reduced T production versus postnatal abnormalities ranged from 23.5% (for % total malformed males) to about 75% (for % inhibition of AGD on PND2) (Fig. 7; Supplementary Fig. S3). The inclusion of nipple retention in adult males in the total malformations in adulthood resulted in a lower ED50 than total malformations in adulthood without nipple retention to reductions in fetal T production (Table 6). Similarly, the number of males with nipple retention on PND13 was the most sensitive of all the malformations evaluated to reductions in fetal T production. In contrast, the total number of possible nipples (or areolae) on PND13 provided a measure of the magnitude of the effect (ie, one areolae or nipple to a maximum of 12 areolae or nipples), and was not as sensitive to

FIG. 6. Fetal testicular T production (A; expressed as % of control) on GD18 in male SD rats prenatally exposed to a dose range of a 5 phthalate mixture on GD8 to 18 (data previously published in Howdeshell et al., 2008), and best fit and goodness of fit (R²) values of the observed data (B) as fit to a sigmoidal dose response (variable slope) logistic equation.

TABLE 6. Percent reduction in fetal T production that altered male reproductive tract development in male SD rats prenatally exposed to a 5 phthalate mixture on GD8 to PND3

	% reduction in fetal T production		
	ED10 ^b	ED50	ED90
AGD at PND2 (% reduction doubled)	11.8	74.9	>100
Nipple/areolae retention at PND13 (% of 12 possible)	19.5	45.6	>100
Epididymal agenesis/underdevelopment	21.5	40.9	77.9
Testicular malformations	21.7	38.3	67.7
Seminal vesicle malformations	60.1	71.6	85.3
Total malformations in adulthood (with nipple retention)	9.0	23.5	61.3
Total malformations in adulthood (without nipple retention)	23.2	38.1	62.7

^aValues interpolated from the logistic regressions with % reductions in fetal T production on the x-axis and % affected males (except where noted otherwise) on the y-axis; ^bEffective dose (ED) to produce malformations in individual males (or litters for AGD and nipple retention).

FIG. 7. Relationship between the percent reduction in fetal T production on GD18 and the induction of reproductive tract malformations in male SD rats prenatally exposed to a 5 phthalate mixture on GD8 to PND3. Data for the percent reduction in fetal T production (Howdeshell *et al.*, 2008). Malformation data are individual means $\pm$ SE, except for reduction in AGD and measures of nipple retention (at PND13 or adulthood) are litter means $\pm$ SE. Data include: total malformed adult males with nipple retention (A) and without nipple retention (B), epididymal malformations (C), testis malformations (D), seminal vesicle malformations (E), % reduction in AGD on PND2 (doubled) (F), % of males with areolae/nipples on PND13 (G), and % total possible nipple retained on PND13 (H).

reductions in fetal T as the incidence of males with areolae or nipples. The percent reduction in fetal T production that lead to an increase in the total possible nipples at PND13 (ED50 affected males = 45.6% reduction in fetal T) was similar to the ED50 for total malformations in adulthood without nipple retention (ED50 affected males = 38.1% reduction in fetal T) (Table 6).

DISCUSSION

The mathematical models predicting the developmental effects of mixtures of chemicals that disrupt androgen-signaling on postnatal reproductive tract signaling have largely been based on data on postnatal effects for the individual chemicals (Christiansen *et al.*, 2009; Hass *et al.*, 2007; Rider *et al.*, 2010, 2009). Our laboratory previously reported that a binary combination of

DBP and DEHP, two phthalates with similar mode of action but different active metabolites, acted in a cumulative dose-additive fashion to reduce fetal testicular T production and induce reproductive malformations as well as decreased androgen-sensitive reproductive organ weights in male rats (Howdeshell et al., 2007). Cumulative and dose-additive effects, respectively, were also observed with binary and more complex mixtures of phthalates with chemicals that disrupt androgen-signaling via different mechanisms (BBP and linuron [Hotchkiss et al., 2004], DBP and procymidone [Hotchkiss et al., 2010], and complex mixtures [Christiansen et al., 2009; Rider et al., 2008, 2010]).

In this study, we extended the results of our previous mixtures studies by testing a phthalate mixture where the ratio of the individual chemicals in the mixture was based on their ability to reduce fetal T production due to the importance of proper androgen-signaling during the period of sexual differentiation. Consistent with our hypothesis, we observed that the phthalate mixture acted in a dose additive manner to suppress androgen-dependent reproductive tract development 11 of 14 endpoints (Tables 4 and 5). We assessed dose addition by using 2 models of dose additivity: one requiring data for each individual phthalate of the mixture (DA_{all}) and another model based on the potency of the individual phthalates to reduce fetal T and a reference chemical for all endpoints (DA_{ref}). DA_{all} more accurately predicted the observed phthalate effect than did RA for all 6 endpoints with a complete set of individual phthalate data. The DA_{ref} model allowed us to expand our evaluation of dose additivity of the phthalate mixture to additional reproductive endpoints that did not have a complete set of individual phthalate data. In total, DA_{ref} provided an accurate prediction of the observed phthalate mixture effect for a total of 11 of 14 endpoints, including all 6 endpoints evaluated by DA_{all}. Thus, the relative potency factors of the phthalates to inhibit fetal T production were useful in accurately estimating the postnatal effects of *in utero* exposure to reproductive toxicant phthalates.

In addition to the postnatal reproductive endpoints, reductions in the fetal testicular mRNA of genes responsible for cholesterol transport, androgen synthesis and *Ins13* were accurately predicted by based on fetal T production (DA_{ref}) (Supplementary Table S10 and S11). Fetal gene expression data of this study corroborate a recent study from our laboratory using the real-time PCR which demonstrated that DA accurately predicted the effects of a 9 phthalate mixture to inhibit fetal gene expression, including *Ins13*, *Star*, and *Cyp11a* among other genes when administered to rat dams on GD14-18 (Hannas et al., 2012). Similar to this study, the ratio of the 9 phthalate mixture dose was based on the ability of the individual phthalates to reduce fetal T production during the period of sexual differentiation.

Phthalate mixture effects on three endpoints were not accurately predicted by the mixture models: hypospadias, ventral prostate agenesis, and ventral prostate weight. Hypospadias and ventral prostate agenesis are generally induced by phthalates at high doses, which makes them difficult to model due to very few doses with a measurable effect. Hypospadias is also a very subtle effect and this malformation can easily be overlooked during necropsy. For example, the 5 phthalate mixture induced hypospadias in only 11.1% of the males at the 60% of the top dose of the 5 phthalate mixture (780 mg/kg/d total phthalates) compared with a 42.9% incidence previously reported for 750 mg DBP/kg/d (Mylchreest et al., 1998). The reduction in ventral prostate weight exceeded the predictions of DA_{ref} in this study. However, in previous mixture studies of phthalates (with each other and/or other anti-androgenic

chemicals), we have observed that DA alone (Rider et al., 2008) or both DA and RA (Howdeshell et al., 2007) accurately predicted the reduction in ventral prostate weight. Inter-laboratory differences in ventral prostate dissection could also have contributed to lack of fit to the mixture model predictions as the mixture models were based on data for DBP collected in another laboratory (Mylchreest et al., 1998). Thus, the incidence of hypospadias and ventral prostate agenesis, and the reduction in ventral prostate weight by phthalate mixtures may be challenging to accurately model due to the high dose required to induce the effect (in the case of hypospadias and ventral prostate agenesis) and possible inter-lab differences in assessing these three malformations.

Fetal mortality has been observed following gestational exposure to high doses of individual phthalates, including: DBP (Ema et al., 2000; Gray et al., 2006a), BBP, DiBP and DPP (Howdeshell et al., 2008b; Tyl et al., 2004). In this study, fetal/neonatal mortality induced by the phthalate mixture was accurately predicted by the DA model and was similar to the fetal/neonatal mortality incidence (58%) observed in the previous phthalate mixture fetal study (Howdeshell et al., 2008b). The increase in fetal/neonatal mortality may be due to direct effects of phthalates on maternal endocrinology and/or on fetal development. Female Long Evans rats chronically exposed to DBP (500 and 1000 mg/kg/d) from weaning through multiple pregnancies exhibited reduced serum levels and ovarian production of progesterone, which was associated with decreased litter size (Gray et al., 2006a). Phthalate-induced reductions in fetal body weights have been reported for BBP at 750 mg/kg/d (Tyl et al., 2004), DBP at 500 mg/kg/d (Saillenfait et al., 1998), and DiBP at 625 mg/kg/d (Saillenfait et al., 2006), when administered over a comparable dosing period as the current study. Such reductions in fetal body weights may contribute to the reduced viability of the fetus. However, other phthalate studies have not observed a decrease in fetal body weights (Gray et al., 2006a; Mylchreest et al., 1998). Further research is needed to understand the mechanism by which phthalates induce fetal mortality.

Relatively high doses of chemicals are used to evaluate whether chemicals exhibit joint action (eg, in this study and others Christiansen et al., 2009; Rider et al., 2010). However, dose-additive effects on male reproductive endpoints have been observed using mixtures of anti-androgenic chemicals (including phthalates) at environmentally relevant levels (Axelstad et al., 2014; Christiansen et al., 2012; Isling et al., 2014). Furthermore, 2 recent cumulative risk assessments reported that current levels of human exposure to 6 phthalates or a mixture of 15 anti-androgenic chemicals (including phthalates) at environmentally relevant levels determined that individuals with the highest exposures to the individual chemicals were exceeding the cumulative Tolerable or Acceptable Daily Intake, respectively (Koch et al., 2011; Kortenkamp and Faust, 2010). Of note, the U.S. Consumer Product Safety Commission's Chronic Hazard Advisory Panel On Phthalates/ Phthalate Alternatives recently used our previously published fetal T production data for the individual phthalates in one of three methods for determining individual hazard indices for specific phthalates; they determine that 10% of pregnant women and 5% of mothers and infants exceed the Hazard Index for phthalate exposure (Lioy et al., 2015).

The biological relevance of the reduction in fetal T production induced by exposure to the phthalate mixture could be useful in predicting the postnatal abnormalities that would be induced by untested phthalates or phthalate mixtures in a quantitative rather than qualitative manner (Fig. 7). In this

study, the ED50 for total malformations in adulthood including the percent of adult males with nipple retention was a more sensitive effect than the total malformations without the percent of adult males with nipple retention for the effects of a reduction in fetal T production (Table 9). Consistent with other studies from our laboratory, we found that nipple retention in juvenile or adult males was a very sensitive and, often, a very subtle effect (eg, 1 faint areolae or 1 nipple) (Fig. 7, Table 7) of exposure to phthalates. In addition, we observed that the percent reduction in fetal T production related to an ED50 for nipple retention in juvenile males was similar to the ED50 for induction of total malformations (not including nipples) in adult males treated with the phthalate mixture. The results of the current study are being compared with similar analyses of reductions in T production versus postnatal outcomes in several additional studies in our laboratory to determine the reproducibility and precision of this relationship (Earl Gray, unpublished data).

In conclusion, dose addition based on fetal T production data of the individual phthalates (DA_{ref}) accurately predicted the effects of *in utero* exposure to a 5 phthalate mixture to suppress expression of steroidogenic pathway and *Ins3* genes and androgen-sensitive postnatal reproductive development in male rats. This study demonstrated that even with limited postnatal reproductive data for individual phthalates, our previously published determinations of phthalate potency ratios for inhibition of fetal T production could be utilized to accurately predict mixture effects. Our laboratory is continuing to study potential of other previously uncharacterized phthalates to inhibit fetal T production in rats (Furr *et al.*, 2014). These fetal T production studies along with further investigations into the biological relevance of fetal T reduction may be useful in assessing the cumulative risk of phthalates in humans (Lioy *et al.*, 2015).

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SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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