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Associations between prenatal phthalate exposure and sex-typed play behavior in preschool age boys and girls

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Abstract

Phthalates, a class of chemicals found widely in consumer products including plastic toys, food contaminants and food packaging, personal care products, cosmetics, air fresheners, and some medications, have been shown to be anti-androgenic in numerous laboratory and epidemiological studies. In a prior cohort enrolled in 2000–2002 we observed associations between prenatal urinary concentrations of di-ethyl hexyl phthalate (DEHP) and dibutyl phthalate (DBP) metabolites and less male-typed play behavior in preschool age boys. The aim of this study was to examine phthalate exposure in pregnancy in relation to play behavior at age 4 years in a larger cohort of pregnant women enrolled in The Infant Development and the Environment Study (TIDES) between 2010 and 2012 at four study sites (Minneapolis, MN; Rochester, NY; San

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Declaration of competing interest

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

Credit author statement

Sarah Felice Evans: Conceptualization, Methodology, Formal analysis, Writing - original draft. Samantha Raymond: Validation, Writing-Review and Edit. Swathi Sethuram: Validation. Nicole R. Bush: Resources, Writing- Review and Edit. Sheela Sathyanarayana: Resources, Writing-Review and Edit. Emily Barrett: Resources, Writing-Review and Edit. Ruby Nguyen: Resources, Writing-Review and Edit. Shanna H. Swan: Funding acquisition, Conceptualization, Supervision, Writing Review and Edit

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2020.110264.

Francisco, CA; Seattle, WA). Maternal urinary metabolites of DEHP, DiBP, DnBP, BBzP, and DEP were measured during the first (n=498) and third trimester (n=468) and mothers completed the Preschool Activities Inventory (PSAI), a validated maternal questionnaire designed to assess child toy preference and sex-typed play behavior when children were 4–5 years of age. After adjusting for child age, maternal education, race, urine dilution, parental attitudes about opposite sex-typed play behavior, and presence of a same sex older sibling, we observed associations between first trimester (mean 10.7±2.1 weeks gestation) (log10) SpG-adjusted MnBP, MiBP, and MBzP and lower masculine scores in boys (β-coefficient [95% confidence intervals]: MnBP −2.18, [−4.16, −0.20]), MiBP −2.1[−4.3,0.1], and MBzP −2.42 [−4.12, −0.71]). In girls, first trimester maternal urinary MBzP was associated with lower masculine scores (−2.12 [−3.98,−0.25]), while third trimester (mean 32.8±3.0 weeks gestation) maternal urinary MiBP was associated with higher masculine scores (2.69 [0.68,4.70]). Third trimester maternal urinary phthalate levels were not associated with play behavior in boys. These findings in boys are largely consistent with previous studies that report that prenatal phthalate exposure is associated with less masculine play behavior. No associations in girls have been previously reported.

Keywords

Phthalate; Play behavior; Sex-dimorphic; Prenatal exposure; Endocrine disruption

1. Introduction

Phthalates are a class of endocrine disrupting chemicals widely used in consumer products as both a plasticizer and a component of synthetic fragrance. Common sources of potential phthalate exposure include toys, building materials, food contaminants and food packaging, nail polish, air fresheners, cosmetics, some medications, and personal care products. The Centers for Disease Control and Prevention (CDC) National Health and Nutrition Examination Survey (NHANES) measured detectable urinary concentrations of several phthalate metabolites in greater than 90% of adult and child participants (CDC, 2019).

Several phthalates have been classified as anti-androgens due to their ability to inhibit fetal testosterone production and disrupt male reproductive development in rodents and humans (Gray et al., 2006; Howdeshell et al., 2008). Our prior studies show an association between first trimester maternal urinary phthalate metabolites and reduced anogenital distance (AGD) in boys at birth and one year of age (Swan et al. 2005, 2015; Swan, 2008). AGD is androgen dependent; females of most mammalian species who normally experience lower *in utero* testosterone exposure have shorter anogenital distances than males (Vandenbergh and Huggett, 1995). *in utero* manipulation of testosterone with pharmacological testosterone antagonists results in shorter AGD in male rats (Kita et al., 2016). These studies provide evidence of anti-androgenic effects of phthalate exposure in early pregnancy.

In addition to affecting reproductive development, *in utero* androgen exposure influences brain development and gender phenotypical behaviors (Hines et al., 2015; Khorashad et al., 2018). Prenatal phthalate exposure is associated with more problem behaviors such as aggression, inattention, and depression, with most associations in boys, suggesting sex-

specific impacts on brain development (Engel et al., 2010; Kobrosly et al., 2014; Whyatt et al., 2012). Given the observed anti-androgenic effects of prenatal phthalate exposure on reproductive development, we asked whether sex-typical play behaviors were also impacted. Previous studies have shown that exposure to testosterone during critical windows of fetal and infant development is robustly associated with sex-typed play behaviors in childhood (Auyeung et al., 2009; Hines, 2006). The testosterone surge that occurs in the male fetus during gestational weeks 8–12 is hypothesized to influence development of sexually dimorphic brain regions (Hines, 2015).

Associations between *in utero* exposure to endocrine disrupting chemicals including phthalates, PCBs, and dioxins and altered sex-typed play behavior in school age children have been reported (Vreugdenhil, 2002; Swan, 2010, Winneke, 2014). Using the Preschool Activities Inventory (PSAI), a maternally-rated survey that assesses maternal perceptions of child activity and toy choice, we previously reported that higher levels of prenatal maternal urinary DEHP and DBP metabolites were associated with less male phenotypical play in boys at age 4 (Swan et al., 2010). This earlier study was limited by the small size of the cohort and availability of a single urine sample obtained relatively late in pregnancy (mean 28.6 weeks). In the current study, we examine the association between prenatal phthalates measured at two different time points during pregnancy and play behavior in boys and girls in a larger cohort. We hypothesized a priori that associations would be stronger in boys than in girls and in early pregnancy compared with later pregnancy.

2. Methods

2.1. Study participants

The Infant Development and the Environment Study (TIDES) is a multi-center prospective pregnancy cohort study. Women were recruited into TIDES between August 2010 and August 2012 at prenatal clinics at San Francisco, CA (University of California, San Francisco, UCSF), Rochester, NY (University of Rochester Medical Center, URMC), Minneapolis, MN (University of Minnesota, UMN), and Seattle, WA (University of Washington/Seattle Children's Hospital, UW/SCH). Women over 18 years old able to read and write English (or Spanish at the CA center), <13 weeks pregnant, whose pregnancy was not medically threatened, and who planned to deliver in a study hospital were eligible to participate. Participants provided urine samples, completed a questionnaire in each trimester, and gave a serum sample in the first trimester. Details on the population and study methods are reported elsewhere (Barrett et al., 2014). The institutional review board (IRB) at all participating institutions approved TIDES prior to study implementation and all subjects provided signed informed consent before starting any study activities. IRB approval was also obtained at the Icahn School of Medicine at Mount Sinai, which served as the TIDES Coordinating Center after 2011. Of 969 pregnant women who enrolled in TIDES, 753 provided a first trimester urine sample and 787 had a live birth. Here we report on 498 mother-child pairs who provided a first trimester urine sample and completed the PSAI when the child was approximately 4 years old. We also report on 468 of these women who also provided a third trimester urine sample. For comparison of study participants included in this analysis with those not included, see Supplemental Tables 1 and 2

2.2. Maternal urinary phthalate concentrations

Urine samples were collected in phthalate-free polypropylene cups and all collection and storage materials were shown to be phthalate-free. Specific gravity (SpG) was measured within 30 min using a hand-held refractometer. (National Instrument Company, Inc., USA) which was calibrated with deionized water before each measurement. Ambient temperature at SpG determination was noted and adjustment was made if required. Samples were stored at −80 °C. All samples from mothers of boys were analyzed at the Division of Laboratory Sciences, National Center for Environmental Health, CDC using an analytical approach that involves the enzymatic deconjugation of the metabolites from their glucuronidated form, automated online solid-phase extraction, separation with high performance liquid chromatography and detection by isotope-dilution tandem mass spectrometry (Silva et al., 2007). Samples from mothers of girls were analyzed in two laboratories: the CDC and the Environmental Health Laboratory at the University of Washington (UW). Most samples from mothers of girls (N =196, 77%) were measured at the UW. At this laboratory glucuronidated phthalate monoesters underwent enzymatic deconjugation, followed by online solid-phase extraction (SPE) coupled with reversed high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) to quantify the simple monoesters in urine (Calafat, 2010). Approximately one quarter of the samples from mothers of girls (N = 59, 23%) were measured at the CDC using the method described above. In both laboratories, procedure blanks were run with each batch of samples and isotopically labeled internal standards were used along with conjugated internal standards to increase precision and accuracy of the measurements. Ten urine samples were selected at random and analyzed at both laboratories for comparison. Phthalate measurements did not vary significantly across laboratories (Supplemental Table 3). For statistical analyses, values below the level of detection (LOD) were assigned the value LOD divided by the square root of 2, as has been recommended (Hornung and Reed, 1990).
In this study, we examined associations between PSAI questionnaire scores and urinary metabolites of phthalates previously shown to be anti-androgenic (Howdeshell et al., 2008; Radke et al., 2018). These include BBzP, DnBP, DiBP, DEP, and the molar sum of the four measured DEHP metabolites (ΣDEHP). Molar sums were calculated using the following equation (Wolff et al., 2008):

$$\text{molar}\sum\text{DEHP} = (\text{MEHP} * (1/278)) + (\text{MEHHP} * (1/294)) + (\text{MEOHP} * (1/292)) + (\text{MECPP} * (1/308))\text{nmol/mL}$$

2.3. Preschool Activities Inventory

The PSAI is a validated parent-rated survey designed to discriminate types of play behavior both within and between the sexes, and has been standardized on children in the UK, the Netherlands, and the US (Golombok and Rust, 1993a; Golombok et al., 2008). When children were approximately 4 years old, TIDES mothers were asked to complete the PSAI as well as a brief questionnaire that included questions on other relevant covariates such as child's age, number and age of siblings, parental education, and questions on parental attitudes towards male phenotypical toy choice. The PSAI consists of 24 items (12 considered 'feminine' and 12 'masculine') addressing three aspects of play behavior: type

of toys, activities, and child characteristics. Answers are given on a 5-point, Likert-type scale ranging from ‘never’ to ‘very often’. Consistent with our previous study (Swan et al., 2010), an item analysis of each of the 24 PSAI questions found no difference by sex in the mean or median values for the “feminine” question ‘avoids taking risks’ (mean ± SD: 4.1±0.8 for boys and 4.1 ± 0.9 for girls) and the “masculine” question ‘likes to explore new surroundings’ (mean ± SD: 2.6 ± 0.9 for boys and 2.7 ± 1.0 for girls). As these items were not sex-dimorphic in our sample, they were dropped in this as in the prior analysis, reducing the total number of items to 22 and forming the modified instrument (PSAI-M), which we used in all analyses. We examined masculine and feminine subscale scores as well as a composite score transformed to a pseudo-T scale using the formula: sum of scores for masculine items - sum of scores for feminine items + (48.1 × 1.1) (Golombok and Rust, 1993b). A higher composite score implies more male-phenotypical play behavior and a lower score implies more female-phenotypical play behavior. Higher scores on the masculine subscale indicates more male-phenotypical play behavior while higher scores on the feminine subscale indicates more female-phenotypical play behavior.

2.4. Parental attitudes scale

Parental attitudes towards opposite sex-typed play may influence toy availability, child’s choice of activities, or maternal PSAI responses (Kollmayer et al., 2018). To account for this, we designed a series of questions to assess parental attitudes towards stereotyped sex-atypical toy choice (Swan et al., 2010). The mother was asked, ‘What would you do if you had a boy who preferred toys that girls usually play with?’ The five possible responses ranged from ‘strongly encourage [him to play in this way]’ to ‘strongly discourage’. She was also asked how she thought the father of the child would respond to these questions. The five possible responses for each parents’ attitude were coded 1–5 and summed for each parent, resulting in a parental attitude-boys (PAB) score. A similar scale (PAG) was constructed for girls. Lower parental attitude scores indicate that the parents are more encouraging of opposite gender play, while higher scores indicate that parents are more discouraging of such behaviors. PAB was included when modeling boys’ behaviors in relation to phthalate metabolite concentrations and PAG when modeling girls’ (Swan et al., 2010).

2.5. Statistical methods

We used specific gravity to adjust for urinary dilution using the formula: $Psg = P[(SG_{cohort} - 1)]/(SG - 1)$, where P_{sg} is the specific gravity-adjusted concentration, P is the metabolite concentration (μg/L), SG_{cohort} is the average specific gravity of all samples in the cohort, and SG is the specific gravity for the sample(Swan et al., 2015). Because phthalate metabolite distributions are extremely skewed, specific gravity-adjusted metabolite concentrations were $\log_{10}$-transformed in all analyses. Sensitivity analyses including the full 24 item PSAI scale were also conducted. Because covariates and phthalate concentrations at one study site (URMC) differed significantly from the other 3 study sites, we conducted center-stratified (URMC vs. Other) sensitivity analyses. After fitting sex-stratified unadjusted linear regression models examining relationships between phthalate metabolites and the PSAI-M masculine, feminine, and composite scores, we fit sex-stratified multiple regression models. Linear regression including phthalate by child sex interaction terms were also evaluated to examine effect modification by sex. Given the sex-specific

patterns of association between maternal education and PSAI scores, we also conducted linear regression using the augmented product interaction term approach recommended by Buckley (Buckley et al., 2017). Covariates initially considered in these analyses were child age at PSAI, maternal age at urine collection, maternal education, presence of same and opposite sex older siblings, race, ethnicity, and parental attitudes. Covariates found to be significantly associated ($p < 0.05$) with both the exposure and outcome in boys or girls (race, parental attitudes, same sex older sibling, and maternal education) were included in the final model. We also adjusted for age given the known association between child age and PSAI (Golombok et al., 2008). To address potential selection bias we used a multiple imputation procedure (Biering et al., 2015; Jannat-Khah et al., 2018) with fully conditional specification to account for missingness in PSAI scores and additional covariates collected at the age 4 visit (older same sex siblings, parental attitude scores, maternal education, and child age). The discriminant function method was used for categorical variables and the predictive mean matching method was used for continuous variables, in order to impute values consistent with observed values. Twenty imputations were run for each model. The imputation model included all variables in the analytical model as well as five auxiliary variables: maternal age, maternal education at birth, center, gestational age at birth, and gestational age at urine collection. These five auxiliary variables were chosen due to their association with missing PSAI scores. This multiple imputation procedure was run for phthalate metabolites and outcomes for which we saw associations in the study sample. We used Pearson's Chi Square to test associations between categorical variables, and two-sided independent sample T-tests to test associations between continuous variables. All analyses were performed using SPSS 25.0 (SPSS Inc. 2017) and verified in SAS version 9.4 (SAS, Cary, NC) by an independent analyst.

3. Results

The final study population consisted of 243 boys and 255 girls for whom demographic data were available and whose mothers provided urine samples during pregnancy and completed the PSAI when the child was approximately 4 years old (mean=4.5 years, Table 1). The majority of mothers had at least a college education (81%) and were Caucasian (69%). Approximately one quarter of boys and girls had an older sibling of the same sex (24.7% of boys and 25.1% of girls). There were no significant differences between boys and girls in child age, center, maternal age, maternal education, race, ethnicity, presence of an older male or female sibling, or gestational age at urine collection (Table 1).

Mean PSAI-M score for masculine, feminine, and composite scores are shown in Table 2. As expected, boys' mean masculine and composite scores were significantly higher than girls' (38.1±6.2 vs. 28.6±6.4; $p < 0.0001$), while girls' mean feminine scores are significantly higher than boys' (41.0±6.6 vs. 25.1±5.7; $p < 0.0001$). Parents of boys were significantly more discouraging than parents of girls towards opposite sex typical play (5.7±1.9 vs. 4.8±1.6; $p < 0.0001$). Mean first trimester maternal urinary concentrations for metabolites of DEHP, DiBP, DnBP, BzBP, and DEP are shown in Table 3. Phthalate metabolite concentrations did not differ significantly in mothers of boys and girls.

We fit sex-stratified univariate regression models to identify predictors of play behavior in boys and girls. Boys with an older male sibling had significantly higher masculine scores (β-coefficient [95% confidence interval]: 2.03[0.23,3.83]). Parental attitudes that were more discouraging of opposite sex-typed play, older child age, and being black were associated with lower feminine scores in boys (−1.10 [−1.46,−0.75], −2.14 [−4.17,−0.12], and −4.51[−6.67,−2.35] respectively) (Table 4). Girls who were Black, whose parents were more discouraging of male-typical play, or who had an older female sibling, had lower masculine scores (−2.77 [−5.27,−0.27], −0.87 [−1.36,−0.38], and −2.41 [−4.21,−0.62] respectively). Girls whose mothers had at least a college education had lower feminine scores than girls whose mothers had less than a college education (−2.41[−4.50, −0.32]) (Table 4).

In boys, first trimester maternal urinary MnBP, MiBP, MBzP, and MEP were significantly associated with lower masculine scores (−2.46 [−4.39,−0.52], −2.53[−4.70,−0.37], −2.61 [−4.28,−0.93], and −1.71 [−3.14,−0.27] respectively), and MnBP was associated with lower feminine scores (−1.91[−3.70,−0.11]) in unadjusted models (Table 5). First trimester associations were similar in magnitude for MnBP, MiBP, and MBzP and masculine scores in boys (−2.18 [−4.16,−0.20], −2.1 [−4.3,0.1], and −2.42 [−4.12,−0.71] respectively) in multivariable models adjusted for child age, race, maternal education, parental attitudes towards opposite gender play, and older male sibling. Before adjustment, MnBP was inversely associated with feminine scores in boys (−1.91 [−2.70,−0.11]), a relationship that was attenuated after adjusting for covariates (−0.7[−2.4,1.1]) (Table 5). No associations were seen for any phthalate metabolite and composite scores in boys, therefore composite score was excluded from all further analyses (Table 5). Sensitivity analyses including PSAI scores calculated from the full 24 item PSAI scale result in similar findings as analyses with PSAI-M scores (Supplemental Table 4).

In girls, maternal first trimester urinary MBzP was associated with lower masculine scores in unadjusted (−2.69[−4.42,−0.96]) and adjusted (−2.12[−3.98,−0.25]) models (Table 6). No associations were observed for any phthalate and feminine scores or composite scores in girls (Table 6). Sensitivity analyses including PSAI scores calculated from the full 24 item PSAI scale yielded similar findings as analyses with PSAI-M scores (Supplemental Table 5).

In analyses of phthalate metabolites and play behavior in boys, regression coefficients were negative and similar in magnitude for all metabolites (Table 5). In order to better characterize the magnitude of change in PSAI score associated with phthalate exposures, we used our multivariable regression model to calculate PSAI score for an average boy or girl in the study as in our prior study Swan et al., 2010. Fig. 1 shows the percent change in masculine score for an increase in phthalate metabolite concentration from the 10th to the 90th percentile for an average participant. Asterisks indicate associations that were significant ($p < 0.05$) in the final multivariable model.

To examine modification by sex, we included a phthalate by sex interaction term in multivariable linear regression models. When pooling boys and girls together, ΣDEHP and MBzP were significantly associated with lower masculine scores (−1.9[−3.7,−0.1] and −3.4[−4.9,−1.9] respectively) (Supplemental Table 6). These associations were attenuated

after adjusting for sex, child age, maternal education, race, parental attitudes, older same sex sibling, and phthalate by sex interaction term. Phthalate by sex interaction terms were not significant in any model.

Given the sex-specific patterns of association between maternal education and PSAI scores (Table 4) we also conducted linear regression using the augmented product interaction term approach recommended by Buckley (Buckley et al., 2017). Again associations were attenuated in the adjusted model that included covariates, and phthalate by sex and maternal education by sex interaction terms. (Supplemental Table 7). Maternal education by sex interaction terms were significantly ($p < 0.01$) associated with feminine score in all phthalate models (Supplemental Table 7).

We also examined associations between third trimester maternal urinary phthalates and play behavior. Third trimester phthalate metabolite concentrations are shown in Supplemental Table 8. There were no significant associations between third trimester urinary phthalate metabolites and PSAI scores in boys (Supplemental Table 9). Third trimester maternal urinary MiBP was associated with higher masculine scores in girl in unadjusted and adjusted models (2.51 [0.46,4.56] and 2.69 [0.68,4.70] respectively) (Supplemental Table 10).

Because participants at the URMC study site differ significantly from participants at other sites in several aspects, we conducted sensitivity analyses stratified by study site. Participants at URMC were younger at time of childbirth (33.0 vs. 37.7 years), more likely to be Black (37.2% vs. 3.2%) or Hispanic (19.0% vs. 7.7%) and to have an older brother (31.4% vs. 24.9%) or sister (35.5% vs. 22.5%), and less likely to have a college education than participants at the other three sites combined (47.1% vs. 92.5%) (Supplemental Table 11). In addition, urine samples were collected slightly earlier at URMC than at other sites (10.0 vs. 11.0 weeks and 31.3 vs. 33.2 weeks), and maternal urinary concentrations of MiBP (6.9 vs. 4.4 ng/mL), MnBP (11.8 vs. 7.0 ng/mL), MBzP (6.4 vs. 3.6 ng/mL), and MEP (41.4 vs. 29.7 ng/mL) were significantly higher at URMC (Supplemental Table 12). Feminine scores in boys were significantly lower (23.6 vs. 25.6), and parents of both boys and girls were more discouraging of opposite sex-typical play than at other study sites (6.7 vs. 5.3 for boys and 5.4 vs. 4.6 for girls) (Supplemental Tables 13 and 14). For these reasons we conducted sensitivity analyses stratified by URMC versus the three other study sites combined. While univariable associations with covariates differed by study site (Supplemental Tables 15 and 16), multivariable associations between MnBP (−3.6 [−7.9,0.8] vs.−2.1[−4.4,0.1]), MiBP (−2.4[−7.2,2.4] vs −2.0[−4.6,0.5]), and MBzP (−3.6[−7.3,0.1] vs. −2.2[−4.1,−0.2]) and masculine score in boys were similar to or slightly greater in magnitude at URMC compared with the other sites combined (Supplemental Table17). Likewise, inverse associations between MBzP and masculine score in girls were observed in both groups, albeit attenuated for URMC (−1.7[−6.7,3.3] vs. −2.2[−4.2,−0.1]) (Supplemental Table 18).

4. Discussion

Sex specific organization of the brain depends in part on exposure to sex hormones *in utero* (Hines et al., 2015). In this study we examined whether prenatal exposure to phthalates,

known anti-androgens, is associated with differences in parent-reported play behavior in boys and girls at age 4–5 years. We hypothesized that exposure to phthalates during hormone-sensitive periods of fetal development would be associated with less masculine play behaviors in boys. We replicated our previous finding that DBP metabolites are associated with less masculine play in boys, and observed associations between BBzP metabolite concentrations and less masculine behavior in both boys and girls that had not been observed previously. In addition, we observed trimester-specific effects, with associations seen in boys in the first trimester only, while third trimester MiBP was associated with higher masculine score in girls.

Sexual differentiation of the brain begins during the prenatal period and is dependent on gonadal hormones. *In utero* testosterone exposure has been shown to influence play behavior in rodents, monkeys, and humans, suggesting that phthalates may alter development of brain regions involved in sex-typical play (Auyeung et al., 2009; Hines, 2006; Thornton et al., 2009). Prenatal phthalate exposure may also act through a non-testosterone dependent mechanism to influence play. In this study we observed for the first time an association between DEP and less masculine play in boys. DEP has been categorized as a weaker anti-androgen then DBP or DEHP in animal models (Radke et al., 2018) and has not previously been shown to be related to either the phthalate syndrome or play behavior in humans (Swan et al., 2010). We also observed associations between first trimester BBzP and less masculine play and third trimester DnBP and more masculine play in girls. Although our previous study did not find associations between phthalates and play behavior in girls, associations between prenatal phthalates and cognition and neurobehavior have been observed in girls (Tellez-Rojo et al., 2013; Zhang et al., 2019). The mechanism by which phthalates may affect gender-stereotyped behavior in girls is not clear, however evidence for a role of prenatal testosterone is shown in Congenital Adrenal Hyperplasia (CAH) females who are exposed to high levels of testosterone *in utero* and engage in more masculine play behaviors (Nordenstrom et al., 2002; Pasterski et al., 2011).

An additional mechanism by which phthalates may alter brain development is disruption of thyroid hormone signaling. Inverse associations between prenatal MEHP, MEP, MBzP, and MBP and thyroid hormone levels in maternal serum and cord blood have been reported, and thyroid hormone disruption has been hypothesized to mediate phthalate effects on behavior and cognition (Huang et al., 2007; Mughal et al., 2018; Romano et al., 2018). Sex-specific associations between child urinary phthalate concentration and thyroid hormone levels have been observed, suggesting that phthalates may act through thyroid hormone to differentially impact fetal brain development in males and females (Morgenstern et al., 2017).

We observed associations between MiBP, MnBP, and MBzP, and masculine play in boys. As shown previously, these metabolites were highly significantly correlated in our population (r=0.63 for MnBP and MiBP; r= 0.56 for MBzP and MnBP; r=0.44, MBzP and MiNP), suggesting possible co-exposure through common sources (Frederiksen et al., 2010). Because MBzP and MnBP are correlated and may share the parent phthalate BBzP, we conducted analyses jointly adjusting for MBzP and MnBP. We found no appreciable difference in phthalate associations with masculine score in boys or girls, suggesting that both MBzP and MnBP are independently associated with play behavior (Supplemental

Table 19). In contrast to our earlier study (Swan et al., 2010), we did not observe a significant association between prenatal maternal DEHP metabolites and masculine play in boys. DEHP exposure has decreased in the general population over time (CDC, 2019), raising the possibility that lower DEHP exposure in the TIDES cohort accounts for the lack of association. A comparison of urinary phthalate metabolite concentrations in the TIDES cohort with those in our Study for Future Families (SFF) cohort (recruited ten years earlier) show a greater than 50% reduction in all metabolites with the exception of the DBP metabolite MiBP, which increased by 48% (Swan et al., 2015). These observations reflect exposure trends in the US population over the same time period (CDC, 2019), likely due to changes in product formulation resulting from consumer pressure and the restriction of phthalates in some children's items implemented by the 2008 Consumer Product Safety Information Act (U.S. Consumer Product Safety Commission, 2017), supporting the efficacy of such measures at reducing harmful exposures and protecting public health.

In addition to lower maternal phthalate exposure in TIDES, there are a number of differences between the SFF cohort and the TIDES cohort that might have contributed to differences in associations in the two studies. The timing of maternal urine collection in SFF was more variable with a range of 7–41 weeks (mean=27.1) weeks) compared with a range of 5–20 weeks (mean=10.7) in the first trimester of TIDES and 26–41 weeks (mean=32.8) in the third trimester. SFF children were somewhat older at the time of PSAI completion (mean ± SD 4.9±0.7 vs. 4.5±0.3 years for SFF and TIDES, respectively, $p < 0.0001$), while the TIDES cohort is considerably larger (n=77 vs. n=243 boys and n=74 vs n=255 girls for SFF and TIDES), more diverse (68.9% vs. 81% Caucasian, $p < 0.0001$), and more educated (81% vs. 73% college graduates, p=0.02). Based on our analysis of predictors of play behavior (Table 4), these factors likely contributed to differences in play behavior scores across the two cohorts. Notably, SFF boys scored higher on the masculine scale than TIDES boys (mean masculine score = 40.9 vs. 38.1, p=0.001), and SFF parents of boys were less encouraging of female typical play (Parental Attitude Score 6.4 vs. 5.7, p=0.001). Similarly, SFF girls scored higher on the feminine scale than TIDES girls (43.3 vs. 41.0, p=0.008), although parental attitudes for girls were similar across the two cohorts. These findings may reflect greater acceptance of opposite gender play over time, particularly for boys, or demographic differences across the two cohorts.

The availability of urinary phthalate metabolite measurements at two different time-points in pregnancy is a strength of this study and allowed us to examine phthalate exposure and the development of gender specific behaviors at two points in pregnancy. We previously demonstrated phthalate associations with first but not third trimester anogenital distance (Martino-Andrade et al., 2016) consistent with animal studies (Welsh et al., 2008). This is consistent with the timing of the testosterone surge that occurs in males during gestational weeks 8–12, the period during which testosterone is hypothesized to influence development of sexually dimorphic brain regions (Hines et al., 2015). Consistent with these findings, the current study is suggestive of a greater impact of phthalate exposures during early pregnancy (mean 10.7±2.1weeks) on gendered play in boys compared with exposures later in pregnancy (mean32.8±3.0 weeks). However we previously observed associations between DEHP and DBP metabolites measured at mean gestational age of 27.1 weeks and masculine play in boys (Swan et al., 2010). Using the Playmate and Play Style Preferences

Structured Interview (PPPSI), a child-based interview, Percy et al. found an association between second trimester maternal MiBP and less child-reported male-typical play in boys at age 8 (Percy et al., 2016). Additional studies are needed to more closely examine the role of timing of exposure in the development of sexually dimorphic behaviors and to confirm our observations in girls. Higher testosterone levels during the postnatal surge at 1–2 months have also been associated with more male-typical toy choice in girls, while lower testosterone during this period is associated with more female-typical toy choice in boys (Lamminmaki et al., 2012). Future studies that examine early postnatal phthalate exposure could provide additional information about the sensitive period for development of sex-typed behaviors.

Previous studies demonstrate the influence of both biological and social factors on sex-typed play behavior (Auyeung et al., 2009; Goldberg and Garcia, 2016). We examined additional factors that might influence play behavior in boys and girls. As reported in other studies, older boys engaged in less feminine play, indicating a tendency towards more sex-typical play with increasing age (Golombok et al., 2008). More educated mothers reported fewer female typical behaviors in girls than less educated mothers, possibly reflecting an effect of education on attitudes towards sex-typed play. Race was also a strong predictor of play, with Black mothers reporting significantly less opposite gender behaviors in their sons and daughters compared with all other races. Because 3 of the 4 TIDES study sites are highly homogenous, with a significantly higher percentage of Black and Hispanic participants at the URMC study site compared to other sites, we conducted stratified analyses for URMC versus other study sites combined. URMC mothers are younger, less likely to have obtained a college education, and have higher levels of urinary phthalate metabolites, suggesting that our findings for race may be confounded by study site and socioeconomic factors. Replacing the race variable with center (URMC vs. non-URMC) resulted in nearly identical associations between phthalate metabolites and PSAI masculine scores (Supplemental Table 20). While associations between phthalates and play were similar across study sites, these findings highlight the importance of examining a wide range of social and demographic factors when examining sex-typed behaviors.

This study is limited by its reliance on maternal reporting of child behavior, which may be influenced by factors such as mother’s age, education, and attitudes about gender atypical play. Some study participants reported that the binary nature (masculine vs. feminine) of the scales was stereotypical and some of the activities are less relevant today than they were when the scale was created. Notably, we did not find an inverse correlation between masculine and feminine play behavior; rather, we observed a significant positive correlation between masculine and feminine scores in boys ($r=0.19$, $p=0.004$). Consistent with other studies, we found that parents of boys were less accepting of gender atypical play than parents of girls (Kollmayer et al., 2018; Spivey et al., 2018). Our parental attitudes scale indicates that boys whose parents are more discouraging of opposite gender typical play report fewer feminine play behaviors. Likewise, girls whose parents were more discouraging of male-typical behaviors had lower masculine scores (Table 4). This could be a result of fewer opposite gender toy and activity choices in the home or indicate that parents who discourage gender atypical play tend to underreport opposite gender behaviors. Sexually dimorphic play behavior, as well as parents’ perceptions and attitudes toward sex-typed play,

may be changing over time as evidenced by our finding of greater parental acceptance of feminine typical play in boys in TIDES compared to the our earlier SFF study. Indeed, parental toy choice and attitudes about gender-atypical toys and behaviors have been shown to influence child play preferences (Eisenberg et al., 1985; Kollmayer et al., 2018; Wood et al., 2002). Recent studies of sex-typed toy choice demonstrate a possible shift in toy choice over time, with both boys and girls selecting more opposite gender and gender neutral toys than in earlier studies (Wood et al., 2002). Changes in the marketplace such as removal of gender specific toy aisles “boy” and “girl” labels on costumes and toys by major retailers, and the creation of a gender neutral Barbie doll reflect greater societal acceptance of opposite gender play (Dockterman, 2019; Tabuchi, 2015). Nevertheless, a recent analysis of playthings present in the homes of boys and girls found that despite apparent changes in parental attitudes about gender roles, sex differences in toy choices changed little since the 1970s (MacPhee and Prendergast, 2019; Rheingold and Cook, 1975; Wood et al., 2002). These findings highlight the need to consider parental influence and changing societal norms in studies of environmental influences on sex-typed behavior.

Additional potential limitations of our study include an exposure assessment based on single spot urine samples collected in the first and third trimesters, which may have resulted in exposure misclassification. We were only able to examine phthalate measurements in the first and third trimesters so had limited data with which to identify a critical exposure window. However, when comparing the two timepoints, associations appear to be stronger with first trimester exposure. Future studies should seek to identify critical windows using exposure assessed at additional timepoints, if possible, and employ methods such as the multiple informants model suggested by Sanchez et al. to more accurately compare associations over time (Sanchez et al., 2011). Although we observed an association between race and play behavior, our study sample was predominantly Caucasian, limiting our ability to accurately assess associations between race and our outcomes. The use of two different laboratories for phthalate analysis, with slightly different analytic methods and limits of detection may have increased variability in metabolite concentrations. However, since all samples from mothers of boys were measured at the CDC laboratory, these differences did not affect associations between measurements in boys and phthalate metabolite concentration. Our study sample includes 498 of the 753 TIDES participants that provided a first trimester urine sample and had a live birth. Participants in the current study were significantly older, more likely to have a college degree, and less likely to be Black or Hispanic than non-participants. A smaller proportion of participants included in this study were from Rochester compared to those who did not participate (Supplemental Table 1). Geometric mean concentration of first trimester urinary MiBP and MEP were slightly higher in non-participants compared to participants (Supplemental Table 2). To address the potential for selection bias in our sample we used multiple imputation to account for missing PSAI scores and covariates. We found slightly attenuated effect sizes for associations between phthalate metabolites and masculine scores in adjusted models in both boys (−1.5 [−3.3,0.4] for MnBP, −1.5[−3.6,0.6] for MiBP, and −1.6[−3.2,0.0] for MBzP) and girls (−1.8[−3.5,−0.1] for MBzP) compared with our study sample, suggesting that demographic differences between the participants lost to follow up and those retained in the study had a modest impact on the observed associations.

In summary, we identified associations between urinary phthalate metabolite concentration and sex typical play behavior, an androgen sensitive endpoint. These relationships differed by phthalate metabolite, sex, and time of exposure, with inverse associations seen for MnBP, MiBP, and MBzP and masculine play in the first, but not third, trimester in boys. We also observed negative associations between DEHP metabolites and masculine play in boys that were not statistically significant, possibly due to lower exposure than observed in previous studies. In girls, we observed associations between first trimester MBzP and lower levels of masculine play, and third trimester MnBP and higher levels of masculine play, findings which warrant further investigation. Future studies should explore more objective methods to examine gender typical behaviors that are reflective of child activities today. Our findings that parental attitudes and demographic factors influence sexually dimorphic play behaviors suggest that further investigation into the interaction between endogenous hormones, endocrine disrupting chemicals, and the social environment is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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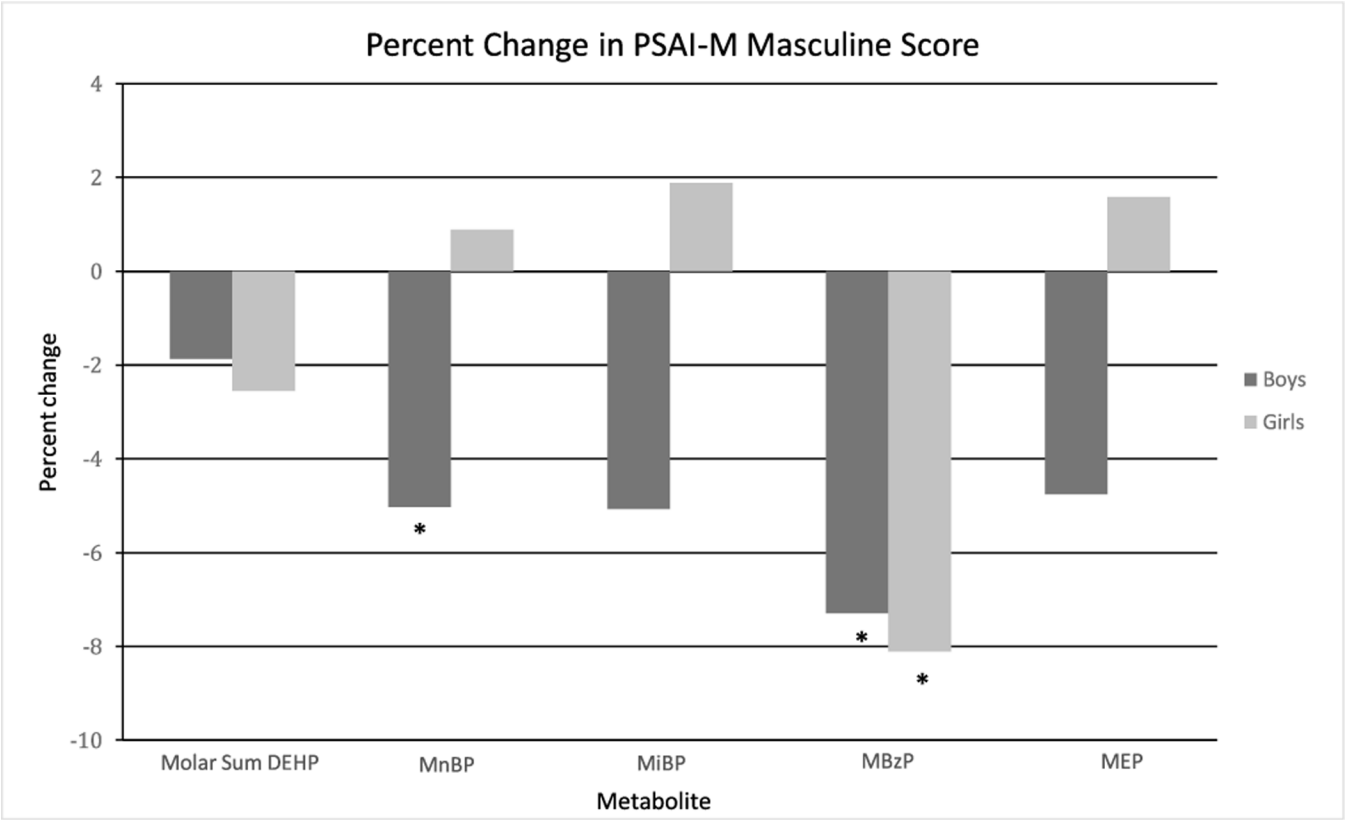

Fig. 1.

Percent change in PSAI-M masculine score in boys and girls associated with an increase in first trimester maternal urinary phthalate metabolite concentration from the 10th to 90th percentile. Asterisks indicate $p < 0.05$ in the final multivariable model.

Table 1

Demographics of 498 TIDES mother-child pairs.

Demographic	Boys (n=243)	Girls (n=255)	p-value[a]
Child age in years; mean (SD)	4.5 (0.3)	4.5 (0.3)	0.80
Center (%)			0.63
Rochester, NY	24.7	23.9	
Minneapolis, MN	31.7	27.1	
San Francisco, CA	25.1	28.6	
Seattle, WA	18.5	20.4	
Mother age in years; mean (SD)	36.5 (5.2)	36.7 (5.4)	0.62
Mother's education (% college graduate)	80.7	81.6	0.80
Race (% Black)	11.9	11.0	0.74
Ethnicity (% Hispanic)	13.2	7.8	0.05
Older male sibling (%)	24.7	28.2	0.37
Older female sibling (%)	26.3	25.1	0.75
Gestational age at T1 urine collection in weeks; mean (SD)	10.7(2.2)	10.7(2.0)	0.93
Gestational age at T3 urine collection in weeks; mean (SD)	32.6(2.9)	32.9(3.2)	0.20

[a] p-values derived by two-sided independent t-tests for continuous variables and Pearson's chi square tests for categorical variables.

Table 2

Summary statistics of PSAI-M scores in boys and girls.

Variable	Boys (n=243)		Girls (n=255)		p-value[a]
	Mean (SD)	Range	Mean (SD)	Range	
PSAI Scores					
Masculine	38.1 (6.2)	18.0–51.0	28.6 (6.4)	12.0–49.0	<0.0001
Feminine	25.1 (5.7)	12.0–43.0	41.0 (6.6)	13.0–53.0	<0.0001
Composite	62.5 (8.4)	38.4–83.5	34.5 (9.5)	10.9–61.5	<0.0001
Parental attitudes; mean (SD)	5.7 (1.9)	2.0–10.0	4.8 (1.6)	2.0–10.0	<0.0001

[a] p-values derived by two-sided independent t-tests.

Table 3

Concentration (ng/mL) of first trimester prenatal urinary phthalate metabolites in 498 TIDES mothers.

Parent Phthalate	Metabolite	Geometric Mean	Percentile			%>LOD
			25th	50th	75th	
DEHP	MEHP	1.9	0.7	2.0	4.0	69
	MEHHP	5.8	2.4	6.0	12.8	98
	MEOHP	4.1	1.9	4.1	9.0	97
	MECPP	7.9	3.0	8.3	17.2	99
DiBP	MiBP	3.7	1.5	4.3	10.0	92
DnBP	MnBP	6.0	2.2	7.0	15.5	97
BBzP	MBzP	3.1	1.0	3.0	8.0	87
DEP	MEP	24.4	8.9	24.0	63.4	99

Table 4

Unadjusted regression coefficients for covariates and PSAI-M scores in boys and girls; β(95% CI)[a].

Covariate	Boys (n=243)		Girls (n=255)	
	Masculine	Feminine	Masculine	Feminine
Child age (years)	−1.4(−3.6,0.8)	**−2.1(−4.2,−0.1)**	0.3(−2.2,2.8)	0.4(−2.2,3.0)
Maternal age (years)	0.0(−0.1,0.2)	0.1(−0.1,0.2)	0.0(−0.2,0.1)	−0.1(−0.3,0.0)
Maternal education[b]	1.0(−1.05,2.95)	1.5(−0.3,3.4)	1.5(−0.5,3.6)	**−2.4(−4.5,−0.3)**
Race[c]	−2.2(−4.6,0.2)	**−4.5(−6.7,−2.4)**	**−2.8(−5.3,−0.3)**	−0.7(−3.3,1.9)
Ethnicity[d]	0.2(−2.2,2.5)	1.5(−0.7,3.6)	1.8(−1.2,4.7)	−2.6(−5.6,0.4)
Parental attitudes	−0.4(−0.8,0.1)	**−1.1(−1.5,−0.8)**	**−0.9(−1.4,−0.4)**	0.3(−0.3,0.8)
Older male sibling	**2.0(0.2,3.8)**	−1.3(−3.0,0.4)	0.0(−1.8,1.8)	−1.0(−2.7,0.9)
Older female sibling	0.8(−1.0,2.6)	0.7(−1.0,2.3)	**−2.4(−4.2,−0.6)**	0.2(−1.7,2.1)

[a] Associations in bold are significant at $p < 0.05$.

[b] College graduate vs. less than college education.

[c] Black vs. not Black.

[d] Hispanic vs. not Hispanic.

Table 5

Unadjusted and adjusted regression coefficients for (log_{10}) specific gravity-adjusted first trimester urinary phthalate metabolites and PSAI-M scores in boys (n=243); β(95% CI)[a].

	Masculine		Feminine		Composite	
Phthalate	**Unadjusted**	**Adjusted[b]**	**Unadjusted**	**Adjusted[b]**	**Unadjusted**	**Adjusted[b]**
ΣDEHP[a]	−0.9(−2.9,1.1)	−0.8(−2.8,1.2)	−0.9(−2.8,0.9)	−0.4(−2.2,1.3)	0.0(−2.7,2.7)	−0.4(−3.1,2.3)
MnBP	**−2.5(−4.4,−0.5)**	**−2.2(−4.2,−0.2)**	**−1.9(−3.7,−0.1)**	−0.7(−2.4,1.1)	−0.6(−3.3,2.0)	−1.7(−4.3,1.0)
MiBP	**−2.5(−4.7,−0.4)**	−2.1(−4.3,0.1)	−2.0(−4.0,0.0)	−0.8(−2.7,1.1)	−0.6(−3.6,2.3)	−1.4(−4.4,1.5)
MBzP	**−2.6(−4.3,−0.9)**	**−2.4(−4.1,−0.7)**	−1.6(−3.1,0.0)	−0.6(−2.1,0.9)	−1.2(−3.5,1.2)	−2.0(−4.3,0.3)
MEP	**−1.7(−3.1,−0.3)**	−1.3(−2.8,0.1)	−0.9(−2.2,0.5)	−0.3(−1.6,1.0)	−0.9(−2.9,1.0)	−1.1(−3.1,0.8)

[a] log_{10} Molar Sum of MEHP, MEHHP, MEOHP, and MECPP.

[b] Adjusted for child age, maternal education, race, same sex older sibling, and parental attitudes.

Table 6

Unadjusted and adjusted regression coefficients for (log_{10}) specific gravity-adjusted first trimester urinary phthalate metabolites and PSAI-M scores in girls (n=255); β(95% CI)[a].

	Masculine		Feminine		Composite	
Phthalate	**Unadjusted**	**Adjusted[b]**	**Unadjusted**	**Adjusted[b]**	**Unadjusted**	**Adjusted[b]**
ΣDEHP[a]	−1.8(−3.9,0.4)	−0.9(−3.1,1.3)	−1.2(−3.5,1.0)	−1.2(−3.5,1.1)	−0.6(−3.8,2.7)	0.3(−3.0,3.6)
MnBP	−0.6(−2.7,1.5)	0.3(−1.9,2.4)	0.5(−1.6,2.6)	0.0(−2.3,2.2)	−1.2(−4.3,1.9)	0.3(−2.9,3.5)
MiBP	0.0(−2.1,2.1)	0.7(−1.3,2.8)	−0.2(−2.3,1.9)	−0.3(−2.5,1.9)	0.2(−2.9,3.3)	1.1(−2.0,4.2)
MBzP	**−2.7(−4.4,−1.0)**	**−2.1(−4.0,−0.3)**	−0.8(−2.7,1.0)	−1.6(−3.5,0.4)	−2.0(−4.7,0.6)	−0.6(−3.4,2.2)
MEP	0.0(−1.5,1.4)	0.3(−1.1,1.7)	−0.1(−1.5,1.4)	−0.2(−1.7,1.3)	0.0(−2.1,2.1)	0.6(−1.5,2.7)

[a] log_{10} Molar Sum of MEHP, MEHHP, MEOHP, and MECPP.

[b] Adjusted for child age, maternal education, race, same sex older sibling, and parental attitudes.