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1 **Prenatal di(2-ethylhexyl) phthalate exposure and disruption of adrenal androgens and**  
2 **glucocorticoids levels in cord blood: The Hokkaido Study**

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33 Disclosure Statement: The authors have nothing to disclose.

34

35 **Abstract**

36 Di(2-ethylhexyl) phthalate (DEHP) is known for its endocrine disrupting properties. We previously  
37 demonstrated that prenatal DEHP exposure is associated with decreased progesterone levels and  
38 testosterone/estradiol ratio in the cord blood. However, evidence of the effects of prenatal DEHP  
39 exposure on adrenal androgen and glucocorticoids in infants is scarce. Thus, the objectives of this  
40 study were to investigate the association between prenatal DEHP exposure and adrenal androgen and  
41 glucocorticoids, and to discuss its effects on steroid hormone profiles in infants. This is part of a birth  
42 cohort study: The Hokkaido Study on Environment and Children's Health, Sapporo Cohort. Among  
43 the 514 participants, 202 mother-infant pairs with available data on maternal mono(2-ethylhexyl)  
44 phthalate (MEHP), adrenal androgen (dehydroepiandrosterone [DHEA] and androstenedione) and  
45 glucocorticoid (cortisol and cortisone) cord blood levels were included in this study. After adjusting  
46 for potential confounders, a linear regression analysis showed that maternal MEHP levels were  
47 associated with reduced cortisol and cortisone levels and glucocorticoid/adrenal androgen ratio,  
48 whereas increased DHEA levels and DHEA/androstenedione ratio. In a quartile model, when  
49 comparing the adjusted least square means in the 4<sup>th</sup> quartile of MEHP with those in the 1<sup>st</sup> quartile,  
50 cortisol and cortisone levels and glucocorticoid/adrenal androgen ratio decreased, whereas  
51 DHEA/androstenedione and cortisol/cortisone ratios increased. Significant p-value trends for cortisol  
52 and cortisone levels, cortisol/cortisone ratio, and glucocorticoid/adrenal androgen ratio were observed.  
53 In combination with the previous results of reduced progesterone levels and testosterone/estradiol

54 ratio, prenatal exposure to DEHP altered the steroid hormone profiles of infants. Further studies  
55 investigating the long-term effects of DEHP exposure on growth, neurodevelopment, and gonad and  
56 reproductive function are required.

57

58

59 **Key Words:** Di(2-ethylhexyl) phthalate (DEHP); mono(2-ethylhexyl) phthalate (MEHP); adrenal  
60 androgen; glucocorticoid; prenatal exposure; fetal blood

61

62 **Abbreviations:**

63 CI, confidence interval

64 CYP11A1, cytochrome P450 family 11 subfamily A member 1

65 CYP11B1, cytochrome P450 family 11 subfamily B member 1

66 CYP17A1, cytochrome P450 family 17 subfamily A member 1

67 CYP19A1, cytochrome P450 family 19 subfamily A member 1

68 CYP21A2, cytochrome P450 family 21 subfamily A member 2

69 CV, coefficient of variation

70 DBP, dibutyl phthalate

71 DEHP, di(2-ethylhexyl) phthalate

72 DHEA, dehydroepiandrosterone

- 73 DHEA-S, DHEA sulfate
- 74 HSD11B2, hydroxysteroid 11-beta dehydrogenase 2
- 75 HSD17B1, hydroxysteroid 17-beta dehydrogenase 1
- 76 HSD3B1, hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
- 77 IQR, interquartile range
- 78 LSM, least square means
- 79 MEHP, mono(2-ethylhexyl) phthalate
- 80 PFOS, perfluorooctane sulfonate
- 81 MBP, Monobutyl phthalate
- 82 PPAR, peroxisome proliferator-activated receptor
- 83 SULT2A1, DHEA sulfotransferase family 2A member 1
- 84 T/E2, testosterone/estradiol
- 85

86 **Introduction**

87 Phthalate diesters (phthalates) have been used as plasticizers for various plastic products, including  
88 toys, food containers, furniture, personal care products, medical devices, and housing materials.  
89 According to a report from the Japan Plasticizer Industry Association and Ministry of Economy, Trade  
90 and Industry in 2012, di(2-ethylhexyl) phthalate (DEHP) constitutes >50% of the phthalates used in  
91 production in Japan. Phthalates are not chemically bonded to polyvinyl chloride in plastic products;  
92 thus, they can leach into the air, dust, foodstuffs, and other materials. Consequently, humans are  
93 constantly exposed to phthalates, and biomonitoring studies have demonstrated the widespread  
94 exposure of the general population to these chemicals (Ait Bamai et al., 2015; Fromme et al., 2007;  
95 Jensen et al., 2015; Koch et al., 2004; Wittassek et al., 2007).

96 DEHP is a potential endocrine-disrupting chemical, and multiple adverse effects on human  
97 health due to DEHP exposure in early life were found. Phthalate exposure has been reported to  
98 shorten the anogenital distance of infants (Bornehag et al., 2015; Swan et al., 2005; Swan et al., 2015).  
99 The associations between phthalates exposure and neurodevelopment and childhood obesity were also  
100 investigated (Ejaredar et al., 2015; Kim and Park, 2014). The underlying mechanisms of their effects  
101 were not clearly understood; however, disruption of steroidogenesis could be one of the contributing  
102 factors because steroid hormones play an important role in homeostasis. Sex steroid hormones  
103 including testosterone, progesterone, and estradiol, have effects predominantly in the gonads; and  
104 dehydroepiandrosterone (DHEA) and androstenedione, which are weak adrenal steroid precursors, are

105 activated to form androgens and estrogens that have important roles in sex differentiation and  
106 maturation (Labrie et al., 2001). Glucocorticoids, including cortisol, and cortisone are synthesized  
107 within the adrenal cortex, and are involved in a wide range of physiological processes.  
108 Glucocorticoids are essential for regulating and/or modulating homeostasis in metabolism, growth,  
109 neurodevelopment, and the immune system (Braun et al., 2013; Reynolds, 2010). As a whole, steroid  
110 hormones in early life play important roles in reproductive growth and neurodevelopment for later life  
111 (Hollier et al., 2014; Quinn et al., 2016).

112 In previous experimental studies in rat, Chinese rare minnow, and zebrafish, DEHP and/or  
113 its primary metabolite, mono (2-ethylhexyl) phthalate (MEHP), were reported to upregulate and/or  
114 downregulate several enzymes in the steroidogenesis pathway (Akingbemi et al., 2001, 2004;  
115 Lehmann et al., 2004; Sekaran and Jagadeesan, 2015; Thompson et al., 2005; Zhu et al., 2016a; Zhu  
116 et al., 2016b). Exposure to DEHP could modify steroidogenesis and disrupt both  
117 hypothalamic-pituitary-gonad and hypothalamic-pituitary-adrenal axes. One animal study  
118 demonstrate that dibythol phthalate initiated a rapid and dynamic change in gene expression unique to  
119 the fetal testis while in the adrenal was unaffected (Thompson et al., 2005). However, there are  
120 fundamental regulation differences of steroidogenesis in the fetal testis between rodent and human  
121 (Scott et al., 2009). In addition to species differences, exposure timing, duration, and dosage  
122 variations between studies are not relevant for the human exposure scenario. Thus, results from  
123 animal studies are limited in predicting the impact of phthalates exposure on adrenal steroid



124 production in human.

125           Despite the importance of DEHP properties on steroidogenesis, epidemiological studies on  
126 DEHP exposure, especially during early life, and its effects on adrenal androgen and glucocorticoid  
127 modulation are limited. In birth cohort studies in Mexico, the DHEA sulfate (DHEA-S) levels were  
128 increased and decreased following prenatal phthalate exposure in pubescent boys and girls,  
129 respectively (Ferguson et al., 2014; Watkins et al., 2014). In a Danish cohort, urinary phthalate  
130 metabolites in children were measured every 6 months from the baseline (aged 5.9 years) to pubertal  
131 age; girls with levels of monobutyl phthalate (MBP) and DEHP metabolites that were above the  
132 geometric group mean had lower levels of DHEA-S and androstenedione, whereas boys with higher  
133 MBP levels had lower DHEA-S levels (Mouritsen et al., 2013). Currently, only one study has  
134 investigated the effects of phthalate exposure on androstenedione and glucocorticoid levels in  
135 amniotic fluid, and the authors did not identify a significant association between phthalate and steroid  
136 hormone levels, including glucocorticoids (Jensen et al., 2015).

137           We recently demonstrated that prenatal DEHP exposure resulted in reduced progesterone  
138 levels and a reduced testosterone/estradiol (T/E2) ratio in cord blood (Araki et al., 2014). The aims of  
139 this study were to examine the effects of prenatal DEHP exposure on adrenal androgens (DHEA and  
140 androstenedione) and glucocorticoids (cortisol and cortisone), and to discuss its effects on the steroid  
141 hormone profile of infants, including adrenal androgens and glucocorticoids, and sex hormones  
142 (progesterone, testosterone, and estradiol) as reported by Araki et al. (2014).

143

144 **Methods**

145 **Participants**

146 This study was based on the Sapporo Cohort of the Hokkaido Study on Environment and Children's  
147 Health. Details of this study, regarding the population, data collection, sampling of the biological  
148 specimens, and contents of the questionnaire, have been previously described (Kishi et al., 2013;  
149 Kishi et al., 2011). Briefly, native Japanese women at an obstetrics and gynecology hospital in  
150 Sapporo (Hokkaido, Japan), who lived in Sapporo City or surrounding areas, were enrolled in the  
151 study at 23–35 weeks of gestation between July 2002 and October 2005. Among the 1796 pregnant  
152 women approached, 25% were excluded as they were enrolled in the Japanese Cord Blood Bank or  
153 planned to deliver the baby at another hospital. Eventually, 514 pregnant women (28.6%) were  
154 enrolled in this study. Selection of participants included in the analysis was described in our previous  
155 study (Araki et al., 2014). Briefly, of the 514 participants, 10 were excluded from the study due to  
156 miscarriage, stillbirth, relocation, or voluntary withdrawal prior to delivery. A total of 493 maternal  
157 blood samples were available for MEHP measurements. However, maternal blood samples collected  
158 during hospitalization after delivery were excluded from this analysis due to the relatively short  
159 biological half-life of DEHP. Steroid hormone measurements were available for 295 infant cord  
160 blood samples. Finally, 202 samples with available MEHP and steroid hormone levels were included  
161 in the statistical analysis.

162

163

164 **MEHP measurement**

165 Maternal blood samples were obtained at the time of their hospital examination following recruitment.

166 If a blood sample could not be taken during pregnancy due to maternal anemia, a blood sample was

167 collected during hospitalization, within a week after delivery. All samples were stored at  $-80\text{ }^{\circ}\text{C}$  until

168 analysis. MEHP, which is the primary metabolite of DEHP, was measured by gas

169 chromatography-mass spectrometry at Nagoya University. The methods for the preparation of samples

170 and standard solutions, and the instrumental analysis have been described previously (Araki et al.,

171 2014; Jia et al., 2015). The detection limit was  $0.278\text{ ng/mL}$  ( $1\text{ pmol/mL}$ ). MEHP levels in a tube

172 containing the same medium as the reaction vial were measured to determine background levels. To

173 exclude the possibility of environmental contamination of DEHP, glassware used for MEHP

174 measurements was heated at  $200\text{ }^{\circ}\text{C}$  for 2 h. A total of 493 maternal blood samples were analyzed for

175 MEHP levels. The coefficient of variation (CV) of MEHP measurements taken within a single day

176 was  $2.0\%–7.8\%$  for 6 days, and the day-to-day CV for 6 days was  $6.2\%$ , at  $5\text{ pmol/mL}$  of

177 concentration, as described previously (Jia et al., 2015).

178

179 **Measurement of steroid hormones**

180 At the time of delivery, a blood sample was collected from the umbilical cord and stored at  $-80\text{ }^{\circ}\text{C}$

181 until analysis. Cord blood samples were available only from the children born vaginally. The  
182 concentrations of adrenal androgens and glucocorticoids were measured using liquid  
183 chromatography–tandem mass spectrometry (LC-MS/MS; Aska Pharma Medical Co., Ltd., Kanagawa,  
184 Japan). The methods for the preparation of samples, standard solutions, and instrumental analysis  
185 were described previously (Yamashita et al., 2007a; Yamashita et al., 2007b). The mean intra-assay  
186 CVs for steroid hormone measurements were as follows: DHEA, 2.1%–5.2%; androstenedione,  
187 2.3%–6.8%; cortisol, 3.9%–10.9%; and cortisone, 1.3%–9.9%. The mean inter-assay CVs for steroid  
188 hormones were: DHEA, 3.3%–4.6%; androstenedione, 6.6%–7.1%; cortisol, 7.6%–11.3%; and  
189 cortisone: 7.8%–9.3% (Mitsui et al., 2016; Mitsui et al., 2015).

190

### 191 **Questionnaire and medical record**

192 The participants completed a self-administered questionnaire regarding information on maternal age,  
193 education level, household income, smoking and alcohol consumption during the first trimester, and  
194 medical history. Medical records were obtained at delivery for information regarding pre-pregnancy  
195 body mass index, pregnancy complications, parity, gestational age, infant sex, infant size, Apgar score,  
196 and congenital anomalies, including hypospadias and cryptorchidism, .

197

### 198 **Statistical analyses**

199 Distribution of adrenal androgen and glucocorticoid levels among boys and girls were

200 compared using the Mann-Whitney U test. Associations between maternal MEHP concentrations and  
201 infant adrenal androgen and glucocorticoid levels were initially calculated using the Spearman's rank  
202 correlation coefficient, followed by multivariate linear regression analysis. MEHP levels and adrenal  
203 androgen and glucocorticoid concentrations were converted to a log<sub>10</sub> scale because they did not fall  
204 into a normal distribution. To evaluate whether the association between hormone and MEHP levels  
205 differed between sexes, a multivariate linear regression model for all study participants was first  
206 constructed with the interaction terms of hormone levels to sex and MEHP interaction. Since the  
207 MEHP and sex interaction was not significant for any of the hormones ( $P_{\text{interaction}} > 0.05$ ), a further  
208 analysis was performed, in which both sexes were included in the same model. The interquartile range  
209 (IQR) for MEHP concentrations and the least square means (LSM) and 95% confidence intervals  
210 (CIs) for hormone levels were calculated. To calculate a p-value for the trend, linear contrast  
211 coefficients of -3, -1, +1, and +3 were assigned to 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>, and 4<sup>th</sup> quartiles, respectively (Goudarzi  
212 et al., 2016; Itoh et al., 2016). The 1<sup>st</sup> quartile was compared to the 2<sup>nd</sup>, 3<sup>rd</sup>, and 4<sup>th</sup> quartile MEHP, and  
213 the p-values were adjusted using the Bonferroni correction. When the levels were below the detection  
214 limits, half of their values were used for individual hormones. Inclusion of covariates was based on  
215 biological considerations, and included maternal age (continuous), maternal smoking during  
216 pregnancy (yes or no), maternal alcohol consumption during pregnancy (yes or no), gestational age  
217 (continuous), the week of gestation at which blood samples were taken (continuous), infant sex (boy  
218 or girl), and Apgar score (ordinal variable). A previous report from our cohort population

219 demonstrated that prenatal perfluorooctane sulfonate (PFOS) levels were associated with steroid  
220 hormone levels (Goudarzi et al., 2016; Itoh et al., 2016). Therefore, PFOS was also included in the  
221 adjusted model. All statistical analyses were performed using JMP Clinical 5.0 software (SAS  
222 Institute Inc., NC, USA).

223

#### 224 **Ethical approval**

225 This study was approved by the Institutional Ethical Review Board for Epidemiological Studies at  
226 Hokkaido University Graduate School of Medicine, Hokkaido University Center for Environmental  
227 and Health Sciences, and Nagoya University Graduate School of Medicine, in accordance with the  
228 principles of the Declaration of Helsinki. All participants provided written informed consents.

229

#### 230 **Results**

231 The characteristics of the participants included in this study are shown in Supplemental Table S1.  
232 Briefly, mean  $\pm$  standard deviation maternal age at delivery (years) was  $29.8 \pm 4.9$ , and 54.5% were  
233 primiparous. For infants, the proportion of the boys was 46.0%, mean gestational age was  $39.5 \pm 1.0$   
234 weeks, and birth weight was  $3138.6 \pm 331.3$  g. Apgar score at one minute after birth was nine for  
235 82.7% children. Concentrations of MEHP in all samples were above the detection limit, and the  
236 median (IQR) concentration was 10.4 ng/mL (5.88–15.3 ng/mL). The concentration of MEHP was not  
237 significantly associated with any maternal or infant characteristics.

238           The adrenal androgen and glucocorticoid levels in infants are shown in Table 1. DHEA levels  
239 were significantly higher in girls than in boys. Correlations between MEHP levels and adrenal  
240 androgen and glucocorticoid concentrations are shown in Table 2, and the results of the linear  
241 regression after adjusting for potential confounders are shown in Table 3. MEHP levels were inversely  
242 associated with cortisol and cortisone levels and glucocorticoid/adrenal androgen ratio, but positively  
243 associated with DHEA levels and DHEA/androstenedione ratios.

244           The associations between MEHP and adrenal androgen and cortisol levels were analyzed for  
245 potential non-linear relationships. The LSM for each hormone in each MEHP quartile is shown in  
246 Figure 1, with details of the data in Supplemental Table S2. The adjusted LSM adrenal androgen and  
247 cortisol levels in relation to the MEHP quartile showed a significant p-value trend for cortisol and  
248 cortisone levels, cortisol/cortisone ratio, and glucocorticoid/adrenal androgen ratio. When comparing  
249 the LSM in the 4<sup>th</sup> quartile of MEHP with those in the 1<sup>st</sup> quartile, cortisol and cortisone  
250 concentrations and glucocorticoid/adrenal androgen ratio were decreased, whereas  
251 DHEA/androstenedione and cortisol/cortisone ratios were increased.

252           Since there were significant differences in DHEA levels in the cord blood with respect to sex,  
253 the associations between MEHP levels and DHEA concentrations were examined separately in boys  
254 and girls for both, linear and quartile models. However, there was no association found between  
255 MEHP and DHEA levels in either boys or girls (data not shown).

256

257 **Discussion**

258 We have investigated the associations between maternal MEHP levels and cord blood adrenal  
259 androgens (DHEA and androstenedione) and glucocorticoids (cortisol and cortisone) in mother-infant  
260 pairs of a prospective birth cohort. Our results demonstrated a significant association between  
261 increased maternal MEHP levels and decreased levels of cortisol and cortisone levels and  
262 glucocorticoid/adrenal androgen ratio, and increased DHEA/androstenedione ratio in linear models. In  
263 quartile models, p-values for trends were statistically significant for cortisol and cortisone levels, and  
264 cortisol/cortisone and glucocorticoid/adrenal/androgen ratios. However, in the 4<sup>th</sup> quartile of MEHP,  
265 cortisol and cortisone levels, glucocorticoid/adrenal androgen ratio were significantly lower, and  
266 DHEA/androstenedione and cortisol/cortisone ratios were significantly higher compared with the 1<sup>st</sup>  
267 quartile of MEHP. We have previously reported that increased maternal MEHP levels decreased  
268 progesterone levels and testosterone/estradiol (T/E2) ratio in the cord blood (Araki et al., 2014). In  
269 combination, the study provided novel evidence regarding the effects of prenatal DEHP exposure on  
270 steroidogenesis disruption in infants, and a threshold for such effects was found.

271 In previous studies, we demonstrated that PFOS levels were inversely associated with  
272 progesterone, T/E2 ratio, cortisol, and cortisone, and positively associated with DHEA (Goudarzi et  
273 al., 2016; Itoh et al., 2016). Nuclear receptors, including peroxisome proliferator-activated receptor  
274 (PPAR) alpha and gamma, are essential regulators of steroidogenesis, and both PFOS and DEHP are  
275 known ligands of these receptors (Lovekamp-Swan et al., 2003; Vanden Heuvel et al., 2006). Thus,



276 the effects of PFOS and DEHP exposure on steroidogenesis could overlap. In addition, there was a  
277 statistically significance correlation between PFOS and MEHP (Spearman's rho = 0.453, P < 0.001).  
278 Therefore, we performed a mutual adjustment with PFOS so that the changes of steroid hormone  
279 levels by MEHP in this study were independent of the effect of PFOS. For further assessment, as an  
280 indicator of infant stress at birth, we have included the Apgar score (one minute after birth, ordinal  
281 variable) into the adjusted model and the results remained consistent (Supplemental Table S3).  
282 Therefore, stress of the birth process cannot be a confounder in our study.

283           Previous studies examining the effects of exposure to DEHP on adrenal androgen and  
284 glucocorticoids in humans are limited. A Danish pregnancy-screening biobank study, which consisted  
285 of cases of cryptorchidism and hypospadias and control boys, reported that increased levels of mono  
286 (2-ethyl-5-carboxypentyl) phthalate, another metabolite of DEHP, was associated with higher levels of  
287 androstenedione and cortisol in the second trimester amniotic fluid (Jensen et al., 2015). Further, a  
288 statistically significant association between DEHP metabolites and hormones seemed more strongly in  
289 cases than controls (Jensen et al., 2015). However, increased MEHP levels in the present study were  
290 associated with reduced androstenedione and cortisol levels, although the p-values did not reach  
291 statistically significant levels. Steroid hormone levels shift during the gestational period, such that the  
292 inconsistent results could be due to the timing of the steroid measurements (Kuijper et al., 2013). In  
293 addition, there were no cases of cryptorchidism or hypospadias in present study. Thus, difference of  
294 the study participants could be another reason for the inconsistencies of the results.

295 This is the first study that examined MEHP exposure and DHEA levels at birth, and reported  
296 that MEHP levels were inversely associated with DHEA levels in a linear model. In birth cohort  
297 studies in Mexico, DHEA-S levels were increased and decreased in pubescent boys and girls,  
298 respectively, following prenatal phthalate exposure (Ferguson et al., 2014; Watkins et al., 2014).  
299 Mouritsen et al.(2013) reported the results of a prospective cohort study in Denmark, in which  
300 phthalate levels in the urine of children were inversely associated with the DHEA-S levels in both  
301 sexes (Mouritsen et al., 2013). These studies in Mexico and Denmark measured hormone levels in  
302 children at puberty, whereas the present study measured hormones at birth. Another explanation for  
303 the inconsistencies between the results of this study and those of previous studies is that DHEA-S,  
304 instead of DHEA, was measured in the Mexico and Denmark studies. The levels of a DHEA  
305 sulfotransferase family 2A member 1 (SULT2A1), which converts DHEA to DHEA-S, were reported  
306 to be downregulated by phthalates in a previous study (Harris et al., 2007). Therefore, the effects of  
307 DEHP exposure on the levels of DHEA and DHEA-S could differ.

308 The findings of this study could be explained by previous experimental studies as shown in  
309 Figure 2. Decreased levels of progesterone following DEHP exposure likely resulted from the  
310 downregulation of hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1  
311 (HSD3B1). Similarly, increases in the DHEA levels and DHEA/androstenedione ratio could also  
312 result from the downregulation of HSD3B1. Increased cortisol/cortisone ratios could be due to  
313 DEHP-mediated downregulation of hydroxysteroid 11-beta dehydrogenase 2 (HSD11B2). Finally,

314 decreased levels of cortisol and cortisone, together with a reduced glucocorticoid/adrenal androgen  
315 ratio, could be due to a shift of steroidogenesis from glucocorticoids to adrenal androgen by  
316 downregulation of HSD3B1 and hydroxysteroid 17-beta dehydrogenase 1 (HSD17B1). Additionally,  
317 cholesterol is a substrate of whole steroid hormones. Thus, changes in the cholesterol profile  
318 following exposure to DEHP may exist as demonstrated in a previous study of elderly patients in  
319 which reduced levels of cholesterol were detected following DEHP exposure (Olsén et al., 2012).

320 Previous animal and in vitro studies have provided evidence that phthalates, including DEHP  
321 and/or MEHP, modify the expression of genes encoding steroidogenesis enzymes. MEHP exposure  
322 decreased HSD11B2, cytochrome P450 family 11 subfamily A member 1 (CYP11A1), HSD3B1, and  
323 HSD17B1 (Akingbemi et al., 2001; Hong et al., 2009; Lehmann et al., 2004; Sekaran and Jagadeesan,  
324 2015; Thompson et al., 2005; Zhao et al., 2010; Zhu et al., 2016a; Zhu et al., 2016b). Although one  
325 experimental study suggested upregulation of CYP17A1 (Wang et al., 2013), other studies found  
326 DEHP and/or MEHP exposure induced inhibition of cytochrome P450 family 17 subfamily A  
327 member 1 (CYP17A1) (Akingbemi et al., 2001; Chauvigne et al., 2011; Lehmann et al., 2004). Thus,  
328 increased levels of DHEA in this study may be better explained by inhibition of SULT2A1, HSD3B1,  
329 and HSD17B1 than activation of CYP17A1. As for cytochrome P450 family 19 subfamily A member  
330 1 (CYP19A1), Wang et al. suggested that direction of up/downregulation differ between sex (Wang et  
331 al., 2013). Nevertheless, many of the in vitro and animal studies were conducted with concentrations  
332 and doses that are not relevant for the human exposure scenario. Moreover, the changes in gene

333 expression of steroidogenic enzymes may vary significantly depending on the experimental conditions,  
334 such as cell lines, species, doses, timing, and duration of exposure. (Akingbemin et al., 2001, 2004;  
335 Ge et al., 2007).

336 As we described in our previous study (Araki et al., 2014), the levels of MEHP in this cohort  
337 were slightly higher than those in American adults (NHANES 1999–2000), elderly Swedish subjects,  
338 and pregnant women in Australia (Hart et al., 2014; Lind et al., 2012; Silva et al., 2004). Slightly  
339 higher MEHP levels in this study can be considered acceptable as the levels of DEHP in house dust in  
340 Sapporo, Japan were higher compared with the studies from other countries, and DEHP intake in the  
341 Japanese population was higher than that of most other studies (Ait Bamai et al., 2014; Ait Bamai et  
342 al., 2015). On the other hand, as for the first limitation, it should be also noted that MEHP in blood  
343 was measured as urine samples were not available in this study. All samples were handled carefully to  
344 avoid *ex vivo* hydrolysis of DEHP; therefore, measurement errors due to contamination were minimal  
345 (Araki et al., 2014). Nevertheless, mono (2-ethyl-5-carboxypentyl) phthalate and mono  
346 (2-ethyl-5-hydroxyhexyl) phthalate have previously been detected in maternal serum (Hart et al.,  
347 2014); thus, these secondary metabolites should be measured in future studies.

348 Another limitation of this study was that the MEHP level was measured only once from  
349 second to third trimester. However, although there are contradicting discussions regarding this in the  
350 literature, several previous reports have found that single measurements can be useful (Hoppin et al.,  
351 2002; Townsend et al., 2013). In addition, the blood sampling week was adjusted to minimize the

352 effect of timing of exposure in this study. Although, we cannot rule out a possibility of the effect from  
353 acute exposure before delivery at the hospital, non-differential errors bias an effect towards the null.  
354 In this study, all participants who delivered vaginally with available cord blood samples were included  
355 in the analysis. Compared to the initial cohort population, the infants had an increased gestational age  
356 and heavier birth weight than those who were excluded from the study. As a result, healthier infants  
357 were included in the analysis; thus, the effects of MEHP may be underestimated in this study. In  
358 addition, the participation rate in our cohort study is rather low, which may limit the extrapolation of  
359 our results to the general population. Finally, the observation of only minimal reductions in the levels  
360 of cortisol in the linear model ( $0.1 > P > 0.05$ ) may have been due to the limited sample size. Future  
361 studies with larger sample sizes are then required to confirm the results. A major strength of the  
362 present study is that this has a prospective birth cohort design, in which the effects of prenatal DEHP  
363 exposure on fetal adrenal androgen and glucocorticoids could be estimated. In addition, steroid  
364 hormones were measured by LC-MS/MS methods, which are considered more accurate than other  
365 methods, such as radioimmunoassay.

366

### 367 **Conclusion**

368 In summary, this study found significant associations between increased maternal MEHP levels and  
369 reduced cortisol and cortisone levels and glucocorticoid/adrenal androgen ratio and increased  
370 DHEA/androstenedione and cortisol/cortisone ratios. In combination with the previous results of

371 reduced progesterone levels (Araki et al., 2014 ), the present results suggested that prenatal DEHP  
372 exposure disrupted the steroid hormone profile of infants, despite the levels of DEHP in the general  
373 population being markedly lower than those in experimental studies. Disrupting the balance of steroid  
374 hormones may cause adverse effects on reproductive growth, development, and other health outcomes  
375 in later life. The clinical significance of these findings is unclear at present because it remains  
376 unknown whether these small hormonal alterations exert any effect on health.

377

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1 **Table 1.** Distribution of adrenal androgen and glucocorticoid concentrations

	Detection limit	>Detection limit (%)	Total (n = 202) Med (IQR)	Boys (n = 93) Med (IQR)	Girls (n = 109) Med (IQR)	<sup>a</sup> P-value
<b>Adrenal androgen</b>						
DHEA (ng/mL)	0.010	100	2.31 (1.79, 3.07)	2.08 (1.59, 2.71)	2.62 (1.99, 3.42)	<0.001
Androstenedione (ng/mL)	0.010	100	0.46 (0.36, 0.58)	0.47 (0.38, 0.59)	0.45 (0.35, 0.58)	0.572
<b>Glucocorticoid</b>						
Cortisol (ng/mL)	0.250	96.5	40.5 (22.7, 66.7)	41.1 (22.3, 66.8)	39.3 (24.1, 66.8)	0.902
Cortisone (ng/mL)	0.100	93.6	96.9 (70.3, 124)	97.2 (72.5, 126)	96.0 (69.2, 125)	0.734

2 DHEA, dehydroepiandrosterone; IQR, the interquartile range

3 <sup>a</sup>p-values were calculated by Mann-Whitney U test comparing boys and girls

4

5

6 **Table 2.** Associations between MEHP and hormone levels

	<sup>a</sup> $\rho$	p-value
DHEA	0.136	0.054
Androstenedione	-0.190	0.007
DHEA/androstenedione	0.178	0.011
Androstenedione/testosterone	0.027	0.699
Cortisol	-0.273	<0.0001
Cortisone	-0.367	<0.0001
Cortisol/cortisone	0.038	0.592
Glucocorticoid/adrenal androgen	-0.297	<0.0001

7 <sup>a</sup>Spearman's  $\rho$

8 MEHP, mono(2-ethylhexyl)phthalate; DHEA, dehydroepiandrosterone

9

10

11 **Table 3.** Adjusted linear regression coefficients of adrenal androgen and glucocorticoid in the cord blood in relation to MEHP

	Crude model			Adjusted model		
	$\beta$	(95% CI)	p-value	$\beta$	(95% CI)	p-value
DHEA	0.246	0.095 0.397	0.002	0.205	0.029 0.381	0.023
Androstenedione	-0.079	-0.199 0.042	0.198	-0.082	-0.224 0.059	0.252
DHEA/androstenedione	0.325	0.147 0.503	<0.001	0.287	0.073 0.501	0.009
Androstenedione/testosterone	0.002	0.000 0.003	0.082	0.001	-0.001 0.004	0.163
Cortisol	-0.527	-0.809 -0.245	<0.001	-0.412	-0.751 -0.072	0.018
Cortisone	-0.734	-1.111 -0.358	<0.001	-0.570	-1.026 -0.114	0.015
Cortisol/cortisone	0.207	0.040 0.375	0.015	0.158	-0.041 0.358	0.119
Glucocorticoid/adrenal	-0.828	-1.258 -0.398	<0.001	-0.649	-1.167 -0.132	0.014

12 ~~Standardized~~ hormone levels and MEHP concentrations were log<sub>10</sub>-transformed and included in the model separately.

13  $\beta$  for linear regression coefficients

14 Adjusted for maternal age, smoking and alcohol consumption during pregnancy, gestational age, blood sampling week, infant sex, and PFOS

15 95%CI, 95% confidence interval; DHEA, dehydroepiandrosterone; MEHP, mono(2-ethylhexyl)phthalate; PFOS, perfluorooctane sulfonate levels,

16

17

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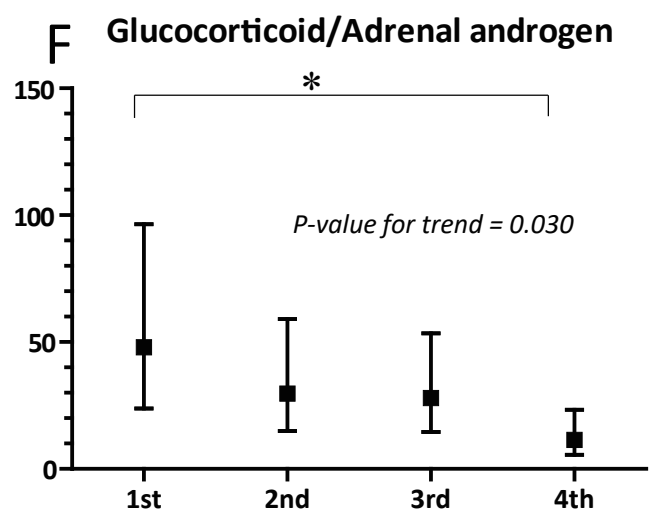
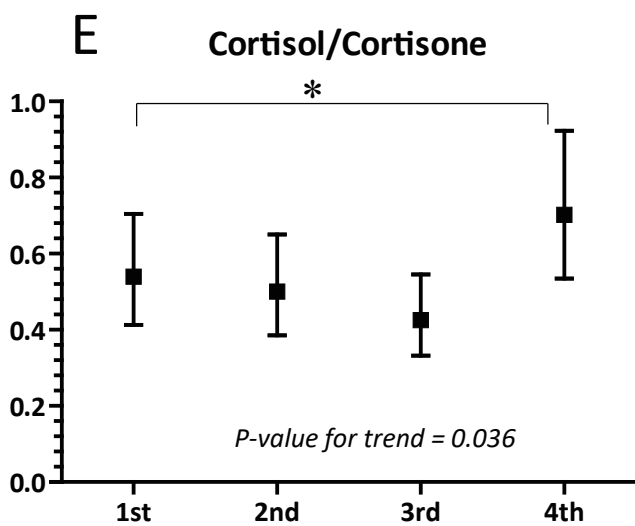
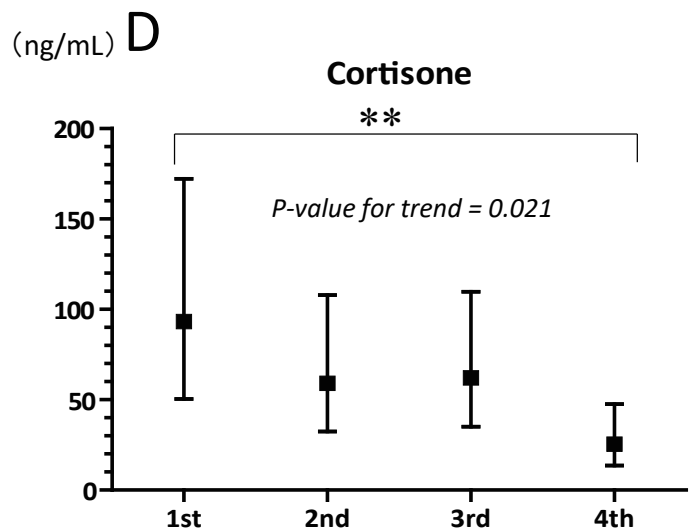
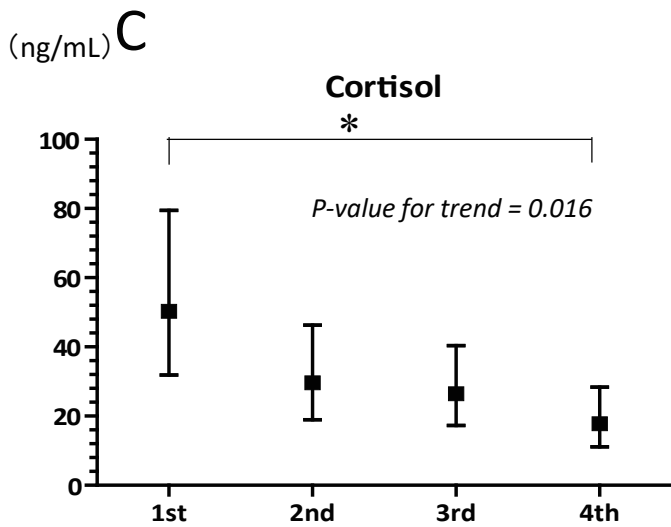
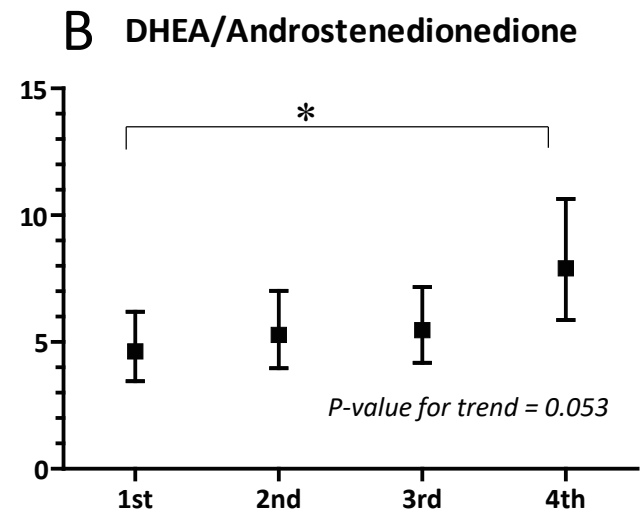
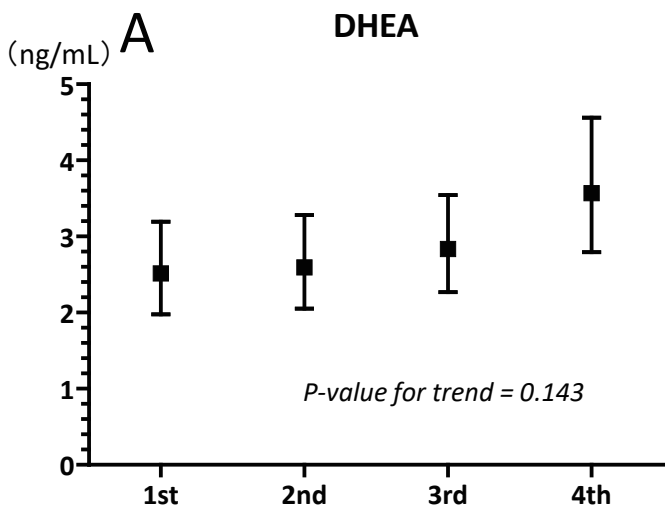


Figure 1

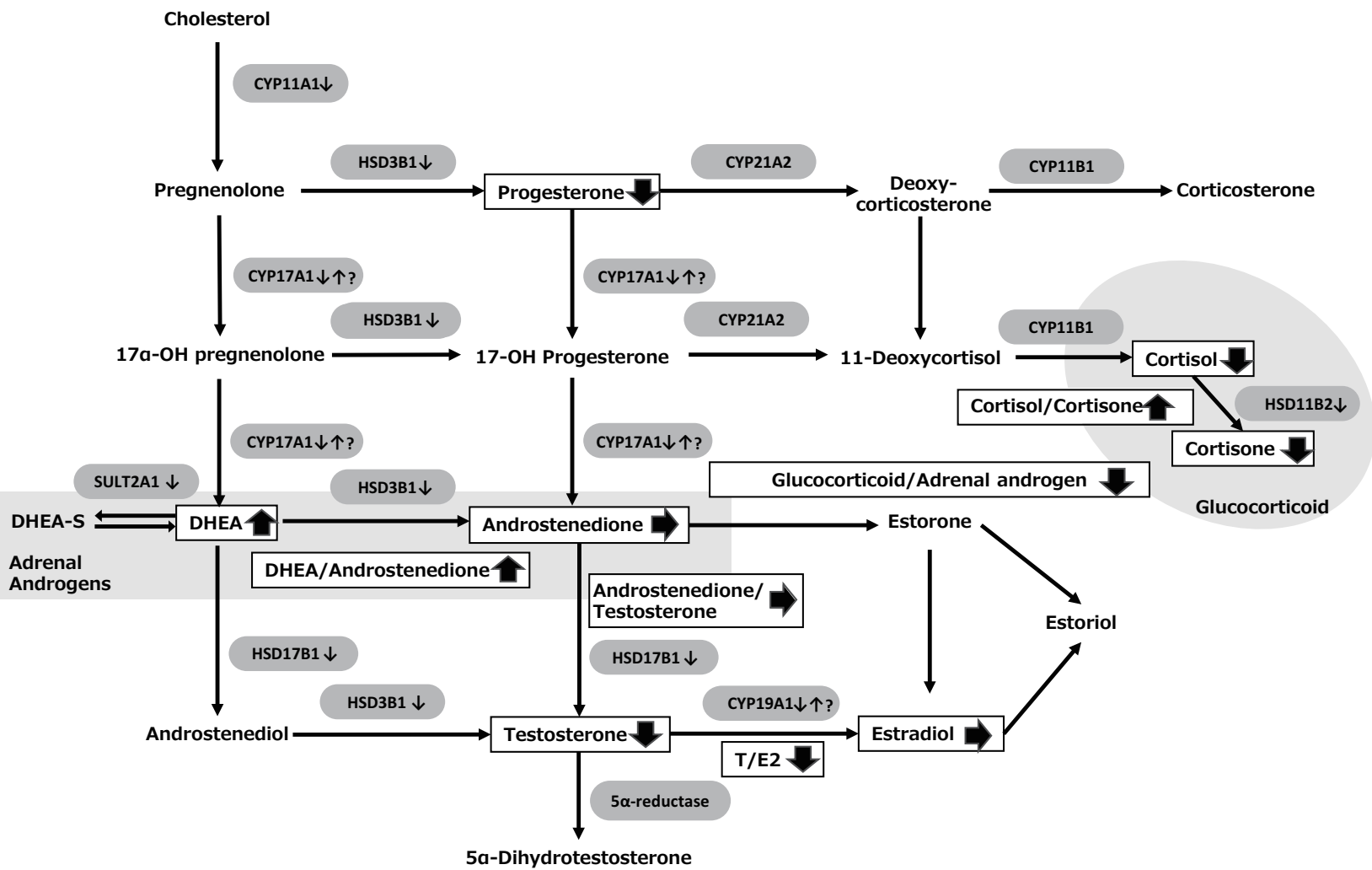


Figure 2



1 **Figure legend**

2 Figure 1

3 X-axis shows the mono(2-ethylhexyl) phthalate (MEHP) quartiles, and Y-axis shows each hormone  
4 level. The adjusted least square means (LSMs) with 95% confidence intervals for each hormone in the  
5 cord blood in relation to the MEHP concentration quartile fit to all study participants are shown with a  
6 *p*-value trend for: (A) DHEA, (B) DHEA/androstenedione ratio, (C) cortisol, (D) cortisone, (E)  
7 cortisol/cortisone ratio, and (F) glucocorticoid (cortisol and cortisone)/adrenal androgen (DHEA and  
8 androstenedione) ratio. There was a significant and inverse association between MEHP levels and  
9 cortisol and cortisone levels, glucocorticoid/adrenal androgen ratio, but a positive association between  
10 MEHP levels and the cortisol/cortisone ratio. The 1<sup>st</sup> quartile ( $\leq 5.88$  ng/mL) was compared with the 2<sup>nd</sup>  
11 (5.88–10.4 ng/mL), 3<sup>rd</sup> (10.4–15.3 ng/mL), and 4<sup>th</sup> ( $>15.3$  ng/mL) quartile MEHP levels. Statistical  
12 significance of the *P* values were  $*p < 0.017$  and  $**P < 0.0033$  based on Bonferroni correction. When  
13 compared with the LSM of the 1<sup>st</sup> MEHP quartile, the 4<sup>th</sup> MEHP quartile of cortisol, cortisone, and  
14 glucocorticoid/adrenal androgen ratios were significantly decreased, whereas the  
15 DHEA/androstenedione ratio and cortisol/cortisone ratio were significantly increased. LSMs were  
16 adjusted for maternal age, smoking and alcohol consumption during pregnancy, gestational age, blood  
17 sampling week, infant sex, and perfluorooctane sulfonate level

18 Abbreviations: DHEA, dehydroepiandrosterone; LSM, least square means, MEHP,  
19 mono(2-ethylhexyl) phthalate

20 Figure 2

21 Steroid metabolic pathways and their disruption following mono(2-ethylhexyl) phthalate (MEHP)  
22 exposure. Squares indicate the hormones measured in this study and our previous report. Black bold  
23 arrows indicate the direction of shift following MEHP exposure. Black characters and arrows in gray  
24 background and rounded rectangles indicate enzymes reported as upregulated or downregulated by di  
25 (2-ethylhexyl) phthalate (DEHP) and/or MEHP in experimental studies.

26 Abbreviations: CYP11A1, cytochrome P450 family 11 subfamily A member 1; CYP11B1,  
27 cytochrome P450 family 11 subfamily B member 1; CYP17A1, cytochrome P450 family 17 subfamily  
28 A member 1; CYP19A1, cytochrome P450 family 19 subfamily A member 1; CYP21A2, cytochrome  
29 P450 family 21 subfamily A member 2; HSD11B2, hydroxysteroid 11-beta dehydrogenase 2;  
30 HSD17B1, hydroxysteroid 17-beta dehydrogenase 1; HSD3B1, hydroxy-delta-5-steroid  
31 dehydrogenase, 3 beta- and steroid delta-isomerase 1; SULT2A1, DHEA sulfotransferase family 2A  
32 member 1