Developmental exposure to the DE-71 mixture of polybrominated diphenyl ether (PBDE) flame retardants induce a complex pattern of endocrine disrupting effects in rats

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Polybrominated diphenyl ethers (PBDEs) are legacy compounds with continued widespread human exposure. Despite this, developmental toxicity studies of DE-71, a mixture of PBDEs, are scarce and its potential for endocrine disrupting effects in vivo is not well covered. To address this knowledge gap, we carried out a developmental exposure study with DE-71. Pregnant Wistar rat dams were exposed to 0, 40 or 60 mg/kg bodyweight/day from gestation day 7 to postnatal day 16, and both sexes were examined. Developmental exposure affected a range of reproductive toxicity endpoints. Effects were seen for both male and female anogenital distances (AGD), with exposed offspring of either sex displaying around 10% shorter AGD compared to controls. Both absolute and relative prostate weights were markedly reduced in exposed male offspring, with about 40% relative to controls. DE-71 reduced mammary gland outgrowth, especially in male offspring. These developmental *in vivo* effects suggest a complex effect pattern involving anti-androgenic, anti-estrogenic and maybe estrogenic mechanisms depending on tissues and developmental stages. Irrespective of the specific underlying mechanisms, these in vivo results corroborate that DE-71 causes endocrine disrupting effects and raises concern for the effects of PBDE-exposure on human reproductive health, including any potential long-term consequences of disrupted mammary gland development.

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ABSTRACT

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human exposure. Despite this, developmental toxicity studies of DE-71, a mixture of PBDEs, are

scarce and its potential for endocrine disrupting effects in vivo is not well covered. To address

this knowledge gap, we carried out a developmental exposure study with DE-71. Pregnant

Wistar rat dams were exposed to 0, 40 or 60 mg/kg bodyweight/day from gestation day 7 to

postnatal day 16, and both sexes were examined. Developmental exposure affected a range of

reproductive toxicity endpoints. Effects were seen for both male and female anogenital distances

(AGD), with exposed offspring of either sex displaying around 10% shorter AGD compared to

controls. Both absolute and relative prostate weights were markedly reduced in exposed male

offspring, with about 40% relative to controls. DE-71 reduced mammary gland outgrowth,

especially in male offspring. These developmental *in vivo* effects suggest a complex effect

pattern involving anti-androgenic, anti-estrogenic and maybe estrogenic mechanisms depending

on tissues and developmental stages. Irrespective of the specific underlying mechanisms, these in

vivo results corroborate that DE-71 causes endocrine disrupting effects and raises concern for the

effects of PBDE-exposure on human reproductive health, including any potential long-term

consequences of disrupted mammary gland development.

Introduction

36   Normal development through gestation and early postnatal life is essential for lifelong health and

37   disruption to developmental processes can result in adverse effects and increased susceptibility to

38   disease. Since the environment where development occurs plays a key role in ensuring proper

39   development, environmental stressors are also major contributors to the development of diseases.

40   This includes endocrine disrupting chemicals, which can disturb molecular and biological

41   pathways that, through their effects on the endocrine systems, can change developmental

42   processes and cause adverse health effects (Gore et al., 2015).

43   Polybrominated diphenyl ethers (PBDEs) are legacy compounds that were widely used as flame

44   retardants in various consumer and industrial products. They have been shown to cause

45   numerous adverse health effects in humans and animals and are now restricted under the

46   Stockholm Convention on Persistent Organic Pollutants (Stockholm Convention, 2019).

47   Unfortunately, humans are still exposed to PBDEs, as they are persistent in indoor and outdoor

48   environments and bioaccumulate.

49   Among the many human health effects for which PBDEs have been implicated, thyroid hormone

50   system disruption, neurotoxicity and reproductive disorders are prevalent (Boas, Feldt-

51   Rasmussen & Main, 2012; Attina et al., 2016; Trasande et al., 2016). Still, their specific

52   endocrine mechanisms of action are not well understood and a characterization of the adverse

53   reproductive effects in perinatally exposed rat offspring remain lacking. Even though PBDEs are

54   largely banned, such information is still valuable as human exposure will continue and since it

55   will help us better understand the current disease burden of these legacy chemical substances as

56   well as of endocrine disruptors in general. Perhaps equally important is its applicability towards

57   improvement of current safety assessment regimens, including considerations for mixture effects.

58   Also experimental studies have shown various potential endocrine effects of DE-71 and its

59   metabolites. Amongst the effects potentially relevant for reproductive health are weak anti-

60   estrogenic and estrogenic effects *in vitro* and *in vivo* (Mercado-Feliciano & Bigsby, 2008a,b) as

61   well as clear anti-androgenic properties *in vitro* (Stoker et al., 2005) and in exposed male rats

62   (Stoker et al., 2004, 2005), while the potential effects of DE-71 in a developmental toxicity

63   scenario are understudied. A recent meta-analysis on PBDEs suggest broad adverse reproductive

64   effects in postnatal male rats (Zhang et al., 2020). Based on this, we surmised that the DE-71

65   mixture could reduce androgen signaling during fetal life and induce effects resembling the

66   testicular dysgenesis syndrome (Skakkebaek et al., 2016).

67   To test our hypothesis, and hence contribute new knowledge to the *in vivo* endocrine modes of

68   action of PBDEs, we conducted a developmental rat toxicity study to determine the effects of

69   early life exposure to DE-71 on endocrine-sensitive endpoints that have not previously been

70   investigated or only investigated at lower doses with smaller statistical power. These endpoints

71   were anogenital distance (AGD) (Schwartz et al., 2019), nipple retention (NR) (Schwartz et al.,

72   2021), and a few selected reproductive postnatal and adult organ weights. We also included

73   analyses of mammary gland development in both male and female offspring by whole-mount

74   assessments. Mammary glands are rarely investigated in reproductive toxicity studies despite

75   their sensitivity to endocrine disruption and importance for reproductive health (Fenton, Reed &

76   Newbold, 2012; Gouesse et al., 2019). Our study corroborates the endocrine activity of DE-71 in

77   vivo and shows how multiple active endocrine mechanisms can result in a complex in vivo effect

78   pattern.

79

80

Materials and Methods

Chemicals

The test compound was a commercial mixture of PBDEs, DE-71 (pentabrominated diphenyl ethers (pentaBDE) from the Great Lakes Chemical Corporation of West Lafayette, IN, CAS No. 32534-81-9, lot 7550OK20A), a kind gift from Dr. Kevin Crofton at the U.S. Environmental Protection Agency. The manufacturer reports DE-71 to contain 50-62% pentaBDE, 24-38% TetraBDE, 4-12% hexaBDE, and 0-1% triBDE (amounting to a possible total of 154 congeners) (ENVIRON International Corporation, 2003). Corn oil (Sigma-Aldrich) was used as control compound and vehicle for all treatments.

Animals and treatment

We conducted two rat toxicity studies (Fig. 1). Study 1 (range-finding) aimed at determining the highest doses of DE-71 that could be given to pregnant and lactating rat dams without causing overt systemic toxicity in either dams or offspring. Study 2 (main study) aimed at assessing a series of endocrine sensitive endpoints to clarify the full potential endocrine effect pattern of DE-71 in a developmental toxicity study. We also assessed effects on the thyroid hormone system and on neurodevelopment (Ramhøj et al., in prep). In both studies, time-mated nulliparous young adult Wistar rats (HanTac: WH, SPF, Taconic Europe, Ejby, Denmark) were received on gestation day (GD) 3 of pregnancy (day of plug-detection designated GD1), randomly distributed for pairwise housing and on GD4 pseudo-randomly divided into groups with similar weight distribution.

103   The expected day of delivery, GD23, was designated PD1 irrespective of the actual day of

104   delivery.

105   In Study 1, dams were divided into 4 groups (n = 10 per group) and exposed to vehicle control or

106   20, 40 or 60 mg/kg body weight (bw)/day DE-71. Study 2 was performed in two balanced blocks

107   with 3 groups (n = 22 per group, in order to have sufficient sample size for mammary gland

108   examinations) exposed to vehicle, 40 or 60 mg/kg bw/day DE-71. Dosing of the dams was

109   performed daily by gavage in the morning from GD7 to GD22, and again from postnatal day

110   (PD)2 – PD14 in Study 1 (study termination) and PD2-16 in Study 2. Dosing was performed

111   with a metal gastric tube with either vehicle (corn oil) or experimental solutions at a constant

112   volume of 2 ml/kg/day, with the individual doses based on the body weight of the animal prior to

113   dosing.

114

115   Dams were housed pairwise until GD17 and individually thereafter. We used semitransparent

116   plastic cages (15 × 27 × 43 cm) with aspen wood hides and with aspen bedding (Tapvei,

117   Gentofte, Denmark) placed on racks (balanced for treatment group) in an animal room with

118   controlled environmental conditions: reversed light/dark cycles of 12 hours (light from 9 pm-9

119   am, light intensity 500 lux), temperature 22±1°C, humidity 55±5%, and ventilation changing air

120   10 times per hour. All animals were fed ad libitum on a standard diet, Altromin 1314 (soy- and

121   alfalfa-free; Altromin GMbH, Lage, Germany), and acidified tap water was provided ad libitum

122   in PSU bottles (84-ACBT0702SU Techniplast).

123   All endpoint examination procedures were carried out with treatment groups represented in a

124   random order (i.e. not examining all controls first, then treated animals). Examinations and

125 outcome measures of live animals were conducted blinded to exposure group while data analysis

126 was conducted unblinded.

127 Animals were observed twice daily for health and signs of overt toxicity (such as hunched

128 posture and raised fur). Criteria followed for euthanasia of animals: if it was considered

129 irresponsible or unethical to let the animal live or if they were in a severe condition (expected to

130 die within 24hrs). Accordingly, some litters and pups in Study 1 (see Table 1) were euthanized,

131 as the dams did not take care of them and they were without milk in their stomachs. This

132 phenomenon only took place in Study 1 and was unrelated to exposure, it also includes some

133 dams that cannibalized their pups. In Study 2, one dam was euthanized on GD23 as she appeared

134 unwell and incapable of giving birth (necropsy did not reveal any explanations for the dystocia).

135 Animal experiments were carried out at the DTU National Food Institute (Mørkhøj, Denmark)

136 facilities. Ethical approval was obtained from the Danish Animal Experiments Inspectorate, with

137 authorization number 2012-15-2934-00089 C4. The experiments were overseen by the National

138 Food Institute's in-house Animal Welfare Committee for animal care and use. All methods in the

139 study were performed in accordance with relevant guidelines and regulations.

140

141 *Birth and postnatal development*

142 On the morning after overnight birth, dam and pup body weights were registered, and the pups

143 were sexed and checked for macroscopic anomalies. Body weights of offspring were recorded on

144 PD6, PD10 and PD14 in Study 1 and on PD6, PD14 and PD27 in Study 2.

145

146 Endocrine sensitive endpoints were investigated in Study 2. Anogenital distance (AGD), the

147 distance between the anus and the genital papilla was measured in all live offspring on PD1,

148 using a stereomicroscope with a micrometer eyepiece. The AGD index (AGDi) was calculated

149 by dividing the AGD with the cube root of the body weight. On PD14 all offspring had their

150 areolas/nipples counted. Nipple retention (NR) of male pups was defined as the number of

151 areolas/nipples (a dark focal area with or without a nipple bud) visible where nipples are usually

152 located (along the milk lines) in female pups (Schwartz et al., 2021). Both AGD and NR were

153 assessed by the same technician, blinded to exposure group.

154

155 On PD27, one male pup from each litter was weaned and housed pairwise with another male

156 from the same group (when no other males were available, housing was with a littermate).

157

158 *Necropsy*

159 Before necropsy was performed, all animals were weighed, anesthetized with CO_2/O_2 and killed

160 by decapitation. Selected reproductive organs were examined in offspring killed on PD16, PD27

161 and PD300 in Study 2. On PD16 one male and one female from each litter were killed and

162 ovaries and ventral prostate were excised and weighed. Mammary glands were collected as

163 whole-mounts from one male and one female on PD27. On PD300, one male offspring from

164 each litter was terminated and prostate together with seminal vesicle was excised and weighed.

165 The ventral prostate was subsequently dissected and weighed alone.

166

167 All dams not giving birth (i.e. with resorbed embryos or never pregnant) were humanely killed

168 on PD3, those with litters were humanely killed, along with remaining pups, on PD14 in Study 1

169   and on PD27 in Study 2. Implantation scars in uteri were counted to determine pregnancy rates

170   and resorptions.

171

172   *Whole-mounts*

173   Mammary glands were excised and spread onto a glass slide, covered with parafilm and placed

174   under pressure for 2 hours. The whole-mounts were subsequently fixed in 4% formalin buffer,

175   stained with alum carmine, dehydrated in alcohol and cleared with xylene. The slides were

176   scanned in a flatbed scanner at 4800 dpi and images of the 4th gland evaluated as described in

177   (Mandrup et al., 2015) for outer area (defined as the smallest polygon enclosing the gland),

178   transverse growth (as defined by (Mandrup et al., 2012)), longitudinal growth, distance to lymph

179   node, distance to 5th gland (n = 13-17, except distance to the 5th gland where n = 11, 4 and 10 in

180   control, PBDE-40 and PBDE-60, respectively) and number of terminal end buds (TEB) (TEB

181   was defined as tear-drop shaped buds with a diameter >100 μm in zone C, as defined by (Russo

182   & Russo, 1996) (n = 17, 11 and 14 in control, PBDE-40 and PBDE-60, respectively). Distance to

183   lymph node was measured and scored (scores 1-3, with a score of 1 representing mammary

184   tissue not reaching the lymph node, score 2 representing mammary tissue reaching the lymph

185   node and score 3 was given to mammary glands where the tissue reaches beyond the lymph

186   node). All data were assessed for males and females separately and blinded to exposure. In

187   addition, for area measurements, longitudinal and transverse growth data from both sexes were

188   pooled for analysis with increased statistical power. Images were analyzed with Image Pro Plus

189   7.0 (Media Cybernetics, Bethesda, MD, USA) and calibration performed for each picture before

190   measurements were made.

191

192   *Statistical analysis*

193   The alpha level for statistical significance was always set to 5% and all samples were included in

194   the analysis. Data with normal distribution and homogeneity of variance were analyzed for

195   treatment-related effect differences relative to the control by analysis of variance (ANOVA)

196   followed by Dunnet's correction for multiple comparisons. Data were transformed if these

197   conditions were not met and data not fulfilling the criteria were analyzed using a non-parametric

198   statistical test (Kruskal-Wallis with Dunn's multiple comparison test). When relevant, body

199   weight was included as a covariate in the analysis (ANCOVA), e.g. for terminal organ weights

200   and whole-mount measurements. Litter effects were accounted for by only analyzing one pup per

201   litter, using litter means (offspring growth) or by including the litter as a random effect variable (

202   AGD, NR and pooled whole mounts). Distance to lymph node was evaluated as the distance and

203   as scores. The scores were analyzed using Kruskal-Wallis and using a two-sided Fishers exact

204   test to compare the number of animals with glands reaching past the lymph node with the

205   number not reaching past. The number of nipples/areolas (NR) was assumed to follow a

206   binomial-distribution with a response range between 0 and 12 (12 assumed to reflect the

207   biologically possible maximal number of nipples in rats). Litter effects on NR and over-

208   dispersion in the data were accounted for by using Generalized Estimating Equations (GEE) as

209   reported in (Christiansen et al., 2012).

210   SAS Enterprise guide 4.3 (2010) (SAS Institute Inc, Cary, NC, USA) and GraphPad Prism 5

211   (Graphpad Software, San Diego, CA; USA) was used for statistical analysis.

212

213 **Results**

214 *Effects on pregnancy, postnatal growth and general toxicity*

215 We administered DE-71 at doses of 20, 40 and 60 mg/kg to the pregnant and lactating dams from

216 GD7 to PD14/16. Pregnancy and litter data from Study 1 and Study 2 are listed in Table 1 and

217 Table 2. In Study 1, there were signs of decreased maternal weight gain at 40 and 60 mg/kg.

218 Pups from the 60 mg/kg bw/day exposure group weighed 8% less than control pups at birth and

219 gained 20% less during the first postnatal week. However, none of these findings were

220 statistically significant. Thus, 60 mg/kg was chosen as the highest dose in Study 2 as it did not

221 induce excessive systemic toxicity in dams and pups.

222 In Study 2, dam weight gain during pregnancy was significantly decreased at 60 mg/kg (Table

223 2). The high dose pups had decreased body weight on PD6 (Table 2) and body weight remained

224 non-significant reduced throughout the postnatal period (Table 2) with non-significant reductions

225 also on PD27 (~7% reduction). Gestation lengths, litter size and pup mortality was not affected

226 by exposure.

227

228 *Effects on markers of early-life endocrine disruption*

229 A shorter anogenital distance (AGD) and retained nipples in male offspring are considered

230 sensitive markers of disrupted androgen action during development (Schwartz et al., 2019). DE-

231 71 exposure induced a significantly shorter AGD and smaller AGDindex (AGDi, AGD adjusted

232 for pup body size) at both 40 and 60 mg/kg. However, the dose-response curve was 'flat' in that

233 reductions in AGD/AGDi were comparable between the two dose groups (10/11% reduced in the

234 low dose group and 8/6% in the high dose group). The effect on AGD was also comparable

235   between both sexes (Fig. 2A and Table 2). Nipple retention was increased in males from the high

236   dose group (p = 0.0249). Notably, the mean number of 0.25 nipple in these high dose males is

237   considered very small (Fig. 2B) and falls well within the range of our historic control data

238   (Schwartz et al 2021, in submission - ref to come).

239

240   *Early- and late-life effects on reproductive tissue weights*

241   Weights of selected reproductive organs were assessed both early in life and in adulthood. DE-71

242   at a dose of 60 mg/kg reduced absolute and relative male PD16 prostate weights with 37% and

243   42%, respectively (Fig. 3 and Table 3). In female offspring, ovary weights were assessed on

244   PD16 and showed no significant differences between controls and exposed animals.

245   On PD300, changes to prostate weights in exposed offspring were no longer apparent, nor were

246   effects on seminal vesicle or ventral prostate weights (Table 3).

247

248   *Effects on mammary gland development*

249   We assessed mammary gland development by whole-mounts in both male and female offspring

250   at PD27 and found that mammary gland outgrowth was stunted by perinatal exposure to DE-71.

251   In male offspring, the transverse growth and mammary gland areas were statistically

252   significantly reduced in the highest exposure group (Fig. 4). Females appeared to display a

253   similar trend, albeit not statistically significant. When analyzing pooled data from both sexes

254   (litter effects were accounted for by including the litter as a random nested factor in the statistical

255   analysis) there was an effect on area in both dose groups and on the transverse growth in the high

256   dose group (Fig. 4). The observed effects are likely not due to reduced body weight of the

257   offspring in the high dose as the effects were observed both on absolute numbers and when

258   accounting for the body weight of the animals in the statistical analysis.

259

260   **Discussion**

261   While production and use of DE-71 is no longer permitted, the constituents of the DE-71 mixture

262   still account for a significant proportion of the brominated flame retardants found in house dust

263   (Bramwell et al., 2016). Humans, including babies and toddlers, continue to be exposed to

264   PBDEs (Klinčić et al., 2020), especially from indoor environments, so a continued focus on the

265   potential detrimental health effects of this exposure is warranted. In this study, we have shown

266   strong endocrine disrupting effects following developmental exposure to DE-71. In contrast to

267   what was expected from existing studies, however, the endocrine disrupting effects seems to be

268   caused by a complex pattern of modalities and not simply anti-androgenic, as discussed in the

269   following.

270

271   Studies on DE-71, including a Hershberger assay, have clearly shown dose-dependent anti-

272   androgenic effects in male rats when exposure occurs during puberty and adulthood. Effects

273   include delayed preputial separation and reduced prostate weights in animals exposed to 30

274   mg/kg and above. Additional reproductive tissues such as seminal vesicle, glandula

275   bulbourethralis and penis showed dose-dependent weight reductions at higher doses (up to 240

276   mg/kg bw/day) (Stoker et al., 2004, 2005). Reductions in reproductive organ weights were seen

277   at 200 mg/kg, but not at 67 mg/kg or lower in a 28-day exposure study (van der Ven et al., 2008).

278   In contrast, a dietary study using a 70-day exposure to a mixture of PBDEs (of which 52% was

279   DE-71), but at maximum dose of 20 mg/kg, found no effects on male reproductive organs,

280   hormone concentrations or sperm count (Ernest et al., 2012). Thus, PBDEs can seemingly elicit

281   anti-androgenic effects in pubertal and adult male rats at doses of 30 mg/kg or higher. With

282   respect to fetal and early postnatal exposure, however, there are not much available data.

283   One study examines effects on anti-androgenic endpoints after developmental exposure (GD21-

284   PND22) to 1, 10 and 30 mg/kg DE-71 administered by gavage (Kodavanti et al., 2010). Here

285   effects in the male offspring included a statistically significant 1.8-day delay in preputial

286   separation, a 5% non-significant shortening of AGD, and a 20% non-significant decrease in

287   testosterone concentration on PND 60. Using a similar experimental setup to Kodavanti et al

288   (2010), but with higher exposure doses, we observed shorter male AGD of around 10% in both

289   dose groups (40 and 60 mg/kg). We also observed a small effect on male nipple retention in the

290   high dose group, with a mean of 0.25 retained nipples - an incidence rate that lies within our

291   historical control data (Schwartz et al., 2021) but is significantly different from the concurrent

292   control. Reduced male AGD is associated with reproductive malformations and reduced sperm

293   count and is usually indicative of an anti-androgenic mode of action (Christiansen et al., 2008;

294   van den Driesche et al., 2011; Thankamony et al., 2016; Schwartz et al., 2019). However, we

295   also observed shorter female AGD to a very similar degree as in the male offspring, which we do

296   not normally see for clear anti-androgenic compounds (Schwartz et al., 2019). Rather, this effect

297   on female AGD suggests additional modalities induced by the DE-71 mixture.

298   We have previously shown that developmental exposure to BPA and butylparaben, which mainly

299   are considered estrogenic compounds, albeit they also have other modes of action, induce some

300   of the same effects as those observed with the DE-71 mixture: moderately shorter AGD without

301   dose response, similar effects on AGD in both sexes, and no or minimal effect on nipple

302 retention (Christiansen et al., 2014; Boberg et al., 2016). This could indicate that some of the

303 same endocrine mechanisms are targeted by BPA, butylparaben and DE-71. However, our

304 understanding of the underlying mechanisms causing this particular effect pattern in males and

305 the reduced AGD in females remain elusive (Schwartz 2019). Thus, weak anti-androgenic effects

306 and other endocrine mechanisms could be responsible for the PBDE induced moderate effects on

307 AGD and NR. Notably, intrauterine exposure to PBDEs has been associated with reduced AGD

308 (Luan et al., 2019) and increased risk of hypospadias in boys (Poon et al., 2018; Koren et al.,

309 2019). Although hypospadias has been linked to anti-androgenic chemicals in both humans and

310 rodents, it has also been linked to several compounds with known estrogenic potentials, which

311 suggests the involvement of disrupted androgen-estrogen balance as a critical factor in phallus

312 development (Mattiske & Pask, 2021). Taken together, this could suggest that the DE-71 mixture

313 induce a more complex disruption to steroid hormone homeostasis which can result in a varied

314 effect pattern.

315 The most marked effect observed in the present study was reduction in prostate weights on

316 PD16, which was around 40% decreased in exposed versus control animals. Again, this effect

317 could be caused by DE-71 having anti-androgenic properties in the postnatal pups as clearly seen

318 in pubertally exposed animals (Stoker 2004, 2005). However, prostate development is also

319 sensitive to estrogen signaling (Gupta, 2000; Prins & Korach, 2008; OECD, 2018) which could

320 also contribute to the observed effect. Interestingly, neither BPA nor butylparaben exposure

321 affected PD16 prostate weights in our studies (Boberg 2016, Christiansen 2014) which could

322 indicate that DE-71 works through a different mechanism or possess different ADME

323 (absorption, distribution, metabolism, excretion) properties than BPA and butylparaben in the

324   postnatal period. Overall, it remains possible that the reduced prostate weight induced by DE-71

325   could have been a result of disrupted androgen-estrogen balance similarly to external genitalia.

326   In addition to the more commonly assessed effect endpoints AGD, NR and prostate weights, we

327   also assessed mammary gland development in both male and female offspring. DE-71 exposure

328   decreased mammary gland area and transverse growth in perinatally exposed offspring,

329   corroborating findings from a DE-71 toxicity study in Long-Evans rats. In the Long-Evans study

330   only female pups were examined, but perinatally exposed female pups showed less outgrowth,

331   fewer lateral branches and limited terminal end bud (TEB) formation on PND 21 (Kodavanti et

332   al., 2010).

333   The delayed mammary gland development in DE-71 exposed offspring is in contrasts to what

334   would be expected for estrogenic compounds, as accelerated or increased mammary gland

335   growth is typically seen after exposure to estrogenic compounds (Mandrup et al., 2012, 2015;

336   Macon & Fenton, 2013) including for BPA and butylparaben (Boberg et al., 2016; Mandrup et

337   al., 2016). Thus the effects of DE-71 on the mammary glands do not appear to support an

338   estrogenic mode of action. The reduced mammary gland outgrowth caused by DE-71 is also not

339   indicative of anti-androgenic mode of action, as such compounds may not induce an effect on

340   mammary growth in prepubertal stages, but only later in life (Škarda, 2003; Peters et al., 2011;

341   Jacobsen et al., 2012; Mandrup et al., 2015). In fact, the observed reductions in mammary gland

342   outgrowth in the present study may best be attributed to an anti-estrogenic mode of action. This

343   fits well with other studies where anti-estrogenic compounds such as ICI 182,780 can cause

344   decreased mammary gland growth and branching in prepubertal female offspring (Silberstein et

345   al., 1994; Cotroneo, 2002).

346   Although mammary gland development is sensitive to estrogen signaling, it is also regulated

347   through other signaling pathways. For instance, 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)

348   given on GD15 can stunt mammary gland growth from PD 4 and persist until PD 68 when the

349   glands still retained undifferentiated terminal structures (Fenton et al., 2002). This effect has

350   been suggested to be mediated through AhR activation (Hushka, Williams & Greenlee, 1998;

351   Fenton et al., 2002; Helle et al., 2016), a mechanism that may also be relevant for DE-71

352   (Hamers et al., 2006). Also 3,3',4,4',5-pentachlorobiphenyl (PCB-126), which can bind AhR and

353   have anti-estrogenic properties has affected mammary glands similarly to DE-71 (Fenton et al.,

354   2002; Muto et al., 2002). Thus various environmental chemicals, including DE-71, seem to cause

355   similar effect patterns on the mammary gland albeit the exact mechanism(s) are still unclear.

356

357

358   In conclusion, we have shown that developmental exposure to DE-71 induces endocrine

359   disrupting effects in rats. We found effects on NR and prostate weights in the male offspring, and

360   effects on AGD and mammary gland development in both males and females. The effect pattern

361   is complex and likely involve various mechanisms of action, which are difficult to pin down. The

362   effect-pattern observed in this study suggest that the mixture acts by anti-androgenic, anti-

363   estrogenic and maybe estrogenic modes of action, possibly in combination with effects on other

364   signaling pathways such as AhR signaling. DE-71 exposure causes endocrine disruption in both

365   male and female rat offspring and thus raises concerns for the long-term consequences of human

366   exposure to PBDEs, especially since associations between PBDE concentrations, AGD, and

367   hypospadias in boys have been reported.

368

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378

Declaration of interests

380 We declare that we have no conflicts of interest.

381

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551

552 **Figure 1.** Study design for developmental toxicity studies with a technical mixture of brominated

553 flame retardants, DE-71. Study 1 (n = 10 dams) was a range-finding study to choose doses for

554 the larger Study 2 (n = 22 dams) that investigated endocrine sensitive endpoints in the perinatally

555 exposed rat offspring. AGD: anogenital distance, Bw: body weight, GD: gestation day, NR:

556 nipple retention, PBDE: polybrominated diphenyl ethers (DE-71), PD: postnatal day.

557

558 **Figure 2.** Anogenital distance (AGD) and nipple retention (NR) in rat offspring after perinatal

559 exposure to brominated flame retardants (DE-71). (A) Reduced AGD in PD1 male and female

560 offspring. (B) NR in male PD14 offspring. n = 19-21 litters. Statistical analysis performed on all

561 pups from each litter with adjustment for litter effects. Litter means+SEM, *p<0.05 and

562 ***p<0.001 compared to control.

563

564 **Figure 3.** Relative ventral prostate weights in PD16 male offspring after perinatal exposure to

565 brominated flame retardants (DE-71). Mean+SEM, n = 16-19. ***p<0.001.

566

567 **Figure 4.** Mammary gland development in PD27 offspring after perinatal exposure to

568 brominated flame retardants (DE-71). (A) Area of the mammary gland was reduced. (B)

569 Transverse growth of the mammary gland was reduced at the highest dose. Mean+SEM. n = 14-

570 17 males, n = 18-20 females, n = 32-37 for males and females pooled and litter effects accounted

571 for in the statistical analysis. Mean+SEM. *p<0.05 (bw as covariate), #p<0.05 using ANOVA.

Figure 1

Study design for developmental toxicity studies with a technical mixture of brominated flame retardants, DE-71.

Study 1 (n = 10 dams) was a range-finding study to choose doses for the larger Study 2 (n = 22 dams) that investigated endocrine sensitive endpoints in the perinatally exposed rat offspring. AGD: anogenital distance, Bw: body weight, GD: gestation day, NR: nipple retention, PBDE: polybrominated diphenyl ethers (DE-71), PD: postnatal day.

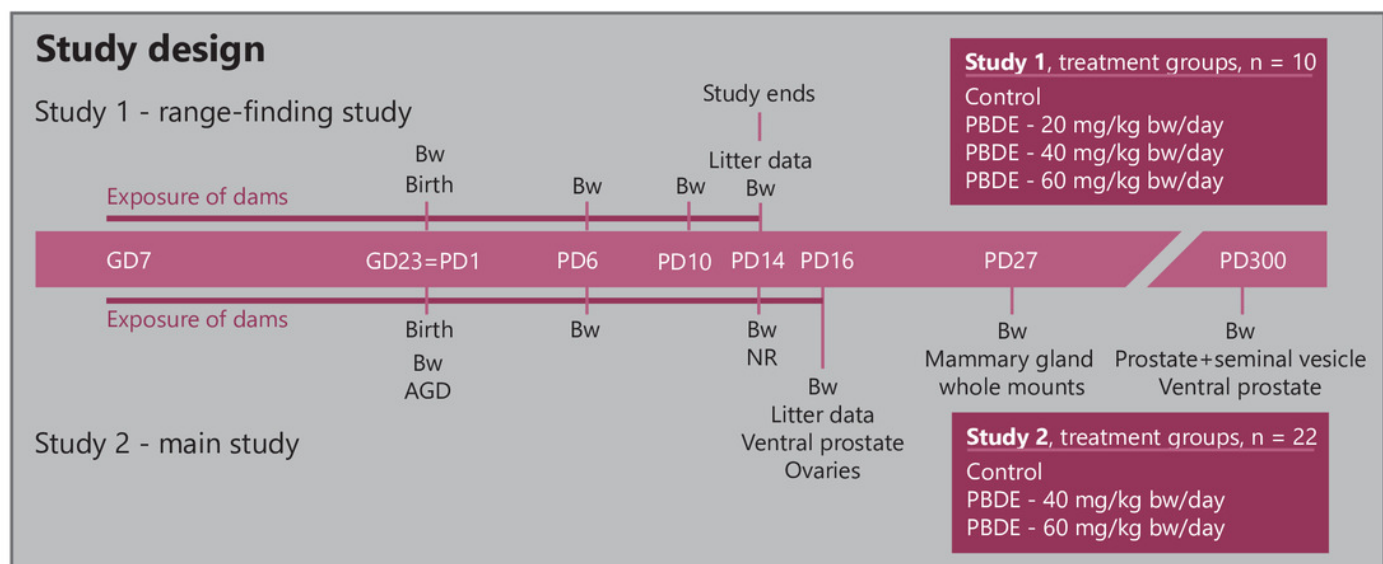



Figure 2

Anogenital distance (AGD) and nipple retention (NR) in rat offspring after perinatal exposure to brominated flame retardants (DE-71)

(A) Reduced AGD in PD1 male and female offspring. (B) NR in male PD14 offspring. n = 19-21 litters. Statistical analysis performed on all pups from each litter with adjustment for litter effects. Litter means+SEM, *p<0.05 and ***p<0.001 compared to control.

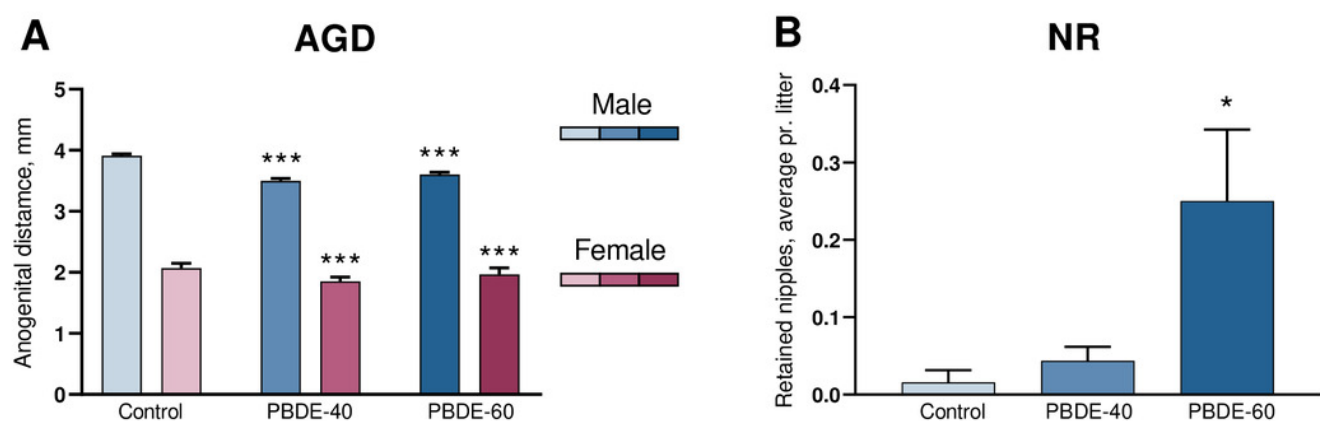



Figure 3

Relative ventral prostate weights in PD16 male offspring after perinatal exposure to brominated flame retardants (DE-71).

Mean+SEM, n = 16-19. ***p<0.001.

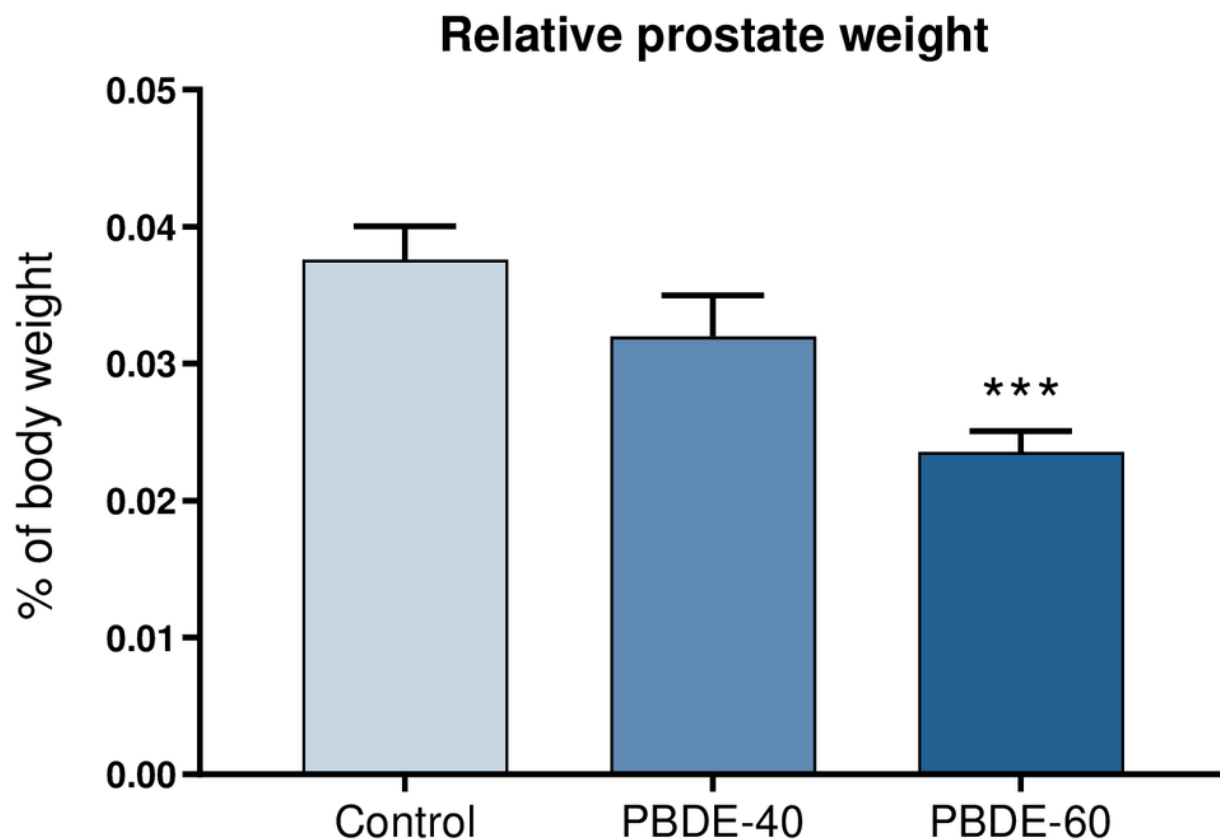
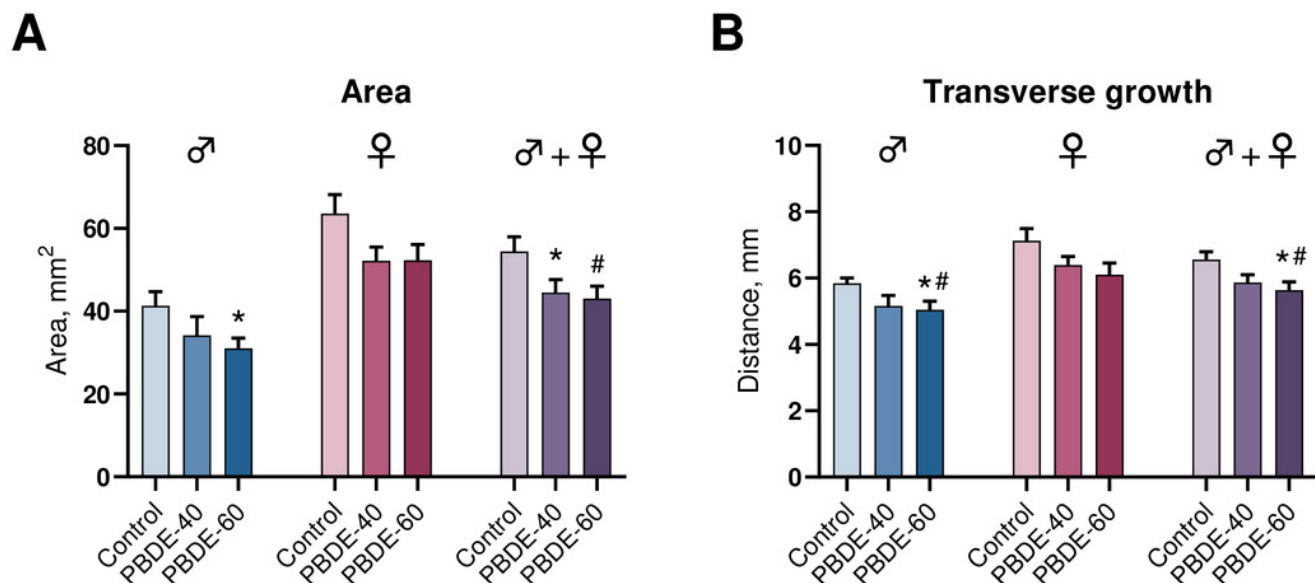



Figure 4

Mammary gland development in PD27 offspring after perinatal exposure to brominated flame retardants (DE-71)

(A) Area of the mammary gland was reduced. (B) Transverse growth of the mammary gland was reduced at the highest dose. Mean+SEM. n = 14-17 males, n = 18-20 females, n = 32-37 for males and females pooled and litter effects accounted for in the statistical analysis. Mean+SEM. *p<0.05 (bw as covariate), #p<0.05 using ANOVA.

Pregnancy and litter data: Study 1

bw: body weight, GD: Gestation day, PD: postnatal day. [a]Unrelated to exposure there were dams cannibalizing or not taking care of their pups in this study. Data represented as Mean±SD.

1   **Table 1.** Pregnancy and litter data: Study 1

Dams and litters	Control	20 mg/kg DE-71	40 mg/kg DE-71	60 mg/kg DE-71
No. of dams (viable litters)	n = 10 (8)	n = 10 (7)	n = 10 (8)	n = 10 (8)
Dam body weight gain				
Dam bw-gain, GD7-GD21 (g)	79.6 ± 13.0	87.1 ± 8.4	82.2 ± 14.6	90 ± 17.2
Dam bw-gain, GD7-PD1 (g)	16.5 ± 8.8	8.9 ± 20.0	2.1 ± 10.9	4.5 ± 9.2
Dam bw-gain, PD1-14 (g)	15.6 ± 13.4	16.6 ± 14.3	26.1 ± 10.2	28.4 ± 10.8
Litters				
Gestation length	22.9 ± 0.6	23.0 ± 0.0	23.0 ± 0.0	23.0 ± 0.0
Postimplantation loss (prenatal mortality) (%)	16.0 ± 12.6	6.0 ± 5.0	4.7 ± 5.5	4.9 ± 6.4
Perinatal loss[a] (pre- and postnatal mortality) (%)	32.3 ± 37.7	21.0 ± 32.0	6.6 ± 6.5	27.6 ± 39.1
Litter size (no.)	9.6 ± 4.3	11.3 ± 2.5	11.8 ± 3.4	11.8 ± 3.4
Postnatal death[a] (%)	21.0 ± 41.8	15.1 ± 34.7	2.0 ± 3.7	23.5 ± 13.0
Sex ratio, males / females (%)	58 /42 ± 17.9	56 / 44 ± 11.7	46 / 54 ± 12.9	52 / 48 ± 9.2
Offspring				
Male birth weight (g)	6.2 ± 0.7	6.1 ± 0.9	6.2 ± 0.9	5.8 ± 0.7
Female birth weight (g)	5.9 ± 0.6	5.9 ± 0.6	5.9 ± 0.7	5.5 ± 0.7
Mean bw-gain PD1-6 (g)	7.4 ± 1.2	6.9 ± 1.7	6.0 ± 1.3	5.9 ± 1.5
Mean bw-gain PD6-14 (g)	23.1 ± 2.0	24.2 ± 1.0	21.7 ± 1.3	22.1 ± 1.8

2   bw: body weight, GD: Gestation day, PD: postnatal day.
3   [a]Unrelated to exposure there were dams cannibalizing or not taking care of their pups in this study.
4   Data represented as Mean±SD.

Pregnancy and litter data: Study 2

* p<0.05. ***p<0.001. Bw: body weight, GD: Gestation day, PD: postnatal day. Data shown as Mean ±SD.

1   **Table 2.** Pregnancy and litter data: Study 2

Dams and litters	Control	40 mg/kg DE-71	60 mg/kg DE-71
No. of dams (viable litters)	n = 22 (21)	n = 22 (20)	n = 22 (20)
Dam body weight gain			
Dam, GD7-GD21 (g)	84.5 ± 13.6	83.8 ± 14.2	78.6 ± 16.6
Dam, GD7-PD1 (g)	13.0 ± 11.1	11.8 ± 12.5	**2.8 ± 11.0***
Dam, PD1-14 (g)	42.4 ± 13.1	37.0 ± 11.4	39.9 ± 17.8
Dam, PD14-27 (g)	-20.9 ± 11.6	-21.5 ± 9.6	**-12.0 ± 10.9***
Litters			
Gestation length	23.0 ± 0.3	23.0 ± 0.2	23.0 ± 0.1
Postimplantation loss (prenatal mortality) (%)	6.6 ± 8.6	6.3 ± 7.9	11.7 ± 23.9
Perinatal loss[a] (pre- and postnatal mortality) (%)	10.3 ± 8.3	7.1 ± 8.0	21.2 ± 31.9
Litter size (no.)	10.9 ± 3.0	10.7 ± 3.1	10.8 ± 3.6
Postnatal death (%)	3.8 ± 6.1	0.8 ± 2.5	9.5 ± 26.0
Sex ratio, males / females (%)	45 / 55 ± 14	46 / 54 ± 16	46 / 54 ± 14
Offspring			
Male birth weight (g)	6.2 ± 0.4	6.3 ± 0.7	5.9 ± 0.6
Female birth weight (g)	5.9 ± 0.5	6.0 ± 0.6	5.8 ± 0.7
Male AGDi	2.13 ± 0.06	**1.90 ± 0.09***	**1.99 ± 0.08***
Female AGDi	1.15 ± 0.04	**1.02 ± 0.03***	**1.09 ± 0.05***
Male bw PD6 (g)	12.5 ± 1.8	12.3 ± 2.7	**11.1 ± 2.1**
Female bw PD6 (g)	12.2 ± 1.9	12.0 ± 2.3	**11.1 ± 2.3**
Male bw PD14 (g)	27.9 ± 4.1	28.1 ± 5.9	25.6 ± 4.4
Female bw PD14 (g)	27.3 ± 4.4	27.5 ± 5.5	25.8 ± 5.2
Male bw PD27 (g)	65.3 ± 1.7	65.1 ± 1.9	61.2 ± 1.6
Female bw PD27 (g)	67.2 ± 1.7	67.5 ± 2.5	62.0 ± 1.8

2   * $p < 0.05$. *** $p < 0.001$. Bw: body weight, GD: Gestation day, PD: postnatal day. Data shown as Mean
3   ±SD.

Absolute organ weights after perinatal exposure to a mixture of brominated flame retardants, Study 2

Mean±SD. ***p<0.001 compared to control with body weight as covariate in the analysis.

1 **Table 3.** Absolute organ weights after perinatal exposure to a mixture of brominated flame retardants,
2 Study 2

Offspring	Control	40 mg/kg DE-71	60 mg/kg DE-71
Male PD16	n = 19	n = 18	n = 17
Body weight, g	30.6 ± 4.2	29.95 ± 5.7	28.60 ± 5.1
Ventral prostate, mg	11.8 ± 4.9	9.8 ± 5.1	**6.8 ± 2.2*****
Female PD16	n = 21	n = 20	n = 19
Body weight, g	30.8 ± 5.0	31.0 ± 6.4	29.1 ± 5.6
Ovary, right, mg	2.4 ± 0.6	2.3 ± 0.5	2.2 ± 0.5
Ovary, left, mg	2.3 ± 0.6	2.3 ± 0.7	2.4 ± 0.6
Male PD300	n = 19	n = 18	n = 18
Body weight, g	516 ± 69.5	549 ± 60.6	529 ± 55.6
Prostate and seminal vesicle, g	3.02 ± 0.38	3.14 ± 0.37	3.10 ± 0.35
Ventral prostate, g	0.74 ± 0.12	0.69 ± 0.17	0.72 ± 0.14

3 Mean±SD. ***$p<0.001$ compared to control with body weight as covariate in the analysis.

Figure 5

Graphical abstract