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

Louise Ramhøj [1], **Karen Mandrup** [1], **Ulla Hass** [1], **Terje Svingen** [1], **Marta Axelstad** [Corresp. 1]

[1] Division of Diet, Disease Prevention and Toxicology, National Food Institute, Technical University of Denmark, Kgs. Lyngby DK-2800, Denmark

Corresponding Author: Marta Axelstad
Email address: maap@food.dtu.dk

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, 20, 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, 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. The various effects that were observed in vivo 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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[1]*Division of Diet, Disease Prevention and Toxicology, National Food Institute, Technical University of Denmark, Kgs. Lyngby DK-2800, Denmark*

* Corresponding author:

Tel: +45 3588 7544

e-mail: maap@food.dtu.dk

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ABSTRACT

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, 20, 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, 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. The various effects that were observed in vivo 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

Normal development through gestation and early postnatal life is essential for lifelong health and disruption to developmental processes can result in adverse effects and increased susceptibility to disease. Since the environment where development occurs plays a key role in ensuring proper development, environmental stressors are also major contributors to the development of diseases. This includes endocrine disrupting chemicals, which can disturb molecular and biological pathways that, through their effects on the endocrine systems, can change developmental processes and cause adverse health effects (Gore et al., 2015).

Polybrominated diphenyl ethers (PBDEs) are legacy compounds that were widely used as flame retardants in various consumer and industrial products. They have been shown to cause numerous adverse health effects in humans and animals and are now restricted under the Stockholm Convention on Persistent Organic Pollutants (Stockholm Convention, 2019). Unfortunately, humans are still exposed to PBDEs, as they are persistent in indoor and outdoor environments and can bioaccumulate.

Among the many human health effects for which PBDEs have been implicated, thyroid hormone system disruption, neurotoxicity and reproductive disorders are prevalent (Boas, Feldt-Rasmussen & Main, 2012; Attina et al., 2016; Trasande et al., 2016). Still, their specific endocrine mechanisms of action are not well understood and a characterization of the adverse reproductive effects in perinatally exposed rat offspring remain lacking. Even though PBDEs are largely banned, such information is still valuable since it will help us better understand the current disease burden of these legacy chemical substances as well as of endocrine disruptors in general. Perhaps equally important is its applicability towards improvement of current safety assessment regimens.

Also experimental studies have shown various potential endocrine effects of DE-71 and its metabolites. Amongst the effects potentially relevant for reproductive health are weak anti-estrogenic and estrogenic effects *in vitro* and *in vivo* (Mercado-Feliciano & Bigsby, 2008a,b) as well as clear anti-androgenic properties *in vitro* (Stoker et al., 2005) and in exposed male rats (Stoker et al., 2004, 2005). A recent meta-analysis on PBDEs suggest broad adverse reproductive effects in postnatal male rats (Zhang et al., 2020). Based on this, we surmised that the DE-71 mixture could reduce androgen signaling during fetal life and induce effects resembling the testicular dysgenesis syndrome (Skakkebaek et al., 2016).

To test our hypothesis, and by so doing contribute new knowledge to the endocrine mode of action of PBDEs *in vivo*, we conducted a developmental rat toxicity study to determine the effects of early life exposure to DE-71 on endocrine-sensitive endpoints: anogenital distance (AGD), nipple retention (NR), and a few selected reproductive organ weights postnatally and in adulthood. We also included analyses of mammary gland development in both male and female offspring by whole-mount assessments. Mammary glands are rarely investigated in reproductive toxicity studies despite their sensitivity to endocrine disruption and importance for reproductive health (Fenton, Reed & Newbold, 2012; Gouesse et al., 2019).

Materials and Methods

Chemicals

The test compound was a commercial mixture of PBDEs, DE-71 (penta-brominated diphenyl ethers (BDE), 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. 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. The expected day of delivery, GD23, was designated PD1 irrespective of the actual day of delivery.

In Study 1, dams were divided into 4 groups (n = 10 per group) and exposed to vehicle control or 20, 40 or 60 mg/kg body weight (bw)/day DE-71. Study 2 was performed in two balanced blocks with 3 groups (n = 22 per group, in order to have sufficient sample size for mammary gland examinations) exposed to vehicle, 40 or 60 mg/kg bw/day DE-71. Dosing of the dams was performed daily by gavage in the morning from GD7 to GD22, and again from postnatal day (PD)2 – PD14 in Study 1 (study termination) and PD2-16 in Study 2. Dosing was performed with a metal gastric tube with either vehicle (corn oil) or experimental solutions at a constant

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

Dams were housed pairwise until GD17 and individually thereafter. We used semitransparent plastic cages (15 × 27 × 43 cm) with aspen wood hides and with aspen bedding (Tapvei, Gentofte, Denmark) placed on racks (balanced for treatment group) in an animal room with controlled environmental conditions: reversed light/dark cycles of 12 hours (light from 9 pm-9 am, light intensity 500 lux), temperature 22±1°C, humidity 55±5%, and ventilation changing air 10 times per hour. All animals were fed ad libitum on a standard diet, Altromin 1314 (soy- and alfalfa-free; Altromin GMbH, Lage, Germany), and acidified tap water was provided ad libitum in PSU bottles (84-ACBT0702SU Techniplast).

All endpoint examination procedures were carried out with treatment groups represented in a random order (i.e. not examining all controls first, then treated animals). Examinations and outcome measures of live animals were conducted blinded to exposure group while data analysis was conducted unblinded.

Animals were observed twice daily for health and signs of overt toxicity. Criteria followed for euthanasia of animals: if it was considered irresponsible or unethical to let the animal live or if they were in a severe condition (expected to die within 24hrs). Accordingly, some litters and pups in Study 1 (see Table 1) were euthanized, as the dams did not take care of them and they were without milk in their stomachs. In Study 2, one dam was euthanized on GD23 as she appeared unwell and incapable of giving birth (necropsy did not reveal any explanations for the dystocia).

Animal experiments were carried out at the DTU National Food Institute (Mørkhøj, Denmark) facilities. Ethical approval was obtained from the Danish Animal Experiments Inspectorate, with authorization number 2012-15-2934-00089 C4. The experiments were overseen by the National Food Institute's in-house Animal Welfare Committee for animal care and use. All methods in the study were performed in accordance with relevant guidelines and regulations.

Birth and postnatal development

On the morning after overnight birth, dam and pup body weights were registered, and the pups were sexed and checked for macroscopic anomalies. Body weights of offspring were recorded on PD6, -10 and -14 in Study 1 and on PD6, -14 and -27 in Study 2.

Endocrine sensitive endpoints were investigated in Study 2. Anogenital distance (AGD), the distance between the anus and the genital papilla was measured in all live offspring on PD1, using a stereomicroscope with a micrometer eyepiece. The AGD index (AGDi) was calculated by dividing the AGD with the cube root of the body weight. On PD14 all offspring had their areolas/nipples counted. Nipple retention (NR) of male pups was defined as the number of areolas/nipples (a dark focal area with or without a nipple bud) visible where nipples are usually located in female pups. Both AGD and NR were assessed by the same technician, blinded to exposure group.

On PD27, one male pup from each litter was weaned and housed pairwise with another male from the same group, avoiding cohabitation with a sibling whenever possible.

158 *Necropsy*

159 Before necropsy all animals were weighed, anesthetized with CO_2/O_2 and killed by decapitation.
160 Selected reproductive organs were examined in offspring killed on PD16, PD27 and PD300 in
161 Study 2. On PD16 one male and one female from each litter were killed and ovaries and ventral
162 prostate were excised and weighed. Mammary glands were collected as whole-mounts from one
163 male and one female on PD27. On PD300, one male offspring from each litter was terminated
164 and prostate together with seminal vesicle was excised and weighed. The ventral prostate was
165 subsequently dissected and weighed alone.

166

167 All dams not giving birth were humanely killed on PD3, those with litters were humanely killed,
168 along with remaining pups, on PD14 in Study 1 and on PD27 in Study 2. Implantation scars in
169 uteri were counted to determine pregnancy rates and resorptions.

170

171 *Whole-mounts*

172 Mammary glands were excised and spread onto a glass slide, covered with parafilm and placed
173 under pressure for 2 hours. The whole-mounts were subsequently fixed in 4% formalin buffer,
174 stained with alum carmine, dehydrated in alcohol and cleared with xylene. The slides were
175 scanned in a flatbed scanner at 4800 dpi and images of the 4^{th} gland evaluated as described in
176 (Mandrup et al., 2015) for outer area (defined as the smallest polygon enclosing the gland),
177 transverse growth (as defined by (Mandrup et al., 2012)), longitudinal growth, distance to lymph
178 node, distance to 5^{th} gland (n = 13-17, except distance to the 5^{th} gland where n = 11, 4 and 10 in
179 control, PBDE-40 and PBDE-60, respectively) and number of terminal end buds (TEB) (TEB

was defined as tear-drop shaped buds with a diameter >100 μm in zone C, as defined by (Russo & Russo, 1996) (n = 17, 11 and 14 in control, PBDE-40 and PBDE-60, respectively). Distance to lymph node was measured and scored (scores 1-3, with a score of 1 representing mammary tissue not reaching the lymph node, score 2 representing mammary tissue reaching the lymph node and score 3 was given to mammary glands where the tissue reaches beyond the lymph node). All data were assessed for males and females separately and blinded to exposure. In addition, for area measurements, longitudinal and transverse growth data from both sexes were pooled for analysis with increased statistical power. Images were analyzed with Image Pro Plus 7.0 (Media Cybernetics, Bethesda, MD, USA) and calibration performed for each picture before measurements were made.

Statistical analysis

The alpha level for statistical significance was always set to 5% and all samples were included in the analysis. Data with normal distribution and homogeneity of variance were analyzed for treatment-related effect differences relative to the control by analysis of variance (ANOVA) followed by Dunnet's correction for multiple comparisons. Data were transformed if these conditions were not met and data not fulfilling the criteria were analyzed using a non-parametric statistical test (Kruskal-Wallis with Dunn's multiple comparison test). When relevant, body weight was included as a covariate in the analysis (ANCOVA), e.g. for terminal organ weights and whole-mount measurements. Litter effects were accounted for by only analyzing one pup per litter, using litter means or by including the litter as a random effect variable (e.g. AGD, NR and pooled whole mounts). Distance to lymph node was evaluated as the distance and as scores. The scores were analyzed using Kruskal-Wallis and using a two-sided Fishers exact test to compare

the number of animals with glands reaching past the lymph node with the number not reaching past. The number of nipple/areolas (NR) was assumed to follow a binomial-distribution with a response range between 0 and 12 (12 assumed to reflect the biologically possible maximal number of nipples in rats). Litter effects on NR and over-dispersion in the data were accounted for by using Generalized Estimating Equations (GEE) as reported in (Christiansen et al., 2012).

SAS Enterprise guide 4.3 (2010) (SAS Institute Inc, Cary, NC, USA) and GraphPad Prism 5 (Graphpad Software, San Diego, CA; USA) was used for statistical analysis.

Results

Effects on pregnancy, postnatal growth and general toxicity

We administered DE-71 at doses of 20, 40 and 60 mg/kg to the pregnant and lactating dams from GD7 to PD14/16. Pregnancy and litter data from Study 1 and Study 2 are listed in Table 1 and Table 2. In Study 1, there were signs of decreased maternal weight gain at 40 and 60 mg/kg. Pups from the 60 mg/kg bw/day exposure group weighed 8% less than control pups at birth and gained 20% less during the first postnatal week. However, none of these findings were statistically significant. Thus, 60 mg/kg was chosen as the highest dose in Study 2 as it did not induce excessive systemic toxicity in dams and pups.

In Study 2, dam weight gain during pregnancy was significantly decreased at 60 mg/kg (Table 2). The high dose pups had decreased body weight on PD6 (Table 2) and body weight remained non-significant reduced throughout the postnatal period (Table 2) with non-significant reductions

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

Effects on markers of early-life endocrine disruption

A shorter anogenital distance (AGD) and retained nipples in male offspring are considered sensitive markers of disrupted androgen action during development (Schwartz et al., 2019). DE-71 exposure induced a significantly shorter AGD and smaller AGDindex (AGDi, AGD adjusted for pup body size) at both 40 and 60 mg/kg. However, the dose-response curve was 'flat' in that reductions in AGD/AGDi were comparable between the two dose groups (10/11% reduced in the low dose group and 8/6% in the high dose group). The effect on AGD was also comparable between both sexes (Fig. 2A and Table 2). Nipple retention was increased in males from the high dose group ($p = 0.0249$). Notably, the mean number of 0.25 nipple in these high dose males is considered very small (Fig. 2B) and falls well within the range of our historic control data (Schwartz et al 2021, in submission - ref to come).

Early- and late-life effects on reproductive tissue weights

Weights of selected reproductive organs were assessed both early in life and in adulthood. DE-71 at a dose of 60 mg/kg reduced absolute and relative male PD16 prostate weights with 37% and 42%, respectively (Fig. 3 and Table 3). In female offspring, ovary weights were assessed on PD16 and showed no significant differences between controls and exposed animals.

On PD300, changes to prostate weights in exposed offspring were no longer apparent, nor were effects on seminal vesicle or ventral prostate weights (Table 3).

245

246 *Effects on mammary gland development*

247 We assessed mammary gland development by whole-mounts in both male and female offspring
248 at PD27 and found that mammary gland outgrowth was stunted by perinatal exposure to DE-71.
249 In male offspring, the transverse growth and mammary gland areas were statistically
250 significantly reduced in the highest exposure group (Fig. 4). Females appeared to display a
251 similar trend, albeit not statistically significant. When analyzing pooled data from both sexes
252 (litter effects were accounted for by including the litter as a random nested factor in the statistical
253 analysis) there was an effect on area in both dose groups and on the transverse growth in the high
254 dose group (Fig. 4). These effects cannot be accounted for by reduced body weight of the
255 offspring in the high dose as the effects were observed both on absolute numbers and when
256 accounting for the body weight of the animals in the statistical analysis.

257

258 ## Discussion

259 While production and use of DE-71 is no longer permitted, the constituents of the DE-71 mixture
260 still account for a significant proportion of the brominated flame retardants found in house dust
261 (Bramwell et al., 2016). Humans, including babies and toddlers, continue to be exposed to
262 PBDEs (Klinčić et al., 2020), especially from indoor environments, so a continued focus on the
263 potential detrimental health effects of this exposure is warranted. In this study, we have shown
264 strong endocrine disrupting effects following developmental exposure to DE-71. In contrast to
265 what was expected from existing studies, however, the endocrine disrupting effects seems to be

266 caused by a complex pattern of modalities and not simply anti-androgenic, as discussed in the
267 following.

268

269 Studies on DE-71, including a Hershberger assay, have clearly shown dose-dependent anti-
270 androgenic effects in male rats when exposure occurs during puberty and adulthood. Effects
271 include delayed preputial separation and reduced prostate weights in animals exposed to 30
272 mg/kg and above. Additional reproductive tissues such as seminal vesicle, glandula
273 bulbourethralis and penis showed dose-dependent weight reductions at higher doses (up to 240
274 mg/kg bw/day) (Stoker et al., 2004, 2005). Reductions in reproductive organ weights were seen
275 at 200 mg/kg, but not at 67 mg/kg or lower in a 28-day exposure study (van der Ven et al., 2008).
276 In contrast, a dietary study using a 70-day exposure to a mixture of PBDEs (of which 52% was
277 DE-71), but at maximum dose of 20 mg/kg, found no effects on male reproductive organs,
278 hormone concentrations or sperm count (Ernest et al., 2012). Thus, PBDEs can seemingly elicit
279 anti-androgenic effects in pubertal and adult male rats at doses of 30 mg/kg or higher. With
280 respect to fetal and early postnatal exposure, however, there are not much available data.

281 One study examines effects on anti-androgenic endpoints after developmental exposure (GD21-
282 PND22) to 1, 10 and 30 mg/kg DE-71 administered by gavage (Kodavanti et al., 2010). Here
283 effects in the male offspring included a statistically significant 1.8-day delay in preputial
284 separation, a 5% non-significant shortening of AGD, and a 20% non-significant decrease in
285 testosterone concentration on PND 60. Using a similar experimental setup to Kodavanti et al
286 (2010), but with higher exposure doses, we observed shorter male AGD of around 10% in both
287 dose groups (40 and 60 mg/kg). We also observed a small effect on male nipple retention in the
288 high dose group, with a mean of 0.25 retained nipples - an incidence rate that lies within our

historical control data (Schwartz et al 2021, in submission - ref to come) but is significantly different from the concurrent control. Reduced male AGD is usually indicative of an anti-androgenic mode of action. However, we also observed shorter female AGD to a very similar degree as in the male offspring, which we do not normally see for clear anti-androgenic compounds (Schwartz et al., 2019). Rather, this effect on female AGD suggests additional modalities induced by the DE-71 mixture.

We have previously shown that developmental exposure to BPA and butylparaben, which mainly are considered estrogenic compounds, albeit they also have other modes of action, induce some of the same effects as those observed with the DE-71 mixture: moderately shorter AGD without dose response, similar effects on AGD in both sexes, and no or minimal effect on nipple retention (Christiansen et al., 2014; Boberg et al., 2016). This could indicate that some of the same endocrine mechanisms are targeted by BPA, butylparaben and DE-71. However, our understanding of the underlying mechanisms causing this particular effect pattern in males and the reduced AGD in females remain elusive (Schwartz 2019). Thus, weak anti-androgenic effects and other endocrine mechanisms could be responsible for the PBDE induced moderate effects on AGD and NR. Notably, intrauterine exposure to PBDEs has been associated with increased risk of hypospadias in boys (Poon et al., 2018; Koren et al., 2019). Although hypospadias has been linked to anti-androgenic chemicals in both humans and rodents, so too have several compounds with known estrogenic potentials, which suggests the involvement of disrupted androgen-estrogen balance as a critical factor in phallus development (Mattiske & Pask, 2021). Taken together, this could suggest that the DE-71 mixture induce a more complex disruption to steroid hormone homeostasis which can result in a varied effect pattern.

311 The most marked effect observed in the present study was reduction in prostate weights on
312 PD16, which was around 40% decreased in exposed versus control animals. Again, this effect
313 could be caused by DE-71 having anti-androgenic properties in the postnatal pups as clearly seen
314 in pubertally exposed animals (Stoker 2004, 2005). However, prostate development is also
315 sensitive to estrogen signaling (Gupta, 2000; Prins & Korach, 2008; OECD, 2018) which could
316 also contribute to the observed effect. Interestingly, neither BPA nor butylparaben exposure
317 affected PD16 prostate weights in our studies (Boberg 2016, Christiansen 2014) which could
318 indicate that DE-71 works through a different mechanismor possess different ADME properties
319 than BPA and butylparaben in the postnatal period. Overall, it remains possible that the reduced
320 prostate weight induced by DE-71 could have been a result of disrupted androgen-estrogen
321 balance similarly to external genitalia.

322 In addition to the more commonly assessed effect endpoints AGD, NR and prostate weights, we
323 also assessed mammary gland development in both male and female offspring. DE-71 exposure
324 decreased mammary gland area and transverse growth in perinatally exposed offspring,
325 corroborating findings from a DE-71 toxicity study in Long-Evans rats. In the Long-Evans study
326 only female pups were examined, but perinatally exposed female pups showed less outgrowth,
327 fewer lateral branches and limited terminal end bud (TEB) formation on PND 21 (Kodavanti et
328 al., 2010).

329 The delayed mammary gland development in DE-71 exposed offspring is in contrasts to what
330 would be expected for estrogenic compounds, as accelerated or increased mammary gland
331 growth is typically seen after exposure to estrogenic compounds (Mandrup et al., 2012, 2015;
332 Macon & Fenton, 2013) including for BPA and butylparaben (Boberg et al., 2016; Mandrup et
333 al., 2016). Thus the effects of DE-71 on the mammary glands do not appear to support an

334 estrogenic mode of action. The reduced mammary gland outgrowth caused by DE-71 is also not
335 indicative of anti-androgenic mode of action, as such compounds may not induce an effect on
336 mammary growth in prepubertal stages, but only later in life (Škarda, 2003; Peters et al., 2011;
337 Jacobsen et al., 2012; Mandrup et al., 2015). In fact, the observed reductions in mammary gland
338 outgrowth in the present study may best be attributed to an anti-estrogenic mode of action. This
339 fits well with other studies where anti-estrogenic compounds such as ICI 182,780 can cause
340 decreased mammary gland growth and branching in prepubertal female offspring (Silberstein et
341 al., 1994; Cotroneo, 2002).

342 Although mammary gland development is sensitive to estrogen signaling, it is also regulated
343 through other signaling pathways. For instance, 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)
344 given on GD15 can stunt mammary gland growth from PD 4 and persist until PD 68 when the
345 glands still retained undifferentiated terminal structures (Fenton et al., 2002). This effect has
346 been suggested to be mediated through AhR activation (Hushka, Williams & Greenlee, 1998;
347 Fenton et al., 2002; Helle et al., 2016), a mechanism that may also be relevant for DE-71
348 (Hamers et al., 2006). Also 3,3',4,4',5-pentachlorobiphenyl (PCB-126), which can bind AhR and
349 have anti-estrogenic properties has affected mammary glands similarly to DE-71 (Fenton et al.,
350 2002; Muto et al., 2002). Thus various environmental chemicals, including DE-71, seem to cause
351 similar effect patterns on the mammary gland albeit the exact mechanism(s) are still unclear.

352

353 Although the effect pattern observed from developmental DE-71 exposure is perplexing, it is not
354 uncommon for environmental chemicals to have several endocrine targets and display different
355 toxicokinetic patterns over the course of development and maternal-fetal-pup compartments.
356 Furthermore, it must be considered that DE-71 is a complex mixture of PBDEs with a large

number of congeners and degradation products that may all have their own targets and specific ADME properties. Thus multiple targets/endocrine modes of action and ADME differences between dams, fetuses, neonates and older offspring as well as in different tissue compartments (Bondy et al., 2011) can all be at play at once. Obviously this complicates the process of determining what the mode of action is in different compartments at different developmental stages when exposing to complex mixtures such as DE-71.

In conclusion, we have shown that developmental exposure to DE-71 induces endocrine disrupting effects in rats. The effect pattern is complex and likely involve various mechanisms of action, which are difficult to pin down. It is however likely that the mixture acts by anti-androgenic, anti-estrogenic and maybe estrogenic mechanisms, possibly in combination with effects on other signaling pathways such as AhR signaling. DE-71 exposure causes endocrine disruption in both male and female rat offspring and thus raises concerns for the long-term consequences of human exposure to PBDEs, especially since associations between PBDE concentrations, AGD, and hypospadias in boys have been reported.

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Declaration of interests

We declare that we have no conflicts of interest.

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532 **Figure 1.** Study design for developmental toxicity studies with a technical mixture of brominated
533 flame retardants, DE-71. Study 1 was a range-finding study to choose doses for the larger Study
534 2 that investigated endocrine sensitive endpoints in the perinatally exposed rat offspring. AGD:
535 anogenital distance, Bw: body weight, GD: gestation day, NR: nipple retention, PBDE:
536 polybrominated diphenyl ethers (DE-71), PD: postnatal day.

537

538 **Figure 2.** Anogenital distance (AGD) and nipple retention (NR) in rat offspring after perinatal
539 exposure to brominated flame retardants (DE-71). (A) Reduced AGD in PD1 male and female
540 offspring. (B) NR in male PD14 offspring. n = 19-21 litters. Statistical analysis performed on all
541 pups from each litter with adjustment for litter effects. Litter means+SEM, *p<0.05 and
542 ***p<0.001 compared to control.

543

544 **Figure 3.** Relative ventral prostate weights in PD16 male offspring after perinatal exposure to
545 brominated flame retardants (DE-71). Mean+SEM, n = 16-19. ***p<0.001.

546

547 **Figure 4.** Mammary gland development in PD27 offspring after perinatal exposure to
548 brominated flame retardants (DE-71). (A) Area of the mammary gland was reduced. (B)
549 Transverse growth of the mammary gland was reduced at the highest dose. Mean+SEM. n = 14-
550 17 males, n = 18-20 females, n = 32-37 for males and females pooled and litter effects accounted
551 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 was a range-finding study to choose doses for the larger Study 2 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.

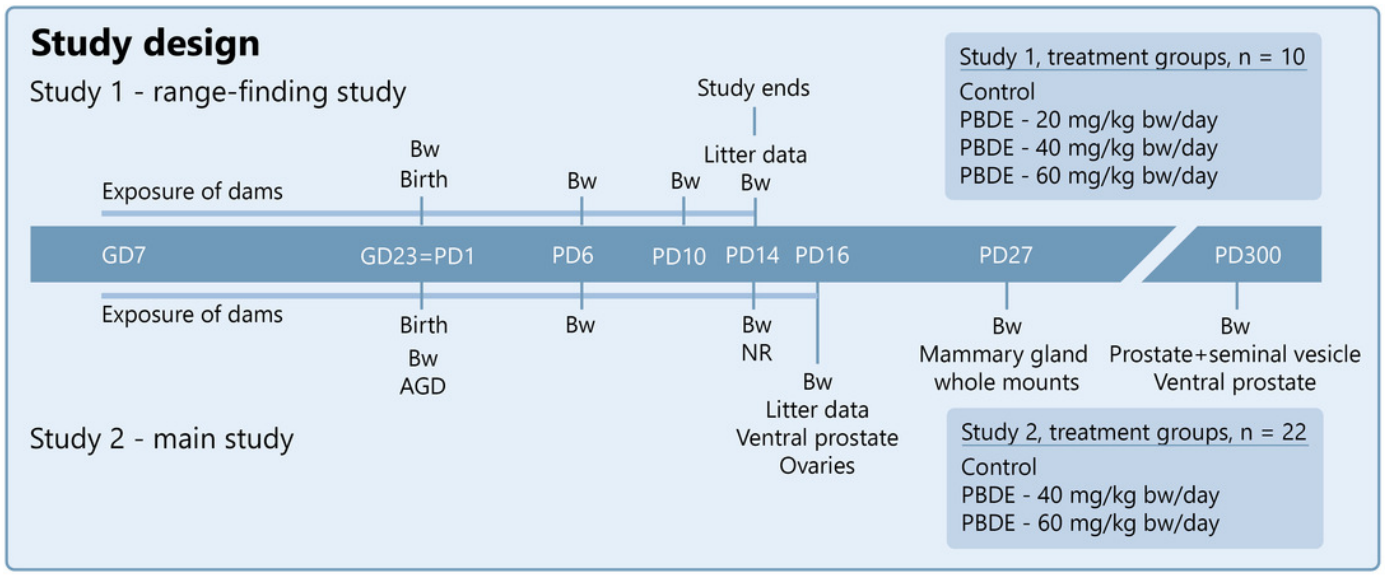

Figure 2

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.

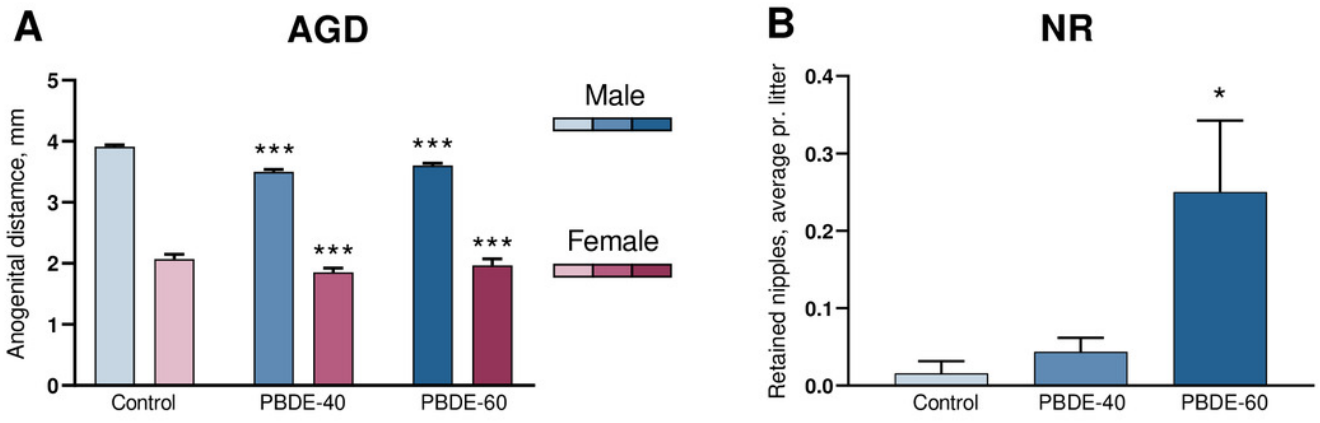

Figure 3

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.

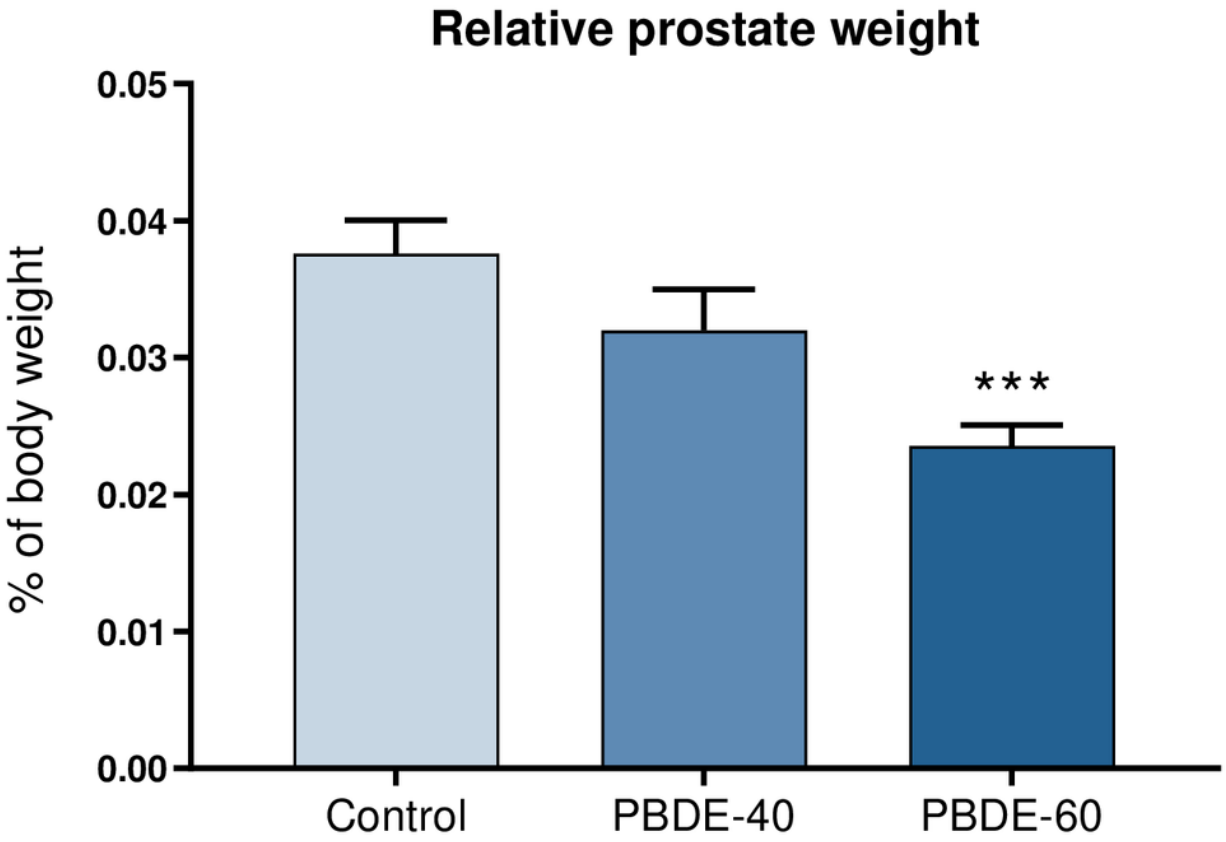

Figure 4

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.

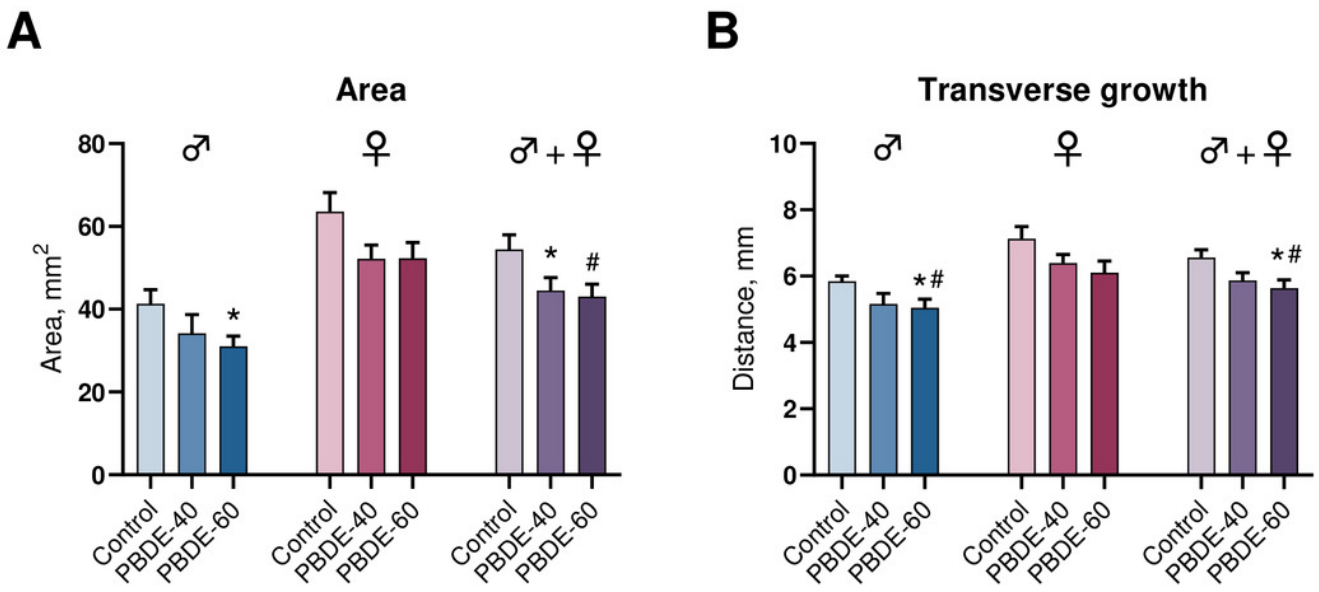

Table 1(on next page)

Pregnancy and litter data: Study 1

* p<0.05, versus control. bw: body weight, GD: Gestation day, PD: postnatal day. [a]Unrelated to exposure there were dams eating or not taking care of their pups in this study. Data represented as Mean±SD.

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

* $p<0.05$, versus control. bw: body weight, GD: Gestation day, PD: postnatal day.

[a]Unrelated to exposure there were dams eating or not taking care of their pups in this study.

Data represented as Mean±SD.

Table 2(on next page)

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.

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

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

Table 3(on next page)

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.

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

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