Low-dose effects of bisphenol A on early sexual development in male and female rats

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Abstract

Bisphenol A (BPA) is widely detected in human urine and blood. BPA has been reported to impair many endpoints for reproductive and neurological development; however, it is controversial whether BPA has effects in the microgram per kilogram dose range. The aim of the current study was to examine the influence of BPA on early sexual development in male and female rats at dose levels covering both regulatory no observed adverse effect levels (NOAELs) (5 and 50 mg/kg bw per day) as well as doses in the microgram per kilogram dose range (0.025 and 0.25 mg/kg bw per day). Time-mated Wistar rats (n = 22) were gavaged during pregnancy and lactation from gestation day 7 to pup day 22 with 0, 0.025, 0.25, 5 or 50 mg/kg bw per day BPA. From 0.250 mg/kg and above, male anogenital distance (AGD) was significantly decreased, whereas decreased female AGD was seen from 0.025 mg/kg bw per day and above. Moreover, the incidence of nipple retention in males appeared to increase dose relatedly and the increase was statistically significant at 50 mg/kg per day. No significant changes in reproductive organ weights in the 16-day-old males and females and no signs of maternal toxicity were seen. The decreased AGD at birth in both sexes indicates effects on prenatal sexual development and provides new evidence of low-dose adverse effects of BPA in rats in the microgram per kilogram dose range. The NOAEL in this study is clearly below 5 mg/kg for BPA, which is used as the basis for establishment of the current tolerable daily intake (TDI) by EFSA; thus a reconsideration of the current TDI of BPA appears warranted.


Introduction

Bisphenol A (BPA) is a high-production volume chemical used mainly as a monomer in the manufacturing of numerous chemical products, including polycarbonate plastics and epoxy resins. BPA is present in many products, and consumer exposure may arise from migration of BPA from polycarbonate or epoxy-lined food and drink containers, from dental sealants, from microwave containers into food during heating (Salian et al. 2011) or from thermal and recycled paper (Vinggaard et al. 2000, Liao & Kannan 2011).

The toxicity of BPA has been extensively investigated by industrial, governmental and academic research groups in short- as well as long-term animal studies, including several reproductive toxicity and multi-generational exposure studies. Health concerns regarding human exposures to BPA have in the past arisen from its well-known oestrogenic properties (Salian et al. 2011). BPA has affinity for nuclear oestrogen receptors (ERα and ERβ) and oestrogenic activity in vivo giving rise to increased uterine wet weights in the uterotrophic assay (Kanno et al. 2001). Furthermore, BPA exposure during development has been shown to affect semen quality (Sakaue et al. 2001, Salian et al. 2009), alter prostate weights (Vom et al. 1998, Timms et al. 2005, Prins & Korach 2008) and increase the incidence of prostate intraepithelial neoplasia (PIN) lesions in the prostate of adult rodents (Prins et al. 2011). Moreover, low-dose effects have been observed for a wide range of endpoints. In rodent studies, adverse effects of BPA doses at or below 50 µg/kg bw per day have been reported for reproductive organ weights (Ashby et al. 1999, 2011).
Chitra et al. 2003), mammary gland development (Munoz-de-Toro et al. 2005) and behaviour, e.g. anxious behaviour and impaired learning (Della Seta et al. 2005, Ryan & Vandenbergh 2006, Gioiosa et al. 2007, Gonçalves et al. 2010, Ayyanan et al. 2011). The majority of these low-dose effects have been observed after exposure during the developmental period when both the reproductive system and the brain are most susceptible to endocrine disturbances.

From multi-generation reproductive toxicity studies in rats, conducted according to standardised toxicity test guidelines (Tyl et al. 2002, 2008), a no observed adverse effect level (NOAEL) of 5 mg/kg bw per day has been established. In these studies, the critical effects were changes in body and organ weights in adult and offspring rats, and liver effects in adult mice. A tolerable daily intake (TDI) of 50 µg/kg bw per day has been obtained by dividing this NOAEL by an uncertainty factor of 100. The TDI was evaluated by EFSA in 2006 and has been reaffirmed in 2008 and 2010 (EFSA 2006, 2008, 2010). However, an increasing number of research studies conducted during the last decade have reported the effects of BPA in animals at doses at or below this NOAEL (Richter et al. 2007). So far these data have not been considered adequately relevant or reliable by regulatory bodies because of either small animal number per group and/or a limited number of dose levels, and thus these studies have not affected the establishment of the TDI or other health-based guidance values for BPA (US FDA 2008, EFSA 2010). However, in 2008 an assessment by NTP-CERHR Expert Panel (National Toxicology Program – Center for the Evaluation of Risks to Human Reproduction) concluded that rodent studies suggest that BPA causes neural and behavioural alterations related to disruptions in normal sex differences in rats and mice at doses below NOAEL (0.01–0.2 mg/kg per day) (Chapin et al. 2008). In 2010, the FDA concurred with the NTP’s assessment on these endpoints (US FDA 2010).

The aim of this study was to investigate early sexual development in male and female Wistar rats after perinatal exposure to BPA using both a sufficient number of litters per group and including several dose levels. The investigated endpoints included anogenital distance (AGD), nipple retention (NR) and prepubertal reproductive organ weights, endpoints which previously have proven sensitive for detecting the effects of endocrine-disrupting chemicals (Gray et al. 1999, Hass et al. 2007, Metzdorf et al. 2007, Christiansen et al. 2008). Also, this study aimed at examining low doses in the microgram per kilogram dose range (25 and 250 µg/kg bw per day) as well as dose levels similar to those that form the basis for establishment of the current TDI.

In adult humans, the daily mean exposure in previous assessments is estimated to be within the range of 0.4–1.5 µg BPA/kg bw per day (EFSA 2006, WHO 2011, ANSES 2011). In a novel EFSA draft report on exposure, dietary exposure for adults (including women of childbearing age) is estimated to be in the range of 0.335–0.388 µg/kg bw per day (95% percentile) (EFSA 2013). A total high exposure (95% percentile) from food and non-food sources is estimated to be in the range of 0.500–0.642 µg/kg bw per day in teenagers and adults (EFSA 2013).

Materials and methods

Chemicals

BPA (purity >99.5%, CAS no. 80-05-7) was purchased from Sigma–Aldrich. Corn oil used both as negative control and as vehicle was purchased from Sigma–Aldrich. The corn oil was provided to the laboratory in glass bottles. The dosing solutions were kept in glass bottles in the dark, at room temperature, and continuously stirred during the dosing period. The levels of BPA in the solutions were verified by chemical analysis.

Animals and treatment

A total of 110 time-mated nulliparous, young adult Wistar rats (HanTac:WH, SPF, Taconic Europe, Ejby, Denmark) were supplied at gestation day (GD) 3 of pregnancy. The day when a vaginal plug was detectable was designated as GD 1 and independently of actual day of delivery, the expected day of delivery, GD 23 was designated as pup day (PD) 1. Thereby, the age of the pups was related to the time of conception, but was rather similar to postnatal age.

The animals were housed in pairs until GD 17 and alone thereafter under standard conditions in semi-transparent polysulfone (PSU) type III cages (PSU 80-1291HOOSU Type III, Tecniplast, Buguggiate, Italy) (15×27×43 cm) with Aspen wood chip bedding (Tapvei, Gentofte, Denmark), Enviro Dri nesting material (Brogaard, Lyngby, Denmark) and Tapvei Arcade 17 (Aspen wood) shelters (Brogaard). They were placed in an animal room with controlled environmental conditions with a 12 h light:12 h darkness cycle with light intensity 500 lux starting at 2100 h, humidity 55%±5, temperature at 21±1 °C and ventilation changing air ten times per hour. All animals were fed on a standard diet with Altromin 1314 (soy- and alfalfa-free, Altromin GmbH, Lage, Germany). Acidified tap water (to prevent microbial growth) in PSU bottles (84-ACBTO702SU Tecniplast) were provided ad libitum. The PSU bottles and cages as well as the aspen wood shelters (instead of plastic) were used to reduce the risk of migration of BPA that potentially could confound the study results.

The study was performed using three blocks (separated by 1 week), and all dose groups were equally represented in the blocks, i.e. the 22 time-mated rats per dose group were allocated among blocks. Dams not giving birth were eliminated from the experiment. On the day after arrival (GD 4), the time-mated dams were pseudo-randomly distributed into the groups with similar body weight distributions. The dams were distributed into five dose groups (0, 0.025, 0.250, 5 or 50 mg/kg bw per day respectively), and gavaged by qualified animal technicians with a stainless steel probe 1.2×80 mm (Scanbur, Karlslund, Denmark) once daily from GD 7 to PD 22 (day of delivery excluded), at a constant volume of 2 ml/kg bw per day. The exposure period was chosen to cover the most

sensitive periods of the development of the reproductive system. The individual doses were based on the body weight of the animal on the day of dosing. The dams were inspected twice a day for general toxicity including changes in clinical appearance. Body weights were recorded on GD 4 and daily during the dosing period to monitor changes in weight gain, to follow pregnancy status and to adjust dose according to weight.

Animal experiments were carried out at the DTU National Food Institute (Mørkhøj, Denmark) facilities. Ethical approval was given by the Danish Animal Experiments Inspectorate. The authorization number given is 2012-15-2934-00089 C4. The experiments were overseen by the National Food Institutes in-house Animal Welfare Committee for animal care and use.

**AGD and NR**

The day after delivery the pups were counted, sexed, weighed and checked for anomalies. Pups found dead were macroscopically investigated for changes when possible. AGD (Fig. 1) was measured the day after delivery in all offspring using an ocular stereomicroscope. All offspring were weighed on PD 6. On PD 14, all male and female pups were weighed and examined for number of areolas/nipples (NR), described as a dark focal area (with or without a nipple bud) located where nipples are normally present in female offspring (Fig. 2). Female rats normally have 12–13 nipples whereas male rats usually have none. The same technician, who is skilled in these measurements and was blinded with respect to exposure groups, recorded both AGD measurements and NR counts.

**Section of male and female offspring PD 16/17**

On PD 16 and 17, one male and one female pup from each litter (n = 18–21) were weighed and decapitated, blinded with respect to exposure groups. From males, the testes, epididymides, ventral prostate, seminal vesicles, levator ani/bulbocavernosus muscles (LABC), bulbouretral glands, adrenals, thyroid, retroperitoneal fat pad, liver and stomach were excised and weighed. From females, the ovaries, thyroid, retroperitoneal fat pad, liver and stomach were excised and weighed.

**Chemical analysis**

The BPA concentrations in the dosing solutions (0, 0.0125, 0.125, 2.5 and 25 mg/ml) were analysed, blinded with respect to exposure groups with an accredited method using liquid chromatography negative electrospray ionisation tandem mass spectrometry LC–ESI–MS/MS (Acquity-Quattro Ultima, Waters, Milford, MA, USA) monitoring the transition reactions m/z 227>212 and m/z 227>133. The samples were extracted with (1:1) n-heptane (HPLC-grade) and methanol:water (3:1) and purified by C18 solid-phase extraction. BPA concentrations in the samples (duplicate) were quantified by external standard calibration (r² > 0.99) with the addition of internal standard of BPA-d16 (from Sigma–Aldrich) into samples, standards and blanks. The relative S.D. was below 11% and the recovery of BPA in the samples was 86%. The limit of detection was 15 μg/l. The analysis confirmed that the BPA concentrations in the dosing solutions were within the range (95% CI) of what was added.

**Statistical analysis**

For all analyses, the α level was set at 0.05 and the litter was the statistical unit. When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor. AGD and organ weights were analysed using body weight as a covariate. Birth weights were analysed using the number of offspring per litter as covariate. Both data on birth weight and data on the dams (pregnancy and lactation data) were analysed using ANOVA, followed by Dunnett’s post hoc test in SAS (SAS Enterprise Guide 4.3 Statistical Software (SAS Institute, Inc., Cary, NC, USA)).
Table 1 Pregnancy and litter data from dams and offspring exposed to 0, 0.025, 0.250, 5 and 50 mg BPA/kg bw per day from GD 7 to PD 22.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.025 mg/kg per day</th>
<th>0.250 mg/kg per day</th>
<th>5 mg/kg per day</th>
<th>50 mg/kg per day</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dams and litters</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Dam BW gain, GD 7–GD 21</td>
<td>82.4±26.4</td>
<td>91.2±14</td>
<td>82.9±22.5</td>
<td>81.1±20.8</td>
<td>78.8±17.1</td>
</tr>
<tr>
<td>Gestation length (days)</td>
<td>23.0±0.6</td>
<td>22.9±0.36</td>
<td>22.9±0.6</td>
<td>22.9±0.2</td>
<td>23.0±0.3</td>
</tr>
<tr>
<td>% postimplantation loss</td>
<td>7.9±11.5</td>
<td>8.0±10.2</td>
<td>11.3±22.5</td>
<td>5.9±4.9</td>
<td>13.0±21.8</td>
</tr>
<tr>
<td>% perinatal loss</td>
<td>8.5±8.0</td>
<td>10.8±12.4</td>
<td>9.2±7.9</td>
<td>6.8±5.4</td>
<td>17.9±26.5</td>
</tr>
<tr>
<td>Litter size</td>
<td>11.8±3.3</td>
<td>11.7±2.3</td>
<td>12.1±3.5</td>
<td>11.4±2.6</td>
<td>11.2±4.1</td>
</tr>
<tr>
<td>% perinatal deaths</td>
<td>3.2±5.4</td>
<td>3.3±7.2</td>
<td>2.5±4.9</td>
<td>1.0±2.8</td>
<td>8.6±23.4</td>
</tr>
<tr>
<td>% males</td>
<td>50.5±18.5</td>
<td>49.8±14.7</td>
<td>45.1±22.5</td>
<td>49.7±15.3</td>
<td>56.2±10.4</td>
</tr>
<tr>
<td><strong>Offspring</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Male birth weight</td>
<td>6.2±0.5</td>
<td>6.3±0.5</td>
<td>6.1±0.5</td>
<td>6.3±0.5</td>
<td>5.9±0.9</td>
</tr>
<tr>
<td>AGD males (mm)</td>
<td>4.1±0.07</td>
<td>3.9±0.1</td>
<td><strong>3.8±0.2</strong></td>
<td><strong>3.8±0.1</strong></td>
<td><strong>3.8±0.2</strong></td>
</tr>
<tr>
<td>AGD females (mm)</td>
<td>2.2±0.1</td>
<td><strong>2.1±0.1</strong></td>
<td><strong>2.0±0.1</strong></td>
<td><strong>2.0±0.1</strong></td>
<td><strong>2.0±0.1</strong></td>
</tr>
<tr>
<td>Nipples males</td>
<td>0.1±0.2</td>
<td>0.2±0.2</td>
<td>0.2±0.2</td>
<td>0.3±0.4</td>
<td><strong>0.4±0.6</strong></td>
</tr>
</tbody>
</table>

Data represent group means based on litter means ± S.D. n = 17–21 (22 time-mated in each group). Statistically significant findings (P<0.05) are written in bold.

The number of nipple/areolas (NR) was assumed to follow a binomial distribution with a response range between 0 and \( n_{\text{max}} \), with \( n_{\text{max}} \) being equal to the biologically possible maximal number of nipples in rats, either 12 or 13. The choice of \( n_{\text{max}} \) was decided by considering the global fit (information criterion of Schwarz). Litter effects on NR and over-dispersion in the data were accounted for by using generalised estimating equation as reported in Christiansen et al. (2012).

**Results**

**Pregnancy data, postnatal growth and general toxicity**

No general maternal toxicity was observed at any of the doses administered in the study. BPA exposure had no significant effect on maternal weight gain during pregnancy (GD 7 to GD 21), or on maternal weight on PND 1, and the mean gestational length was also unaffected (Table 1). Moreover, post-implantation loss, litter size, birth weights, sex ratio and perinatal loss were unaffected after in utero and lactational exposure to BPA and so were maternal weight gains, neonatal deaths and offspring body weights in the postnatal period (Table 1).

**Anogenital distance**

The prenatal exposure to BPA significantly decreased AGD in both male and female offspring (Figs 3 and 4). The decrease was statistically significant at all doses in the females (P<0.0001 for all groups except for the 0.025 mg/kg group for which \( P=0.002 \)) and from 0.25 mg/kg bw per day and above in the males (P<0.0001).

In order to evaluate whether the observed effects could be due to unusually high control values, the AGD in both male and female controls was compared with historic control data from two recent similar studies from our laboratory. There were no statistically significant differences between control levels of AGD in the present and in previous studies (Fig. 5).

**NR in males**

The number of nipples in female rats is usually 12 vs zero in males. Perinatal BPA exposure induced NR in male offspring in a dose-related manner and the increase in NR was statistically significant at the highest dose of 50 mg/kg (Fig. 6). There were no changes in number of nipples in females (data not shown).

The effect in the males was small, as the mean value at the highest dose of 50 mg/kg was below 0.6 nipples.

**Autopsy and organ weights at PD 16**

Weight of the testes, uterus, ovaries, epididymides, ventral prostate, seminal vesicles, LABC, bulbouretral glands, adrenals, thyroid, liver, and stomach was unaffected by BPA exposure (Table 2). However, the weight of the retroperitoneal fat pad was significantly increased in male offspring from the highest dose group of 50 mg/kg, when data were analysed with body weight as a covariate and also when the relative weight of the fat...
Discussion

In this study, clear effects of BPA exposure in the microgram per kilogram dose range were detected on the AGD of male and female offspring, an effect which is a well-known predictor for adverse effects on reproductive health in male offspring. The study was designed as a high-quality study, in order to be applicable by regulatory authorities. This means that the study included a large number of rats per treatment group and four dose levels. Moreover, an effort was made to avoid potential confounding of the results, such as water bottles and animal cages were made of PSU to reduce the risk that BPA or related compounds would leach from the plastic and the animal diet was soy- and alfalfa-free.

AGD was significantly decreased in both male and female offspring at most of the tested BPA doses; however, for both sexes the dose–response curves were very shallow. No clear increase in response was seen with increasing dose, i.e. the AGDs were almost equally lowered in males exposed to 250 μg/kg and 50 mg/kg and in females exposed to 25 μg/kg and 50 mg/kg. Reduced AGD in male rats is usually a sign of anti-androgenic exposure during sexual development. When exposed to potent anti-androgens, AGDs in the male rodents are usually reduced in a dose-related manner, and for some chemicals, such as certain drugs or pesticides, perinatal exposure can even lead to males having female-like AGDs (Ostby et al. 1999, Parks et al. 2000, Hass et al. 2007, Christiansen et al. 2010). However, other anti-androgenic chemicals show more shallow dose–response curves. One example of such a substance is the anti-androgenic drug and 5α-reductase inhibitor finasteride (Clark et al. 1990, Bowman et al. 2003, Christiansen et al. 2009). Interestingly, a new study that examines the effects of short-term exposure to BPA on mRNA levels of 5α-reductase isozymes in prostate of adult castrated rats show that BPA significantly decreased the mRNA levels of both 5α-reductase isozymes (Sánchez et al. 2013). This indicates that BPA is a 5α-reductase inhibitor and could be one explanation for the shallow dose–response similarly as for finasteride. Another example of such a shallow dose–response curve is the effect on AGD in male rats after in utero exposure to the oestrogenic antimicrobial preservative butylparaben (Boberg J, Axelstad M, Christiansen S, Isling LK, Geyic G, Mandrup K & Hass U 2013. Butylparaben alters anogenital distance, mammary development, and reproductive organ weights in perinatally exposed rats. Personal communication). In female rats, AGDs can in some cases also be affected by exposure to endocrine disruptors. In most studies of anti-androgenic exposure, female AGDs have not been affected (Hass et al. 2007, Christiansen et al. 2009); however, androgen exposure has been shown to increase female AGD (Ostby & Gray 2001) and exposure to oestrogenic agents like ethinyl estradiol (EE2) and genistein have been shown to increase or decrease female AGDs, depending on study design (Levy et al. 1995, Casanova et al. 1999, Delclos et al. 2009, Golub et al. 2010, Mandrup et al. 2013).

Due to the shallow dose–response curves observed in this study, it was evaluated whether unusually high control values could have caused the observed statistically significant differences. However, both male and female control AGD values in this study were similar to control values in recent studies from the same laboratory. The fact that the AGDs were evaluated blindly to exposure group,
the large numbers of litters included in the study and the low P values, consolidate that the observed AGD effects were unlikely to be random findings.

A number of studies have investigated the effects of perinatal BPA exposure on AGDs in rat offspring. Some of them corroborate the present findings, whereas others contradict them. In a developmental toxicity study, SD rats (n = 18–20) were administered by gavage to doses of 0.1 and 50 mg BPA/kg bw per day, from GD 6 to GD 21, and AGDs were assessed in male offspring only (Talsness et al. 2000). Significantly shorter AGDs were seen in rats exposed to 50 mg/kg bw per day, whereas no effects were seen at 100 µg/kg; however, this dose was lower than the lowest effective dose found in this study, so these results fit well with the present ones. In another developmental toxicity study, Long–Evans rats (n = 6–9 in exposed groups) were exposed orally to either EE2 or 2, 20, 200 µg BPA/kg bw per day from GD 7 to PND 18 (Howdeshell et al. 2008, Ryan et al. 2010). In females, no effect on AGD was seen (Howdeshell et al. 2008, Ryan et al. 2010), whereas AGDs appeared reduced in male offspring at the highest dose level (7% shorter than control values). However, no statistically significant change was detected (Howdeshell et al. 2008). In this study, male AGDs were very similarly decreased, i.e. by 6% at 250 µg/kg bw per day. The lack of a statistically significant effect in the Howdeshell et al. (2008) study could be due to the relatively small group size of 6–9 l, compared with the 17–22 l per group in this study. In another study, pregnant Sprague–Dawley rats (n = 10) were fed a diet containing BPA from GD 6 to PND 21, at doses of 0.017, 0.17 or 1.7 mg/kg bw per day, and AGD was examined in 5-week-old and 3-month-old males and females. No effect was seen on male AGDs, whereas AGDs were significantly shorter in 1-month-old females exposed to 0.17 and 1.7 mg/kg, but not at 3 months of age. As the effects were only seen in the younger females, the authors did not consider the results to be biologically significant (Kobayashi et al. 2012). However, these reductions in female AGDs corroborate the findings from this study, and it is possible that measurement of AGD in newborn male and female rats, instead of 1- and 3-month-old rats, would have yielded some even more similar results to the present ones. This study has only examined AGD at the time of birth and not at a later age. However, even if such a measurement would have shown that the decreases in AGD were transient we do not find

**Table 2** Body and organ weights for BPA-exposed male and female pups at PD 16/17. n = 15–20 (litter).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.025 mg BPA</th>
<th>0.250 mg BPA</th>
<th>5 mg BPA</th>
<th>50 mg BPA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of males*</td>
<td>18</td>
<td>20</td>
<td>14</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Body weights (g)</td>
<td>28.6±4.6</td>
<td>29.9±4.3</td>
<td>28.3±2.3</td>
<td>28.6±3.0</td>
<td>28.7±5.3</td>
</tr>
<tr>
<td>Right testis (mg)</td>
<td>51.3±7.6</td>
<td>51.4±7.8</td>
<td>51.0±4.6</td>
<td>50.5±7.0</td>
<td>51.0±9.8</td>
</tr>
<tr>
<td>Left testis (mg)</td>
<td>50.9±7.4</td>
<td>51.3±7.6</td>
<td>50.3±4.3</td>
<td>49.9±6.6</td>
<td>50.7±9.2</td>
</tr>
<tr>
<td>Ventral prostate (mg)</td>
<td>11.7±3.6</td>
<td>11.2±2.8</td>
<td>11.1±2.4</td>
<td>11.5±2.6</td>
<td>10.8±2.9</td>
</tr>
<tr>
<td>Epididymides (mg)</td>
<td>22.8±3.8</td>
<td>21.7±3.1</td>
<td>21.1±2.7</td>
<td>21.0±5.7</td>
<td>21.9±3.4</td>
</tr>
<tr>
<td>Seminal vesicles (mg)</td>
<td>7.1±2.0</td>
<td>7.5±2.3</td>
<td>7.1±1.8</td>
<td>7.5±2.8</td>
<td>7.1±1.8</td>
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<tr>
<td>LABCb (mg)</td>
<td>26.0±4.4</td>
<td>25.7±5.6</td>
<td>23.9±3.8</td>
<td>27.2±6.5</td>
<td>24.3±5.0</td>
</tr>
<tr>
<td>Bulbo urethral glands (mg)</td>
<td>1.7±0.4</td>
<td>1.6±0.3</td>
<td>1.5±0.4</td>
<td>1.6±0.4</td>
<td>1.6±0.4</td>
</tr>
<tr>
<td>Liver (mg)</td>
<td>753.7±114.7</td>
<td>778.2±136.4</td>
<td>722.5±67.4</td>
<td>739.2±75.3</td>
<td>746.6±150.1</td>
</tr>
<tr>
<td>Adrenals (mg)</td>
<td>7.5±2.1</td>
<td>7.3±1.8</td>
<td>7.2±1.5</td>
<td>7.9±1.4</td>
<td>7.6±1.6</td>
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<tr>
<td>Retroperitoneal fat (mg)</td>
<td>38.0±11.2</td>
<td>42.5±13.2</td>
<td>38.7±11.6</td>
<td>36.6±12.3</td>
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</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of females*</td>
<td>15</td>
<td>20</td>
<td>16</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>Body weights (g)</td>
<td>29.1±2.6</td>
<td>31.2±4.0</td>
<td>29.4±4.7</td>
<td>30.9±3.0</td>
<td>29.6±5.3</td>
</tr>
<tr>
<td>Right ovary (mg)</td>
<td>2.9±0.77</td>
<td>2.8±0.73</td>
<td>2.9±0.81</td>
<td>2.8±0.66</td>
<td>2.9±0.74</td>
</tr>
<tr>
<td>Left ovary (mg)</td>
<td>3.2±1.04</td>
<td>3.1±0.7</td>
<td>3.2±0.77</td>
<td>3.1±0.59</td>
<td>3.3±1.22</td>
</tr>
<tr>
<td>Liver (mg)</td>
<td>798.8±66.7</td>
<td>847.8±141.0</td>
<td>780.4±127.1</td>
<td>822.8±100.6</td>
<td>846.2±178.6</td>
</tr>
<tr>
<td>Thyroid (mg)</td>
<td>4.7±0.9</td>
<td>5.2±1.2</td>
<td>4.7±0.9</td>
<td>5.0±1.3</td>
<td>4.8±0.9</td>
</tr>
<tr>
<td>Retroperitoneal fat (mg)</td>
<td>29.5±12.3</td>
<td>31.1±9.7</td>
<td>31.0±12.5</td>
<td>32.3±10.3</td>
<td>30.3±10.0</td>
</tr>
</tbody>
</table>

Data represent means±s.e.m. Statistically significant findings (*P*<0.05) are written in bold.

*One male/female pup per litter. bLevator ani/bulbocavernous muscles abbreviated LABC. cThe retroperitoneal fat pad was significantly increased in the males from 50 mg BPA when data were analysed with body weight as a covariate and also when the relative weight of this fat pad was compared with the controls.
that this would weaken the findings seen at birth. An altered AGD in male or female offspring at the time of birth indicates that the endocrine signalling, which is necessary for normal reproductive development, has been altered (Bowman et al. 2003, Hotchkiss et al. 2004). Such altered programming can have adverse consequences on reproductive functions later in life (Hotchkiss et al. 2004, Christiansen et al. 2008, Welsh et al. 2008), even if AGD is no longer affected.

In a small toxicity study treating Sprague–Dawley rats \((n=4)\) with BPA in drinking water during gestation and lactation (estimated dose from 0.1 to 1.2 mg/kg bw per day), no difference in AGD during the neonatal period was seen in neither male nor female pups (Rubin et al. 2001); however, the low sensitivity of this study means that no clear conclusion should be drawn from it. In an effort to set a reliable NOAEL, Tyl et al. (2002) performed a multi-generational study in Sprague–Dawley rats \((n=26–30)\), using dietary BPA exposure at doses of 0.001, 0.02, 0.3, 5, 50 and 500 mg/kg bw per day. AGD was assessed in the F2 and F3 generations, and in male offspring no significant effects were seen. In females, increased neonatal AGD was seen at some doses in the F2, but not in the F3, and therefore the findings were considered to be not biologically relevant by the authors. As this study had high statistical power, the discrepancies in AGD result between the study by Tyl et al. (2002) and the present one are difficult to explain, but they could be caused by differences in rat strain (Sprague–Dawley vs Wistar), exposure (dietary vs gavage) and/or the generation of studied offspring (F2 vs F1).

Effects of developmental BPA exposure on AGD have also been evaluated in mice. Honma et al. (2002) subcutaneously dosed pregnant mice \((n=10)\) with 2 or 20 µg/kg bw BPA and found increased AGD (measured on PND 21) in female offspring exposed to 2 µg/kg, but not to 20 µg/kg. AGDs were no longer significantly increased at day 60. In males, increased AGD was seen in both dose groups; however, this was only significant at PND 60. These altered AGDs were reported at lower doses than those observed in this study; however, the different species and dosing scheme make it difficult to compare the results directly. BPA exposure was also evaluated in a murine two-generation study. Tyl et al. (2008) found that in male offspring, absolute AGDs were significantly reduced at the highest tested dose of 600 mg/kg per day on PND 21, and at both 600 mg/kg per day and the second highest dose of 50 mg/kg, when adjusted for terminal body weight. However, no significant reductions in male AGD were seen at the lower doses of 3, 30, 300 or 5000 µg/kg. According to the authors, these effects were not considered to be treatment related because of the absence of effects on PND 0 for F1/F2 males and on PND 21 for F2 females. However, the findings of reduced male AGDs after BPA exposure may corroborate the results from this study, even though the studies are difficult to compare directly due to differences in tested species and exposure scheme.

Overall, it can be concluded that AGD effects in rodents after BPA exposure are conflicting, probably due to varying study designs, tested species and strains, routes of exposure, dose levels and statistical power for detecting effects. However, the overall weight of evidence precludes dismissing the findings of decreased AGD at low doses in this study.

In this study, BPA also caused retention of areolae or nipples in male offspring in a dose-related manner; however, the effect was small and only statistically significant in the highest dose group (50 mg/kg). Previous studies have examined areolas/nipples in rats and found no effects (Tyl et al. 2002, Howdeshell et al. 2008). As with the effects on AGD, differences in study design may explain why an effect was seen in this study but not in other studies. It is, however, important to note, that even though the observed effects on NR was quite small, the increased number of areolas/nipples seen in the BPA-exposed male offspring supports that the perinatal BPA seems to adversely affect sexual differentiation.

During the last decade, it has become evident that assessment of AGD and NR in rodent offspring can be used as markers of impaired androgen action within the critical programming windows of sexual differentiation (Welsh et al. 2008, 2010). These endpoints have been shown to be highly predictive of increased risk of adverse reproductive toxicity effects later in life, including increased incidence of hypospadias and cryptorchidism, decreased penile length and seminal vesicle weight (Bowman et al. 2003, Christiansen et al. 2008, Welsh et al. 2008), and assessment of both AGD and NR has been recognised for regulatory purposes. Both measurements are mandatory in the newly accepted OECD Test guideline (Extended one-generation reproductive toxicity study (OECD 2012)), and statistically significant effects are considered adverse and useful for setting a NOAEL (OECD 2013).

In humans, recent studies have reported shorter AGD in boys with hypospadias or cryptorchidism as compared with boys with normal genitalia (Hsieh et al. 2008), and decreased AGD in adult men has been correlated to changes in semen parameters and decreased testosterone level (Eisenberg et al. 2011, 2012a, 2012b, Mendola et al. 2011). The reproductive effects of prenatal BPA exposure in humans was evaluated by Miao et al. (2011), who compared AGD in sons of workers who had or had not been occupationally exposed to BPA during pregnancy. The results showed that parental occupational exposure to BPA during pregnancy was associated with shortened AGD in male offspring. This data indicates that BPA can potentially disturb the development of the foetal reproductive systems in humans (Miao et al. 2011). An inverse association between shorter male AGD in human infants and prenatal phthalate exposure (particularly di-(2-ethylhexyl) phthalate (DEHP) and di-n-butyl...
phthalate (DBP)) has also been reported (Swan et al. 2005, Swan 2008). Similarly, girls androgenised due to congenital adrenal hyperplasia have longer AGD compared with healthy girls (Bongiovanni & Kellenbenz 1962). This suggests that AGD can also be a predictor of persistent disturbances in human sexual differentiation.

Another marker for disruption of sexual development in rodents is reproductive organ weights. When measured in prepuberty, male reproductive organ weights have in numerous studies been shown to be reduced by anti-androgenic compounds (Metzdorff et al. 2007, Christiansen et al. 2009, Jacobsen et al. 2012). In this study, no significant changes in reproductive organ weights were seen in offspring at PD 16/17. This is in contrast to some studies showing increased prostate weights were seen in offspring at PD 16/17. This is in this study, no significant changes in reproductive organ weights. When measured in prepuberty, male reproductive organ weights have in numerous studies been shown to be reduced by anti-androgenic compounds (Metzdorff et al. 2007, Christiansen et al. 2009, Jacobsen et al. 2012). In this study, no significant changes in reproductive organ weights were seen in offspring at PD 16/17. This is in contrast to some studies showing increased prostate weights were seen in offspring at PD 16/17.

An increased weight of the retroperitoneal fat pad was seen in males at the highest dose level. Finding of enlarged fat deposits is in agreement with many studies showing BPA-induced effects on obesity-related parameters and adipocyte differentiation, thereby indicating a link between BPA exposure and obesity development (vom Saal et al. 2012).

The mode of action of BPA leading to the effects in this study is not clarified and may involve several differential modes of actions. In the ToxCast programme, launched by US-EPA to develop ways to predict potential toxicity of chemicals and to develop a cost-effective approach for prioritising the thousands of chemicals that need toxicity testing, BPA was shown to have an effect in 101 out of 467 in vitro toxicity assays (Reif et al. 2010). Thus, BPA's toxicological effects are not limited to the classical oestrogenic mechanism (Shelby 2008) and comprise several other signalling pathways. In addition to being an ER agonist, BPA also show activity on the androgen receptor (AR), other nuclear receptors and xenobiotic metabolising enzymes (including aromatase) (Reif et al. 2010). In the literature, BPA has shown oestrogenic properties, the ability to antagonise AR activation with an IC_{50} in the micromolar range (Paris et al. 2002, Bonefeld-Jorgensen et al. 2007) and the ability to inhibit aromatase (Bonefeld-Jorgensen et al. 2007). In addition, BPA has been found to inhibit testosterone production and E_2 metabolism in H295R cells (Zhang et al. 2011).

Consequently, interpretation of BPA effects only in terms of an oestrogen-mimicking effect would be too simplistic. The involvement of several of these mechanisms could explain the effects observed in this study at low doses of BPA. It is conceivable that the BPA-induced effects on AGD and nipples in rat offspring are caused by an interference of BPA with the effect, synthesis and/or metabolism of oestriadiol and testosterone. According to the available in vitro data, BPA has the potential to affect all these pathways. The reduced AGD in males is a sign of demasculinisation and may be caused by inhibited foetal testosterone levels and/or an AR antagonistic effect. In females, the reduced AGD is a sign of super-feminisation and can be hypothesised to be linked to BPA-induced ER agonism and/or metabolism. However, further studies would be needed to elucidate this.

In adult humans, the daily mean exposure in previous assessments is estimated to be within the range of 0.4–1.5 μg BPA/kg bw per day (EFSA 2006, WHO 2011, ANSES 2011). In a novel EFSA draft report on exposure, dietary exposure for adults (including women of childbearing age) is estimated to be in the range of 0.335–0.388 μg kg bw per day (95% percentile) (EFSA 2013). A total high exposure (95% percentile) from food and non-food sources is estimated to be in the range of 0.500–0.642 μg kg bw per day in teenagers and adults (EFSA 2013). These estimated exposure levels to BPA are only somewhat lower than the dose that caused adverse effects, which leaves little margin of exposure.

The presented data are the first results from this large study on BPA, which will be extended to include assessment of sexually dimorphic behaviours, mammary gland development, semen quality and reproductive organ weight and histopathology in adolescent and adult offspring.

**Conclusions**

Perinatal BPA exposure caused adverse effects on both male and female rat sexual development at low doses. At doses from 250 μg/kg and above, male AGD at birth was significantly decreased, whereas decreased female AGD was seen from 25 μg/kg bw per day and above. Moreover, the incidence of NR in males appeared to increase dose relatedly and the increase was statistically significant at 50 mg/kg per day. The decreased AGD in both sexes indicates endocrine effects on sexual development and provides new evidence of adverse effects of BPA in rats in the microgram per kilogram dose range.

The NOAEL in this study is clearly below the NOAEL of 5 mg/kg for BPA that is used as the basis for establishment of the current TDI by EFSA, thus a reconsideration of the current TDI of BPA appears warranted.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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