Low-dose effect of developmental bisphenol A exposure on sperm count and behaviour in rats

Hass, Ulla; Christiansen, Sofie; Boberg, Julie; Rasmussen, M. G.; Egebjerg, Karen Mandrup; Petersen, Marta Axelstad

Published in:
International Journal of Andrology

Link to article, DOI:
10.1111/andr.12176

Publication date:
2016

Document Version
Publisher's PDF, also known as Version of record

ANDROLOGY

ISSN: 2047-2919

ANDROLOGY

ORIGINAL ARTICLE

Correspondence:
Ulla Hass, National Food Institute, Division of Diet, Disease Prevention and Toxicology, Technical University of Denmark, Mørkhøj Bygade 19, DK-2860 Søborg, Denmark.
E-mail: ulha@food.dtu.dk

Keywords:
behaviour, bisphenol A, endocrine disruption, low dose, rat, sperm count

Received: 14-Oct-2015
Revised: 25-Jan-2016
Accepted: 28-Jan-2016

doi: 10.1111/andr.12176

Low-dose effect of developmental bisphenol A exposure on sperm count and behaviour in rats

U. Hass, S. Christiansen, J. Boberg, M. G. Rasmussen, K. Mandrup and M. Axelstad

National Food Institute, Division of Diet, Disease Prevention and Toxicology, Technical University of Denmark, Søborg, Denmark

SUMMARY

Bisphenol A is widely used in food contact materials and other products and is detected in human urine and blood. Bisphenol A may affect reproductive and neurological development; however, opinion of the European Food Safety Authority (EFSA) on bisphenol A (EFSA J, 13, 2015 and 3978) concluded that none of the available studies were robust enough to provide a point of departure for setting a tolerable daily intake for bisphenol A. In the present study, pregnant Wistar rats (n = 17–21) were gavaged from gestation day 7 to pup day 22 with bisphenol A doses of 0, 25 μg, 250 μg, 5 mg or 50 mg/kg bw/day. In the offspring, growth, sexual maturation, weights and histopathology of reproductive organs, oestrus cyclicity and sperm counts were assessed. Neurobehavioural development was investigated using a behavioural testing battery including tests for motor activity, sweet preference, anxiety and spatial learning. Decreased sperm count was found at the lowest bisphenol A dose, that is 25 μg/kg/day, but not at the higher doses. Reproductive organ weight and histology were not affected and no behavioural effects were seen in male offspring. In the female offspring, exposure to 25 μg/kg bw/day bisphenol A dose resulted in increased body weight late in life and altered spatial learning in a Morris water maze, indicating masculinization of the brain. Decreased intake of sweetened water was seen in females from the highest bisphenol A dose group, also a possible sign of masculinization. The other investigated endpoints were not significantly affected. In conclusion, the present study using a robust experimental study design, has shown that developmental exposure to 25 μg/kg bw/day bisphenol A can cause adverse effects on fertility (decreased sperm count), neurodevelopment (masculinization of spatial learning in females) and lead to increased female body weight late in life. These results suggest that the new EFSA temporary tolerable daily intake of 4 μg/kg bw/day is not sufficiently protective with regard to endocrine disrupting effects of bisphenol A in humans.

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 several reproductive toxicity and multi-generational exposure studies. Health concerns regarding human exposures to BPA have in the past arisen from its well-known estrogenic properties (Salian *et al.*, 2011). BPA has affinity for nuclear oestrogen receptors (ERα and ERβ) and estrogenic activity in vivo giving rise to increased uterine wet weights in the uterotrophic assay (Sik Kim *et al.*, 2005). Furthermore, BPA exposure during development has been shown to affect semen quality (vom Saal *et al.*, 1998; Salian *et al.*, 2009), alter prostate weights (vom Saal *et al.*, 1998; Timms *et al.*, 2005; Prins & Korach, 2008) and increase the incidence of 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 250 μg/kg bw/day have been reported for reproductive organ weights (Ashby *et al.*, 1999; Chitra *et al.*, 2003), mammary gland development (Munoz-de-Toro *et al.*, 2005) and behaviour, for example anxious behaviour and impaired learning (Della Seta *et al.*, 2005; Ryan & Vandenbergh,

2006; Gioiosa *et al.*, 2007; Gonçalves *et al.*, 2010; Xu *et al.*, 2010, 2011a; Ayyanan *et al.*, 2011; Jasarevic *et al.*, 2013). 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. The European Food Safety Authority (EFSA) have concluded in their opinion on bisphenol A (BPA) in January 2015 that none of the available studies showing effects at low doses were robust enough to provide a point of departure for setting a Tolerable Daily Intake (TDI) for BPA.

The aim of the present study was to investigate reproductive development in male and female Wistar rats after perinatal exposure to BPA using a sufficient number of litters per group and low doses in the μg/kg dose range (25 and 250 μg/kg bw/day) as well as dose levels similar to those that formed the basis for establishment of the earlier TDI from EFSA.

From this study we have earlier reported decreased anogenital distance (AGD) in the newborn male and female offspring from dose levels of 250 and 25 μg/kg bw/day, respectively, indicating endocrine disrupting effects on reproductive development at low doses of BPA (Christiansen *et al.*, 2014). Also, effects of BPA on mammary gland development at 25 and 250 μg/kg are reported in Mandrup *et al.* (2016). The present study reports the results with regard to effects of BPA on sexual maturation, weights and histopathology of reproductive organs, oestrus cyclicity and sperm counts as well as sexually dimorphic behaviour.

MATERIALS AND METHODS

Chemicals

Bisphenol A (purity >99.5%, CAS no. 80-05-7) was purchased from Sigma-Aldrich (Brøndby, Denmark). 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

The animal study was carried out at the DTU National Food Institute facilities. Ethical approval was given by the Danish Animal Experiments Inspectorate (Council for Animal Experimentation). The authorization number given was 2012-15-2934-00089 C4. The experiments were overseen by the National Food Institutes in-house Animal Welfare Committee for Animal Care and Use.

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 gestation day (GD) 1 and the expected day of delivery, GD 23, was designated as pup day (PD) 1. The study was performed in three blocks (separated by 1 week), and all dose groups were equally represented in these blocks. The dams were given 4 days after arrival to adapt to the reversed light–dark cycle before beginning the exposure. The animals were housed in pairs until GD 17 and alone thereafter under standard conditions in semi-transparent polysulfone cages, as described in detail in Christiansen *et al.* (2014). The polysulfone bottles and cages, as well as the aspen wood shelters (instead of polycarbonate), were used to reduce the risk of migration of BPA that potentially could confound the study results.

The day after arrival (GD 4), time-mated dams were distributed into five groups of 22 rats with similar body weight (bw) distributions. The dams received vehicle (controls) or the following doses of BPA: 25 μg, 250 μg, 5 mg or 50 mg//kg bw/day. Test compounds and vehicle were administered by oral gavage with a stainless steel probe 1.2 × 80 mm (Scanbur, Karlslunde, Denmark) once daily in the morning from GD 7 to the day before expected birth (GD 21) and again after birth from PD 1 to 22 at a constant volume of 2 mL/kg bw/day. Dams that did not give birth were omitted from the study. The exposure period was chosen to cover the most sensitive periods of the development of the reproductive system in the offspring, that is beginning after the time of implantation and continuing throughout the lactation period. Throughout the study only dams were gavaged, meaning that offspring exposure was indirect, first through placental transfer and hereafter through the milk. The individual doses were based on the body weight of the dams 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. The number of live-born litters in the five dose groups, respectively, was 17, 19, 15, 16 and 16. Details regarding pre- and early postnatal development are presented in Christiansen *et al.* (2014).

On PD 22, two male and two female pups per litter were weaned. One subset of animals (one male and one female from each litter) was terminated around 3 months of age (PD 105–116) and sperm count and reproductive organ weight were assessed. The other subset was used for behavioural studies, assessment of oestrus cyclicity and was terminated around 9 months for the males and 14 months for the females.

Section of dams and offspring PD 22

The male and female pups which were not to be kept after weaning were decapitated on PD 22 in CO_2/O_2 anaesthesia. Testes and ventral prostate from one male pup per litter and ovaries from one female per litter were dissected and weighed. Testes were fixed in Bouin's fixative and ventral prostate were fixed in formalin and embedded in paraffin. Dams were decapitated in CO_2/O_2 anaesthesia and the number of uterine implantations was counted.

Onset of puberty

Onset of puberty was registered in all weaned male and female offspring. In female offspring sexual maturity was assessed by determining day of vaginal opening (VO) and the females were examined daily from PD 25 until all were positive. In male offspring the onset of puberty was assessed as time of preputial separation (PPS). Males were examined daily from PD 38 until all were positive. On the day of VO or PPS the age and weight of the animals were recorded.

Oestrus cyclicity

Vaginal smears were collected every morning for 21 consecutive days, starting when the female offspring were around 12 months old. The smear samples were air-dried, fixed in ethanol, stained, and examined by light microscopy by an

investigator blinded to the exposure groups. Stages were recognized in terms of the presence, absence, or proportional numbers of epithelial cells and cornified cells and were classified as oestrus or not oestrous. The animals were categorized as either being regularly cycling (cycles lasting 4–5 days), or as being irregularly cycling which was defined as having cycles lasting less than 4 days or more than 5 days. Furthermore, episodes of 3–4 consecutive days of vaginal oestrus and 5 days or more between vaginal oestrous were considered extended.

Section of 3–4 months old offspring (males: PD 97–107, females: PD 110–125)

The adult pups were weighed and decapitated in CO_2/O_2 anaesthesia. Vaginal smears were performed on females in the morning, and females in proestous or oestrous were selected for autopsy over a 2-week period. Ovaries, retroperitoneal fat pads, livers and thyroid glands were dissected from females and weighed.

Male reproductive organs were examined macroscopically for anomalies. Testis, epididymis, ventral prostate, seminal vesicle, levator ani/bulbocavernosus muscle (LABC), bulbourethral gland, retroperitoneal fat pads and liver were dissected from one male pup per litter and weighed. Ventral prostates were fixed in formalin and embedded in paraffin.

Section of 8 months old male offspring

The adult males were weighed and decapitated in CO_2/O_2 anaesthesia. Testis, ventral prostate, seminal vesicle (weighed with ventral and dorsolateral prostate), epididymis, levator ani/bulbocavernosus muscle (LABC), bulbourethral glands and liver were excised and weighed. Testes were fixed in Bouin's fixative, and ventral prostates were fixed in formalin and embedded in paraffin.

Section of 14 months old female offspring

Vaginal smears were performed on females in the morning, and females in proestous or oestrous were selected for autopsy over a 1-week period. A few females were not cycling and were sacrificed at the end of the study period. Females were weighed and decapitated in CO_2/O_2 anaesthesia. Ovaries, pituitary gland, liver and retroperitoneal fat pad were excised and weighed, and ovaries were fixed in formalin and embedded in paraffin.

Histopathology and morphometry

At PD 22, one section per testis and ventral prostate from the control group, the lowest dose group and the highest dose group were stained with haematoxylin and eosin (H&E). Immunohistochemical staining for the proliferation marker Ki67 was performed on one ventral prostate section for all males from the control group, the lowest dose group and the highest dose group as described by Boberg *et al.* (2015). Quantification of Ki67-positive epithelial cells, calculation of proliferation index and morphometrical analyses of lumen:epithelium ratios and distribution of compartments of the ventral prostate were performed as described in Boberg *et al.* (2015). In testes, lumen formation and seminiferous tubular diameter was examined in four randomly selected fields per section at 10× magnification.

At 3 and 8 months of age, histological examination was performed on one H&E stained section per ventral prostate and one H&E stained section per testis from the control group and the low-dose group. In testes from 3 months old males, seminiferous tubular diameter was examined in four randomly selected fields per section at 10× magnification.

Ventral prostates were scored according to Isling *et al.* (2014) for presence of epithelial atrophy, epithelial cell height, interstitial inflammation, epithelial atypical hyperplasia, presence of cribriform patterns in areas of hyperplasia and luminal concretion. Testes were evaluated for spermatid retention, tubular dilation, degeneration of germ cells and Leydig cell hyperplasia. Ovaries from 4 months old offspring were examined for presence of corpora lutea to evaluate reproductive senescence.

Sperm count

For sperm count analysis, samples were analysed using computer-assisted sperm analysis (CASA). From 3 months old male offspring, alternately left or right cauda epididymis including 1 cm of ductus deferens was frozen in liquid nitrogen and stored at −80 °C for sperm count analysis. The cauda epididymis was thawed, weighed and prepared as described by Jarfelt *et al.* (2005), and samples were analysed using a 10× UV fluorescent objective and IDENT OPTIONS on the CASA. Ten fields were analysed for each sample and three counts were performed for each suspension. Counts were averaged and data are presented as number of spermatozoa per gram cauda.

Behavioural testing

The behavioural testing was performed during the animals' dark cycle, that is their active period, from 9 AM to 4 PM, in dimly lit rooms. There were one male and one female from each litter and 18–22 litters per group. The experimenter was unaware as to which group an individual rat belonged and exposed and control animals were tested alternately.

Elevated plus maze. The animals were tested at the age of 3–4 months (PND 90–112) in an elevated plus maze. This maze was made of grey PVC and consisted of two open arms, 50 × 10 cm, and two opposite enclosed arms, 50 × 10 × 40 cm, with an open roof. Metal ledges (1 cm) were made along the edges of the open arms to minimize the risk of falling down during testing. The enclosed arms were closed at the end opposite to the centre. The centre of the maze was represented by an area 10 × 10 cm, thus the maze formed the shape of a plus sign. It was elevated to a height of 50 cm above the floor. An infra red camera was mounted vertically above the central square recorded the rat behaviour in dark. All the test animals were initially placed in the centre of the plus maze facing the same direction towards one of the open arms. The experimenter left the room just after placing the rat. The maze was cleaned with ethanol following each trial. The behaviour was scored by an observer blind to the treatment using a manual software timer with split time function. Scored parameters included time spent in open/close/centre and number of entries to each of the arms as well as latencies to each arm in the maze. The entries of an arm were registered when the animal entered an area with both front paws.

Learning and memory (Morris water maze). The animals were tested at the age of 4–6 months in a maze with a diameter of 220 cm, filled with water at room temperature, as described earlier (Hass *et al.* 1995, 1999; Axelstad *et al.*, 2008). A circular

transparent platform was situated on a solid support and submerged below the water surface. When the rat swam to and climbed onto the platform, the trial was completed. If the animal failed to locate the platform within 60 sec, it was led to the platform. A video-tracking device (ViewPoint VideoTrack System, Sandown Scientific, Middlesex, UK) was used to collect data about latencies to find the platform, the path lengths and swimming speeds of the animals.

Learning was tested with the platform situated at the centre of the south west quadrant of the pool, and the animals were tested in four trials per day for 8 days (five consecutive days, 2 days break and then three more days).

Motor activity and habituation. The animals were tested 2 days after the last day of Morris water testing at the age of 4–6 months. The motor activity of the animals was recorded in activity boxes with photocells for 10 × 3 min (as described in Axelstad *et al.*, 2008). The total activity during the 30 min was used as a measure of general activity. In order to assess habituation capability, the activity within the 30 min was also divided into two time periods of 15 min and analysed separately.

The sweet preference test. At the age of 6–7 months, the offspring were tested in the sweet preference test. One week prior to the test, the rats were housed individually, their weight was recorded and each rat was given two water bottles with acidified tap water to acclimatize to more than one bottle to choose from. During the saccharin preference test, each rat was provided with one bottle containing deionized tap water, while the other bottle contained a 0.1% saccharin solution dissolved in acidified tap water. The saccharin bottles were placed alternately right and left in relation to water bottles, to avoid position preference. The consumption of water and saccharin solution was recorded daily at 10:00 AM for 4 days and bottles were refilled when needed. At the fifth and last test day bottles were weighed and the rats were rehoused in pairs and only given one water bottle.

The saccharin preference score was calculated as the relative saccharin intake [intake of saccharin water (g) per 100 g bw] and as the absolute saccharin intake (with bw was used as a covariate in the statistical analysis). All rats maintained ad libitum soy-free Altromin diet throughout the behavioural test.

Statistical analysis

For all analyses, the level of significance was set at 0.05. Data with normal distribution and homogeneity of variance were analysed using analysis of variance (ANOVA). For analysis of organ weights, ANCOVA, using body weight as a covariate, was used. For body weights of the adult offspring, repeated measures analysis were used. When more than one pup from each litter was examined, statistical analyses investigating the effect of litter were performed. When appropriate, the model was adjusted using litter as an independent, random and nested factor in ANOVA, whereas the data were analysed without the nested design, when this resulted in the best fitted model. Where an overall significant treatment effect was observed, two-tailed comparison was performed using least square means or Dunnett's post hoc test. Testing for non-monotonic dose–response relationships was performed using a Tukey's test for LSMeans Dichotomous histological data were evaluated using the 2 × 2 Fisher's exact test. All statistical analyses were performed using (SAS Enterprise Guide 4.3 Statistical Software (SAS Institute, Inc., Cary, NC, USA)).

RESULTS

Reproductive development and body weights

Body weights of male pups were not altered by the BPA exposure at any age (Table 1). Female pup body weights were similar among groups until the age of approximately 6 months, but were significantly increased compared to controls in the 25 μg/kg dose group at 9–13 months of age, and at the final sacrifice (Fig. 1, repeated measures analysis, $p = 0.025$).

On PD 22, weights of the testes, ovaries and ventral prostate were unaffected by BPA exposure (Table 1). BPA exposure did not affect lumen formation or seminiferous tubule diameters in testes, proliferation index of prostate epithelium or the morphometric parameters analysed in ventral prostates at PD 22 (data not shown).

In 3 months old males, significantly decreased sperm count was seen at the lowest tested BPA dose of 25 μg/kg bw/day, ($p = 0.0373$), but not at the higher doses (Fig. 2). The effect was only statistically significant when an outlier in the 50 mg/kg bw/day dose group was excluded from the analysis. This one male showed a sperm count that was approximately 10 times lower than seen in the rest of the males, and the seminal vesicles of this male were small and underdeveloped. This effect was judged not to be a treatment-related effect, since such malformations have previously also been seen in control males in our laboratory (Axelstad *et al.*, 2011; Isling *et al.*, 2014). Thus, because of the observed malformations, the sperm count data from this one male were excluded from the analysis, as they were not considered representative.

Sperm count in the males from the 25 μg/kg bw/day group were also significantly lower than sperm count in males in the 50 mg/kg bw group, indicating a non-monotonic dose–response relationship.

Ventral prostate weight appeared increased at 25 μg/kg bw/day, but the increase was not statistically significant compared to controls ($p = 0.08$). No changes in weight of testes, epididymides, bulbourethral glands, LABC, liver, retroperitoneal fat pad or body weight were seen in the 90-day-old offspring (Table 1). Overall, histological examination of testes and measurement of tubule diameter showed no significant differences between controls and males from the 25 μg/kg bw/day group (data not shown). In one testis from the low-dose group, an area of dysgenetic tubules with impaired spermatogenesis was seen inside a Leydig cell cluster, and another animal from the low-dose group had spermatid retention and multinucleated spermatid aggregates (as described in Creasy, 2001) in a few tubules scattered throughout the testis. As these findings were only seen in two of 22 examined testes from the low-dose group, this was not considered to be clearly related to BPA exposure.

In 4 months old female offspring, no changes in weights of ovaries (right and left combined), liver, thyroids or retroperitoneal fat pad were observed.

Organ weights and histological examination in 8 months old male offspring and 14 months old female offspring

At 8 months of age, the prostate and seminal vesicle were dissected and weighed together and showed an increased weight at

Table 1 Organ weights. Effect of BPA on body weights (BW) and weight of reproductive organs

	Control	25 μg/kg	250 μg/kg	5 mg/kg	50 mg/kg
Male, PND 22					
N	17	19	14	14	16
Body weight (g)	44.1 ± 4.2	45.1 ± 4.1	41.1 ± 4.7	42.7 ± 3.5	43.8 ± 6.8
Right testis (mg)	116 ± 11	119 ± 13	108 ± 13	110 ± 9	115 ± 19
Left testis (mg)	118 ± 13	121 ± 12	111 ± 12	112 ± 8	115 ± 20
Prostate (mg)	29.8 ± 4.4	32.7 ± 4.8	29.9 ± 5.8	31.3 ± 2.8	30.1 ± 5.3
Female, PND 22					
N	14	17	15	16	14
Body weight (g)	40.9 ± 5.0	43.3 ± 4.3	41.4 ± 4.8	43.6 ± 4.6	41.3 ± 6.2
Right ovary (mg)	7.71 ± 1.39	8.22 ± 1.36	7.67 ± 1.45	8.05 ± 1.97	7.71 ± 1.84
Left ovary (mg)	7.81 ± 1.72	8.34 ± 1.14	8.09 ± 1.48	8.65 ± 1.22	7.87 ± 1.61
Male, 3 months					
N	20	22	17	19	18
Body weight (g)	352 ± 40	365 ± 30	334 ± 30	341 ± 32	340 ± 34
Right testis (g)	1.76 ± 0.10	1.79 ± 0.15	1.73 ± 0.11	1.76 ± 0.13	1.75 ± 0.13
Left testis (g)	1.80 ± 0.11	1.81 ± 0.15	1.76 ± 0.11	1.80 ± 0.15	1.82 ± 0.14
Prostate (g)	0.45 ± 0.08	0.51 ± 0.07	0.48 ± 0.07	0.44 ± 0.07	0.48 ± 0.09
Epididymis (g)	0.56 ± 0.06	0.56 ± 0.05	0.54 ± 0.02	0.55 ± 0.02	0.54 ± 0.05
Seminal vesicle (g)	1.32 ± 0.18	1.40 ± 0.22	1.40 ± 0.13	1.40 ± 0.20	1.26 ± 0.18
LABC (g)	0.96 ± 0.14	0.99 ± 0.11	0.93 ± 0.09	0.96 ± 0.10	0.94 ± 0.12
Bulbourethral gland (g)	0.103 ± 0.021	0.107 ± 0.029	0.103 ± 0.019	0.105 ± 0.031	0.102 ± 0.029
Liver (g)	10.72 ± 1.53	11.67 ± 1.59	10.19 ± 1.04	10.31 ± 1.07	10.47 ± 1.32
Retroperitoneal fat pad (g)	1.37 ± 0.54	1.74 ± 0.50	1.48 ± 0.50	1.39 ± 0.41	1.42 ± 0.45
Female, 4 months					
N	21	20	17–18	17–19	15
Body weight (g)	213 ± 14	218 ± 12	208 ± 15	214 ± 17	218 ± 36
Ovaries (mg)	83.6 ± 16.4	87.4 ± 15.8	83.9 ± 11.8	83.1 ± 12.3	89.4 ± 16.1
Retroperitoneal fat (mg)	717 ± 172	747 ± 240	670 ± 193	641 ± 175	727 ± 196
Liver (g)	6.35 ± 0.98	6.49 ± 0.56	6.18 ± 0.65	6.45 ± 0.77	6.45 ± 0.79
Thyreoidea (mg)	15.6 ± 6.2	14.7 ± 2.0	13.6 ± 2.2	15.7 ± 5.9	15.0 ± 3.4
Male, 8 months					
N	20	22	18	20	18
Body weight (g)	461 ± 49	476 ± 44	464 ± 46	462 ± 37	463 ± 39
Right testis (g)	1.98 ± 0.16	1.88 ± 0.14	1.89 ± 0.16	1.89 ± 0.15	1.92 ± 0.21
Left test (g)	1.98 ± 0.15	1.91 ± 0.14	1.95 ± 0.16	1.95 ± 0.17	1.93 ± 0.16
Prostate/seminal vesicle (g)	2.96 ± 0.44	3.05 ± 0.46	3.08 ± 0.30	**3.38 ± 0.50***	3.02 ± 0.37
Ventral prostate (g)	0.71 ± 0.14	0.75 ± 0.16	0.76 ± 0.15	0.76 ± 0.14	0.70 ± 0.13
Liver (g)	12.4 ± 1.7	13.0 ± 1.7	12.4 ± 1.7	12.4 ± 1.4	12.6 ± 1.5
Epididymis (g)	1.32 ± 0.17	1.30 ± 0.10	1.31 ± 0.10	1.39 ± 0.17	1.29 ± 0.19
Bulbourethral gland (g)	0.198 ± 0.062	0.205 ± 0.059	0.220 ± 0.058	0.212 ± 0.070	0.176 ± 0.071
LABC (g)	1.30 ± 0.14	1.30 ± 0.18	1.27 ± 0.12	1.36 ± 0.15	1.31 ± 0.16
Female, 14 months					
N	20	21	18	19	18
Body weight (g)	280 ± 25	304 ± 35	291 ± 27	282 ± 31	277 ± 28
Ovaries (mg)	72.8 ± 21.3	85.9 ± 23.9	83.7 ± 24.0	79.1 ± 28.8	75.5 ± 21.5
Pituitary (mg)	17.7 ± 4.7	18.3 ± 7.1	16.8 ± 2.19	17.8 ± 4.0	15.3 ± 2.7
Liver (g)	7.47 ± 0.78	7.73 ± 1.34	7.57 ± 1.16	7.46 ± 1.29	7.09 ± 0.92
Retroperitoneal fat pad (g)	2.00 ± 0.82	2.31 ± 0.74	2.08 ± 0.63	1.97 ± 0.77	2.22 ± 1.01

*$p < 0.05$ compared to control. Data represent group means ± SD. BW was used as a covariate in the statistical analyses. All significant results are written in bold.

5 mg BPA/kg compared to controls ($p = 0.013$, Table 1). No changes were seen in weights of testis, ventral prostate, epididymides, bulbourethral glands, LABC, liver or body weight at 8 months of age (Table 1). In the 14 months old female offspring, no changes were observed in the weights of ovaries (right and left combined), liver, pituitary gland or retroperitoneal fat pad.

Histological examination of ventral prostates revealed a higher prevalence of interstitial inflammation at 3 months than at 8 months of age, and an increased incidence of epithelial atrophy with increasing age. No differences between controls and low-dose males were seen for these or other endpoints examined in ventral prostates. In testes, one male from the low-dose group showed degeneration of seminiferous tubules, but no differences between dose groups were observed. In ovaries, 2–3 females per dose group had no corpora lutea (CL), whereas 1–3 females per dose group had 1–3 CL in the evaluated ovary section, indicating no or impaired oestrous cyclicity. There were no statistically differences between dose groups for these observations or in the mean number of CL (data not shown).

Onset of puberty

No effects of BPA were found with regard to the time for sexual maturation of male offspring or the body weight at puberty onset (data not shown). The puberty onset in the BPA-exposed female offspring appeared to be 0.5–1.2 days earlier in all exposed groups, but no significant differences were found (data not shown).

Oestrus cyclicity

The results of the scoring of the vaginal smears taken for evaluation of oestrus cyclicity at 1 year of age showed as expected

Figure 1 Body weights in female rats exposed to 0, 25 μg, 250 μg, 5 mg or 50 mg BPA/kg bw/day from GD 7 to PD 22. Group mean values ± SEM are shown, $n = 17–22$. *$p < 0.05$ in the 25 μg/kg group compared to the control group.

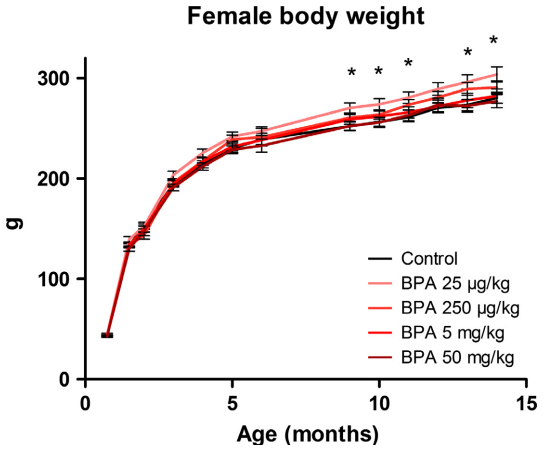
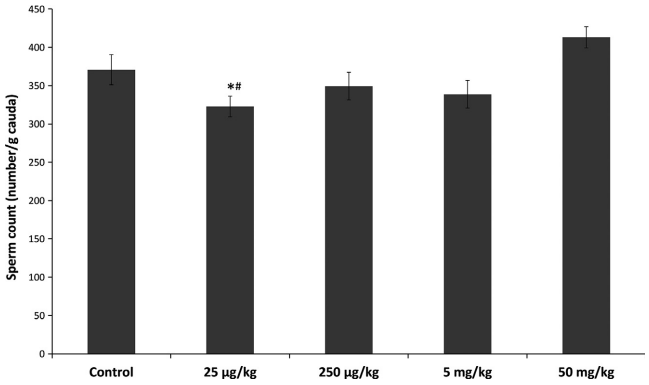


Figure 2 Number of spermatozoa per gram cauda epididymis in 3 months old male rats exposed to 0, 25 μg, 250 μg, 5 mg or 50 mg BPA/kg bw/day from GD 7 to PD 22. Group mean values ± SEM are shown, $n = 17–22$. Sperm count is significantly lower, indicated by * with $p < 0.05$, in the 25 μg/kg group compared to the control group, when an outlier in the highest dose group (50 mg/kg) is excluded from the statistical analysis (see text for details). # represents a statistical significant difference from the 50 mg/kg group ($p < 0.05$).



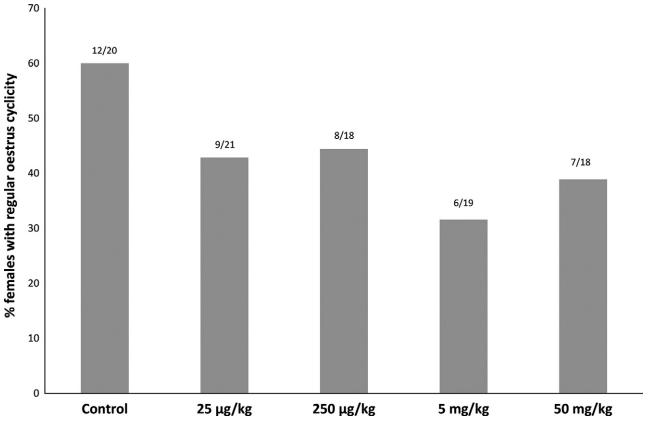
based on our previous study (Isling *et al.*, 2014) that only around 60% of the control animals were still regularly cycling (Fig. 3). Somewhat lower frequencies ranging from 32 to 44% were seen in the BPA-dosed animals. Statistical analysis showed, however, that none of the dosed groups were significantly different from the control group (*p*s = 0.11 or higher).

Behavioural effects

Anxiety in elevated plus maze

Results from the elevated plus maze test showed that in both male and female offspring this behaviour was not significantly affected by BPA exposure in any of the tested dose groups (data not shown). Large variations in the data were seen. Moreover, the expected sex difference in control rats with female rats being significantly more in the open arms than control males was not found.

Figure 3 The percentage of females with regular oestrus cyclicity at approximately 1 year of age, female offspring exposed perinatally to 25 μg, 250 μg, 5 mg or 50 mg BPA/kg bw/day. Lower frequencies ranging from 32 to 44% were seen in the BPA-dosed animals compared to 60% in the control, but no statistically significant differences from controls were observed (*p*s = 0.11 or higher).



Spatial learning in the Morris water maze

Over the 8-day testing period, the expected sex difference in control rats was seen ($p < 0.01$) with male rats swimming a shorter distance before reaching the platform ($p = 0.001$, Fig. 4A). The control males also found the platform faster (i.e. had shorter latencies) ($p = 0.004$) (Fig. 4C) compared to control females, although they swam significantly slower than the control females ($p = 0.003$) (Fig. 4B).

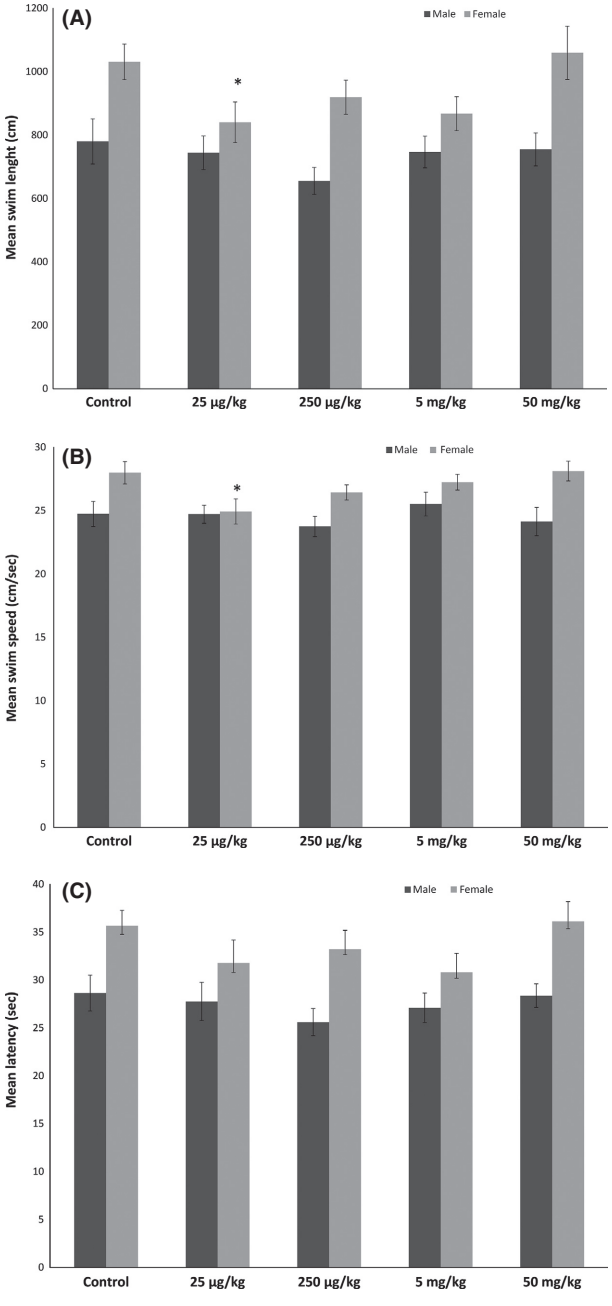
In the female offspring, exposure to the low dose of BPA (25 μg/kg) caused significantly shorter swim lengths ($p = 0.024$) (Fig. 4A) and significantly slower swim speeds ($p = 0.0045$) (Fig. 4B) than in control females, making the behaviour of these low-dose BPA-exposed females resemble the male maze performance.

With regard to swim length, the female offspring from the low dose of BPA group (25 μg/kg) were not significantly different from the two next dose groups (i.e. 250 μg/kg and 5 mg/kg) (Fig. 4A), but their swim lengths were significantly shorter than in the 50 mg/kg group ($p = 0.011$), indicating a non-monotonic dose–response relationship. Swim speed in the females from the 25 μg/kg group were not only significantly different from control females but also from females in the 5 and 50 mg/kg bw groups ($p = 0.028$ and 0.0036 respectively), again indicating a non-monotonic dose–response relationship.

BPA exposure did not significantly affect female latency to reach the platform, and did not significantly affect maze performance in the male offspring.

As described earlier, significant sex differences were seen in the control group for all three measured endpoints, that is swim length, swim speed and latency. This was not the case for the animals from the 25 μg/kg group, where there was no significant sex difference in any of these three measurements ($p = 0.18$, $p = 0.80$ & $p = 0.068$ respectively), corroborating that the performance of the females in this dose group resembled that of males. A significant difference between the sexes, similar to the one seen in the control animals, was seen in the groups exposed to 250 μg/kg ($p = 0.0018$, $p = 0.022$, $p = 0.004$) and 50 mg/kg ($p = 0.0003$ $p = 0.0008$ &

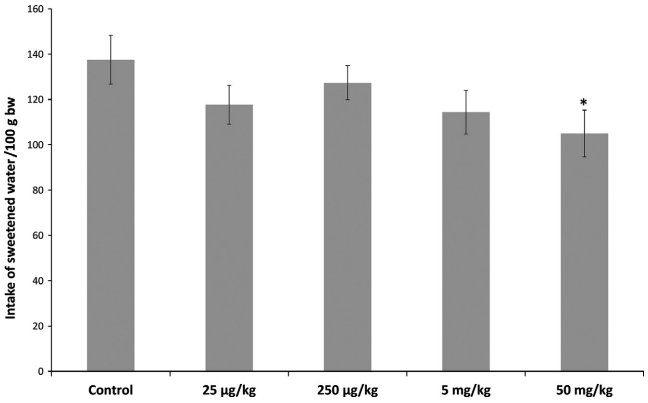
Figure 4 (A–C) Effects of BPA on (A) mean swim length (B) mean swim speed and (C) mean latency to reach the platform in the Morris water maze, in 4–6 months old male and female offspring exposed perinatally to BPA. Mean values ± SEM are shown, n = 18–22. Significant effects in low-dose females compared to controls are indicated by * with $p < 0.05$. Significant difference from 50 mg/kg females is indicated by # ($p < 0.05$). ¤ represents a statistically significant difference from both the 5 and 50 mg/kg groups. No statistically significant effect was seen in male offspring. For all three endpoints, a significant difference between male and female performance was seen in the control group, and in the groups exposed to 250 μg/kg and 50 mg/kg. This sex difference was not present in the animals from the 25 μg/kg and the 5 mg/kg groups. See text for more details on these sex differences, including the respective p values.



$p = 0.0002$), but was missing for all three endpoints in the group exposed to 5 mg/kg bw/day ($p = 12.9$, $p = 11.8$ & $p = 13.7$).

Motor activity levels in male and female offspring were not affected in any dose groups (data not shown).

Figure 5 Effect of BPA on sweetened water intake per 100 gram of body weight in 6–7 months old female offspring exposed perinatally to BPA. Group mean values ± SEM are shown, n = 18–22. Intake of saccharin solution is significantly lower, indicated by * with $p < 0.05$ in the highest exposed group compared to the control group. No effect of BPA exposure was seen in the male offspring. In the control males the intake of sweetened water was between 55 and 75 g per100 g bw, with SEM values between 4 and 7 (data not shown).



Sweet preference test

The expected gender difference in saccharin consumption between male and female offspring with females consuming more sweet water than males was seen, when analysing the relative intake of sweet water (mL intake of saccharin solution per 100 g of body weight) ($p < 0.01$). In adult female offspring, a decreased intake of saccharin water was seen with increasing BPA exposure, with a statistically significant downwards trend ($p < 0.025$) and females from the highest dose group showed significantly decreased intake of sweetened water compared to controls ($p < 0.05$) (Fig. 5). BPA exposure had no effect on sweet preference in male offspring (data not shown),

DISCUSSION

In the present study, the developmental BPA exposure caused reproductive toxicity effects manifested as decreased sperm count. Also, female body weight was increased during the last part of the study, that is at 9–14 months of age. No clear signs of effects on male behaviour were found, however, spatial learning of the females was affected. These effects were all found after exposure to the lowest dose of BPA, that is 25 μg/kg, but not at higher doses.

Sperm count

The decreased sperm count at the lowest tested BPA group (25 μg/kg) is to some extent supported by other studies reporting decreased sperm count in rodents after low-dose developmental exposure to BPA (Vom *et al.*, 1998; Salian *et al.*, 2009). Salian *et al.* (2009) performed a three-generation study to assess the effects of very low doses of BPA (1.2 or 2.4 μg/kg bw/day administered by gavage) in Holtzman rats (n = 8 litters per group/generation). Sperm count and motility were significantly reduced in the F1, F2 and F3 male offspring, with a dose-related reduction in sperm count. In Vom *et al.* (1998), pregnant CF-1 mice (n = 5 per group) were fed with 2 and 20 μg BPA/kg bw from GD 11–17. The daily sperm production was impaired in the group exposed to 20 μg/kg BPA, that is at a dose level that is rather similar to the 25 μg/kg in the present study. Additionally,

a recent study has shown that pre- and postnatal exposure of SD rats (n = 3) to 5 μg/kg bw/day of BPA also decreased epididymal sperm count and motility at 70 days of age (Yang *et al.*, 2015). In that study no other doses of BPA were investigated.

Other studies have not found significant effects of low-dose BPA on sperm count (Tinwell *et al.*, 2002; Kato *et al.*, 2006; Howdeshell *et al.*, 2008; Delclos *et al.*, 2014). In Kato *et al.* (2006), neonatal male Sprague Dawley rats (N = 8) were given subcutaneous injections of BPA doses at 2, 11, 56, 277 and 97.000 μg/kg bw/day from PND 0 to PND 9 and no effects of BPA on sperm count was found. Tinwell *et al.* (2002) administered BPA (0.02, 0.1, 50 mg/kg bw/day) by oral gavage from GD 6 to 21 to two strains of rat (Sprague Dawley and Alderley Park). In males at PND 90, the highest BPA dose of 50 mg/kg induced significantly reduced daily sperm production in the Alderly Park strain. However, at 20 μg/kg, the sperm production in the Alderly Park strain appear around 7% lower than control values and the lack of statistical significance for this difference may be related to the limited group size of 7 litters per group. In Howdeshell *et al.* (2008), Long-Evans hooded rats were gavaged with corn oil vehicle or BPA (2, 20 and 200 μg/kg/day) during pregnancy through lactation from GD 7 to PND 18. A significantly increased incidence of testicular degeneration was reported in the first block of the study, but not in the second block of the study. When the histopathology results from the two blocks were pooled, the total incidence of testicular degeneration induced by BPA was not statistically different from controls. Treatment with BPA did not significantly lower sperm counts at the doses used in this study. However, the sperm counts generally appear around 5% lower than control values at all doses and the lack of statistical significance for these difference might be related to the limited number of exposed litters analysed in each of the four sub-blocks in this study, that is N = 4–8. In Delclos *et al.* (2014), Sprague Dawley rat dams were dosed by gavage from gestation day 6 until the start of labour, and their pups were directly dosed from day 1 after birth to termination to seven equally spaced BPA doses (2.5–2700 μg/kg bw/day) and two high BPA doses (100 000 and 300 000 μg/kg bw/day). No effect of BPA was reported on male reproductive organ weights and sperm production. The Delcos study included a sufficient number of males per group (18–23) and a dose level similar to the dose where decrease in sperm count was seen in the present study, that is 25 μg/kg. However, the sperm count data are not shown in the Delclos paper and it is therefore not possible for us to perform any further evaluations of these data.

Overall, there is conflicting evidence with regard to low-dose effects of BPA on sperm count. The unclear picture may to some extent be due to the use of different dosing regimen, different rat strains or species and especially the limited group sizes of 4–8 in several studies.

The lowered sperm count observed in the present study was not associated with changes in testis weight. It has been proposed that perinatal exposure to estrogenic compounds may alter Sertoli cell numbers (Atanassova *et al.*, 2000, but this does not seem to be the case for BPA in this study. Lower Sertoli cell numbers would be expected to be associated with reduced testis weight, reduced seminiferous tubule diameter and possibly altered timing of seminiferous tubule lumen formation, which was not seen in the current study. Other causes of BPA-induced effects on epididymal sperm counts may be hypothesized, and further histological, immunohistochemical and morphometrical analyses on testes as well as epididymides may give further insight into possible causes of low sperm counts. Occasional findings in a few testes from the low-dose group could not be clearly associated with BPA exposure, but call for further histological examination of testes from the other dose groups.

Prostate development

Early exposure to weak and strong oestrogens can induce adverse changes in prostate development. A recent study revealed an increased incidence of multifocal inflammation and reactive hyperplasia in Sprague Dawley rat ventral prostate at 6 months of age after prenatal exposure to 25 and 250 μg/kg bw/day of BPA (Bernardo *et al.*, 2015). In contrast to the current study, BPA exposure induced increased ventral prostate weight, increased proliferation of epithelial cells and changes in distribution of compartments at PD 21 (Bernardo *et al.*, 2015). It is possible that different effects arise after pre- and postnatal exposures, as no effects on inflammation in adult ventral prostate were seen in the low-dose group of the current study, and as a study on neonatal exposure to BPA revealed no pathological changes at 6 months of age (Ho *et al.*, 2006). However, a 'second hit' of estradiol and testosterone in adult rats exposed neonatally to BPA induced an increase in incidence of prostatic intraepithelial neoplasia in ventral and dorsal prostate (Ho *et al.*, 2006). This indicates that more sensitive models using, for example, hormone treatment may be necessary to detect effects of early exposures on carcinogenesis. Chemically induced changes in prostate development appears to be lobe specific (Prins *et al.*, 2011), and different results may be achieved by examination of other prostate lobes or other BPA dose groups in the current study.

Obesogenic effect

The body weight of the female offspring exposed to 25 μg/kg was increased during the last part of our study, that is at 9–14 months of age. A number of other studies in pre- and postnatally exposed rats and mice indicate that BPA exposure could have an effect on metabolic function as evidenced by effects on glucose or insulin regulation or lipogenesis, and body weight gain (reviewed by EFSA 2015).

Rubin *et al.* (2001) have found increased body weights in male and female offspring of Sprague Dawley female rats exposed via drinking water to approximately 100 or 1200 μg BPA/kg bw/day from GD 6 throughout the period of lactation. The increase persisted longer in females than in males and on days 87 and 110 the low-dose BPA females showed higher body weights than both control and high-dose females.

In the study of Somm *et al.* (2009) in rats receiving a dose of approximately 70 μg/kg bw/day via drinking water from GD 6 until PND 21, body weight on PND 1 was increased in males and females, whereas body weight was increased only in females after weaning to 14 weeks of age.

Wei *et al.* (2011) administered doses of 0, 50, 250 or 1250 μg BPA/kg bw/day orally by gavage in corn oil to pregnant Wistar rats from GD 0 to PND 2. The offspring exposed prenatally to 50 μg BPA/kg bw/day showed increased weight gain from week 17 (females) or week 19 (males). No effects of BPA were observed at doses of 250 or 1250 μg BPA/kg bw/day.

In the study of Delclos *et al.* (2014), Sprague Dawley rats were treated with BPA by oral gavage from gestation day 6 through the start of labour. BPA was then given directly to pups from PND 1 until termination at PND 90 ± 5 at doses of 2.5, 8, 25, 80, 260, 840, 2 700, 100 000, and 300 000 μg/kg bw/day. Reduced body weight was found on day 90 at the dose of 300 mg/kg bw/day, whereas no signs of effect on body weight were found in the adult offspring from the lower dose groups.

In mice exposed to BPA in drinking water with doses corresponding to 0, 260, 2720 μg/kg bw/day from GD 10 to PND 21, body weights of female offspring were increased at both doses, but for the males only at the high-dose group (Miyawaki *et al.* 2007).

Anderson *et al.* (2013) exposed mice 2 weeks before mating, during gestation and lactation to BPA via the diet in doses corresponding to 0, 10.75 ng, 10.75 μg and 10.75 mg/kg bw/day. One male and one female/litter were followed until 10 months of age, and were given standard diet or diets containing BPA at the same levels as administered to the dams. Body weight and body fat was not statistically different from control in either sex.

Overall, several studies indicate that especially low doses of BPA, that is 50–260 μg/kg, can cause increased body weight in rats and mice and that this effect in adulthood can be more persistent or marked in females than in males. This picture is supported by the decreased body weight of the female offspring exposed to 25 μg/kg at 9–14 months of age found in the present study. There are, however, also studies where low doses of BPA have not lead to increased body weight. The unclear picture may to some extent be due to the use of different dosing regimen, different rat strains or species and especially that very few studies have assessed body weight later in life, for example after 4 months of age.

Oestrus cycles

No significant effects on oestrus cyclicity were seen in the 1-year-old BPA-exposed females, at any of the tested dose levels, even though the percentage of regularly cycling females seemed lower in the BPA-exposed animals. In a previous study from our group, significantly lower proportion of regularly cycling females was seen at 1 year of age after developmental exposure to a mixture of 13 endocrine-disrupting chemicals including BPA (Isling *et al.*, 2014). This finding was interpreted as a sign of premature reproductive senescence. The mixture that affected oestrus cyclicity included a BPA dose of 675 μg/kg bw/day. This is an intermediate dose compared to the BPA doses used in the present study and where no effect was seen. Thus, it seems that exposure to the other estrogenic and anti-androgenic compounds included in the mixture probably caused or contributed to the effect on oestrus cyclicity, resulting in a statistically significant effect in the mixture study, but not in the present study on BPA alone. The mechanisms behind the effects of endocrine disrupters on reproductive ageing are still not well understood and generally effects on reproductive ageing may be overlooked, since the evaluations addressed in the current OECD test guidelines for reproductive toxicology testing are focused on effects in young adult animals and do not include investigations of effects in old age after developmental exposure.

Anxiety behaviour

No significant effects of the BPA exposure were seen on motor activity or anxiety behaviour in the elevated plus maze (EPM), in either males or females. The lack of effect on motor activity in this study is in accordance with other data showing no effect on motor activity in BPA-exposed offspring (e.g. Stump *et al.*, 2010; Kuwahara *et al.*, 2013). A number of studies have reported effects of developmental exposure to BPA on anxiety-like behaviours in EPM in rodents (e.g. Jones & Watson, 2012; Patisaul *et al.*, 2012; Gioiosa *et al.*, 2013). Both increases and decreases in anxiety levels have been reported in these studies, however they consistently report that BPA-exposed adult offspring showed disappearance of the normal sexual dimorphism in exploratory behaviour in the EMP. A new study conducted as part of the CLARITY-BPA (Consortium Linking Academic and Regulatory Insights on BPA Toxicity) programme do not indicate BPA-related effects on anxiety or exploratory activity using assessment in open field, EMP and zero maze (Rebuli *et al.*, 2015). However, similarly as in the present study, the expected sex differences in EPM performance were not consistently observed in the control groups, and thus considered a potential limitation of both studies.

Spatial learning and memory

The spatial learning in Morris water maze was affected in the female offspring as females from the lowest dose group (25 μg/kg bw/day) showed significantly decreased swim length and swim speed. Also, the females showed male-like performance as the normal sexual dimorphism seen on the control group was not observed in the BPA-exposed animals.

Several animal studies have reported that developmental exposure to BPA can interfere with learning and memory capacities in different learning tasks in rodents, including spatial learning, passive avoidance learning and object recognition (for review, see EFSA 2015). Some studies report significant impairment of either learning and/or memory capacities (both in spatial and non-spatial learning tasks), whereas other do not.

Studies of effects of low doses of BPA on spatial learning in Morris water maze or other spatial learning mazes show conflicting results. In Xu *et al.* (2010) male ICR mice were perinatally exposed to BPA (0.05, 0.5, 5 and 50 mg/kg bw) and tested as weanlings and as adults in the Morris water maze. Increased swim lengths were seen for the upper two doses in weanlings and the upper three doses in adult males. In another study from the same group (Xu *et al.*, 2011a), control females showed longer swim length to the hidden platform (i.e. slower learners) than control males. The lowest BPA dose (40 μg/kg/day) caused increased swim length in males, whereas no effects were seen in females at any of the tested dose levels (i.e. 40 and 400 μg/kg/day). Thus, the sex differences in spatial learning and memory were abolished at 40 μg/kg/day. Jasarevic *et al.* (2013) reported that male but not female deer mice (n = 6–10) orally exposed during pre- and postnatal development to BPA (0.05, 5 or 50 mg/kg feed, equivalent to 0.25, 25 or 250 μg/kg bw/day) had impaired learning performance in the Barnes maze at 25 and 250 μg/kg bw/day. Also, the typical sex differences in spatial learning, of adult deer mice were diminished following developmental exposure to BPA. In Ferguson *et al.* (2012), low doses of BPA (2.5 or 25 μg/kg bw/day) given to Sprague Dawley dams

(n = 11–12) by oral gavage on gestational days 6–21 and then to the offspring from birth to weaning, did not show any significant effects on spatial learning in adolescent/adult offspring neither in the Barnes maze nor in the Morris water maze. Jones & Watson (2012) used the Morris water maze and found no spatial learning effects in adult offspring (PND 90–150) of Long-Evans rat dams orally exposed to BPA (5, 50, 500, or 5000 μg/kg bw/day) during gestation and lactation. However, the number of offspring studied was very limited as the study only included 2–3 litters per group. Also, Viberg *et al.* (2011) found no effect on learning and memory in the Morris water maze in 6-month-old mice orally exposed neonatally to single doses of BPA (0, 0.32, 3.2 or 4.8 mg/kg bw). The group study included 3–4 litters per group.

Kuwahara *et al.* (2013) assessed spatial learning in male Sprague Dawley rats orally administered BPA (50 or 500 μg/kg/day) from GD 10 to PND 14. In an appetite-motivated maze test male offspring exposed to 50 μg/kg used significantly more time to reach the reward, whereas no significant differences were seen in the Morris water maze test. The number of males tested per group was 12, but the number of litters per group was not stated.

The unclear picture for effects of BPA on spatial learning may to some extent be due to the use of different exposure regimen, different species (mice and rats) or age, and different strains as well as different dose levels of BPA in the studies. The choice of dose levels may be especially important, as the present study indicates that the effect of BPA on female spatial learning is non-monotonic. Also, only some of the studies have included focus on gender-related effect on the normal sexual dimorphism in spatial learning. Last but not the least, the unclear picture may also be due to underpowered sample size, that is the use of small group sizes of 2–6 in most of these studies. This is in contrast to the present study where a group size of 18–20 was used.

In Johnson *et al.* (2015), three doses of BPA (2.5, 25 and 2500 μg/kg bw/day) or a 0.5 μg/kg/day ethinyl estradiol dose were given to Sprague Dawley dams (as part of the CLARITY-BPA programme mentioned above). Dams were dosed by oral gavage on gestational days 6–21, whereas offspring were dosed directly from birth to weaning. The study examines effect of perinatal BPA exposure in a Barnes Maze and found that the 2500 BPA, and to a lesser extent the 2.5 BPA dose, prolonged latency in females, and the lowest dose (2.5 μg/kg bw/day) improved responses (shorter latency) in males. These findings indicate that developmental exposure to BPA can disrupt aspects of spatial navigational learning and memory in a sex-dependent manner. The developmental neurotoxicity study from Stump *et al.* (2010) have used group sizes similar those in the present study and examined spatial learning and memory in the Biel Maze test. BPA was offered to female Crl:CD(SD) rats (24 per dose group) and their litters at dietary concentrations of 0 (control), 0.15, 1.5, 75, 750, and 2250 ppm daily from GD 0 through PND 21. The females in the 0.15 and 2250 ppm feed groups showed significantly improved performance (i.e. they made fewer errors than the controls) at PND 22 in Path B (trials 5–10). The 0.15 ppm in feed equals a dose of 10–30 μg/kg bw/day which is dose very similar to the dose of 25 μg/kg bw/day, where the present study have also found improved spatial learning in female offspring. Also, at PND 62 the male offspring from the 0.15 ppm feed exposure group showed impaired learning manifested as a significant increase in the overall mean number of errors. The conclusion in Stump *et al.* (2010) is that there were no effects of BPA on spatial learning. The argument given is that the statistically significant differences noted were considered spurious and not BPA related because they did not occur consistently between or within testing periods and did not demonstrate any evidence of a dose-related trend. However, we find that lack of consistency within and between tasting periods is not a valid argument for dismissing significant effects on learning, as learning is an ongoing process and, for example initial learning can only be assessed once. Also, we find that the lack of dose-related trend is an insufficient argument for dismissing significant effect as several studies have shown non-monotonic dose–response for some effects of BPA and other endocrine disrupting substances. We therefore find that the significant effect on female spatial learning at 10–30 μg/kg bw/day in the Stump study strongly supports the very similar effect on female spatial learning at 25 μg/kg bw/day in the present study.

Sweet preference

In the sweet preference test, no effects were seen in the male offspring. However, the female preference for sweetened water decreased with increasing BPA exposure, and the females in the highest exposed group (50 mg BPA/kg bw/day) had significantly lower intake compared to the control group, that is these female showed signs of masculinized or defeminized behaviour. These results appear different from the findings by Xu *et al.* (2011b), where Sprague Dawley rats were perinatally exposed to BPA doses of 0.01, 0.1 or 1.0 mg/L in drinking water from GD 11 to PND 21. The male rats in the two highest dose groups increased their intake of the saccharin solution compared to the controls, whereas the females were unaffected by exposure. However, the paper does not report the dose levels calculated into mg/kg bw/day and there were only 6–8 animals per group which complicates the comparison to the present findings. Sweet preference has also been investigated in female Long-Evans rat offspring exposed to EE2 (0.05–50 μg/kg/day) or low doses of BPA (2, 20 and 200 μg/kg/day) from GD 7 to PD 18 (Ryan *et al.*, 2010). A statistically significant difference between male and female control rats was observed, but offspring exposed to BPA were unaffected. This does not contradict the present finding as the effect in the present study was found at a much higher dose of BPA. Interestingly the sweet preference test conducted with offspring exposed to EE2 showed reduced saccharin solution intake in females, indicating that another estrogenic chemical masculinized the behaviour of the female rat (Ryan *et al.*, 2010). Overall, the significant effect on female sweet preference observed in the present study after high-dose BPA exposure indicates that BPA may interact with the sexually dimorphic development of the brain leading to masculinized or defeminized behaviour of the female offspring.

Monotonic vs. non-monotonic dose–response relationships

The present study has found several statistically significant adverse effects only at the lowest tested dose of BPA (25 μg/kg bw/day). These include decreased sperm count, effect on spatial learning in adult female offspring and increased body weight late in female life as well as altered mammary gland development in 22-day-old males (Mandrup *et al.*, 2016). Seen

separately, these findings indicating non-monotonic dose–response relationship could be interpreted as random findings. However, the fact that several endpoints were only significantly affected at this low dose indicates that these effects were not random findings, and that some aspects of sexual- and neurobehavioural differentiation were affected only after exposure to this low dose of BPA.

Statistical analysis can aid to elucidate whether the low-dose effects found in the present study and other studies represent 'real' non-monotonic dose–response relationships, that is the effects in the low-dose group were both significantly different from controls and from the other higher exposure groups. For the observed effects on both sperm count and female spatial learning at 25 μg/kg this appeared to be the case as the results at 25 μg/kg were also significantly different from the results in the highest BPA dose groups, that is 5 and 50 mg/kg. Thus, for these endpoints the exposure to BPA during development does seem to result in non-monotonic dose–response relationships in the μg/kg dose span.

The present study also showed that some other endocrine sensitive endpoints were not affected (e.g. almost all of the investigated reproductive organs) or were affected showing a monotonic dose–response relationship (e.g. sweet preference in the present paper as well as male and female anogenital and distance nipple retention in Christiansen *et al.*, 2014). That different endpoints within the same study can be affected in different ways, for example show monotonic or non-monotonic relationships, is important to keep in mind when evaluating effects of endocrine disrupters.

Mode of action

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^{TM} programme, launched by US-EPA to develop ways to predict potential toxicity of chemicals and to develop a cost-effective approach for prioritizing 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 shows activity on the androgen receptor (AR), other nuclear receptors, and xenobiotic metabolizing enzymes (including aromatase) (Reif *et al.*, 2010). In the literature, BPA has shown estrogenic properties, the ability to antagonize AR activation with an IC50 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 E2 metabolism in H295R cells (Zhang *et al.*, 2011). Moreover, a study by Maamar *et al.* (2015) investigated possible direct endocrine disruption by BPA of the foetal testes of two rat strains (14.5–17.5 days post-coitum) and humans (8–12 gestational weeks) under different culture conditions. The authors reported that BPA can display anti-androgenic effects both in rat and human foetal testes (Maamar *et al.*, 2015).

Consequently, interpretation of BPA effects only in terms of an oestrogen-mimicking effect would be far too simplistic. The involvement of several of these mechanisms could explain the non-monotonic dose–response relationship seen in this study and other studies at low doses of BPA. It has for example been suggested that at low doses, BPA almost exclusively binds to the oestrogen receptor, but at high doses, it can also bind weakly to other hormone receptors such as the androgen receptor and thyroid hormone receptor (Vandenberg *et al.*, 2012). This property of BPA might contribute to the non-monotonic dose–response observed in our study.

CONCLUSION

In conclusion, the present results show that exposure to a low dose of BPA (25 μg/kg bw/day) during development may cause adverse effects on male reproductive development leading to decreased sperm count, affect sexually dimorphic development of the brain leading to masculinization of spatial learning in female offspring and lead to increase female body weights late in life. The results indicate non-monotonic dose–response relationships for these effects.

These new results do not change the lowest observed effect level (LOAEL) in our BPA study, as anogenital distance in new born females (reported in Christiansen *et al.*, 2014) and male mammary gland differentiation on PD 22 (Mandrup *et al.*, 2016) were also significantly affected at the 25 μg/kg bw/day. However, the severity of the adverse effects observed in the adult offspring adds further to the concern for effects of low doses of BPA. The multitude of adverse effects observed at 25 μg/kg bw/day in rats suggest that the new temporary TDI of 4 μg/kg bw/day proposed by EFSA is not sufficiently protective with regard to endocrine disrupting effects of BPA in humans.

The aggregated exposure to BPA for highly exposed humans are according to the recent exposure assessment from EFSA 1.01 to 1.06 μg/kg bw/day for men and women and 1.26 to 1.45 μg/kg bw/day for children (3–10 years) and teenagers (EFSA 2015). These exposure levels are only somewhat lower than the 25 μg/kg that caused adverse effects in rats in the present study as well as in other studies. This indicates that highly exposed humans may not be sufficiently protected with regard to endocrine disrupting effects of BPA.

ACKNOWLEDGEMENTS

This study was part of a large project funded by the Centre on Endocrine Disrupters in Denmark and the Danish Environmental Agency. The presented research was made possible with contributions of laboratory technicians and assistants of whom we wish to thank for their excellent technical assistance Lillian Sztuk, Dorte Lykkegaard Korsbech, Sarah Grundt Simonsen, Ulla El-Baroudy, Vibeke Kjær, Bo Oscar Herbst and Sidsel Brandt, and also Anne Ørngreen and Co-workers from the animal facility.

FUNDING

The work presented here was supported by grants from the Danish Environmental Protection Agency.

DISCLOSURES

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

MEETING COMMENTS*

Monica Lind (Uppsala, Sweden)

We are performing low-dose BPA studies at Uppsala independent of the CLARITY-BPA study described by Jerry Heindel at this Workshop. We use Fischer strain rats, you use Wistar rats, and Sprague–Dawley rats are used in CLARITY. The different strains of rat have marked differences in their response to BPA and I would like to know why laboratories choose to use a specific strain. Your studies on sperm count show results different from other laboratories and this might also be explained by strain differences.

Ulla Hass (Søborg, Denmark)

Strain is probably relevant to sperm counts after exposure to BPA. I think CD rats and Long–Evans rats were used in other laboratories. I do not know why Sprague–Dawley rats were selected for CLARITY.

Richard Sharpe (Edinburgh, UK)

Your study is impressive and the design is excellent. You suggested that BPA is possibly exerting an oestrogenic effect on your rats because of the behavioural changes observed. The exact mechanism of action is not known. It might be possible to determine if the effects are genomic ER-mediated by using low-dose BPA +/− an ER antagonist. However, an antagonist might be too much of a confounder.

Ulla Hass

In the first instance, we could see if treatment with oestrogen itself has the same effect on spatial learning as BPA. We would also have to look at the effect of the ER antagonist on its own to check for toxicity and its effect on the pups. BPA might also be acting directly as oestrogen during brain development but not acting through ER, for example, it might cause an increase in ER receptor numbers in the brain. There are other effects of oestrogen whose mode of action is not ER agonism. Your suggestion is valid and should be investigated.

REFERENCES

Anderson OS, Peterson KE, Sanchez BN, Zhang Z, Mancuso P & Dolinoy DC. (2013) Perinatal bisphenol A exposure promotes hyperactivity, lean body composition, and hormonal responses across the murine life course. *FASEB J* 27, 1784–1792.

Ashby J, Tinwell H & Haseman J. (1999) Lack of effects for low dose levels of bisphenol A and diethylstilbestrol on the prostate gland of CF1 mice exposed in utero. *Regul Toxicol Pharmacol* 30, 156–166.

Atanassova N, McKinnell C, Turner KJ, Walker M, Fisher JS, Morley M, Millar MR, Groome NP & Sharpe RM. (2000) Comparative effects of neonatal exposure of male rats to potent and weak (environmental) estrogens on spermatogenesis at puberty and the relationship to adult testis size and fertility: evidence for stimulatory effects of low estrogen levels. *Endocrinology* 141, 3898–3907.

Axelstad M, Boberg J, Hougaard KS, Christiansen S, Jacobsen PR, Mandrup KR, Nellemann C, Lund SP & Hass U. (2011) Effects of pre- and postnatal exposure to the UV-filter Octyl Methoxycinnamate (OMC) on the reproductive, auditory and neurological development of rat offspring. *Toxicol Appl Pharmacol* 250, 278–290.

*Correction added on 16 June 2016, after first online publication: Meeting Comments added.

Axelstad M, Hansen PR, Boberg J, Bonnichsen M, Nellemann C, Lund SP, Hougaard KS & Hass U. (2008) Developmental neurotoxicity of Propylthiouracil (PTU) in rats: relationship between transient hypothyroxinemia during development and long-lasting behavioural and functional changes. *Toxicol Appl Pharmacol* 232, 1–13.

Ayyanan A, Laribi O, Schuepbach-Mallepell S, Schrick C, Gutierrez M, Tanos T, Lefebvre G, Rougemont J, Yalcin-Ozuysal O & Brisken C. (2011) Perinatal exposure to bisphenol a increases adult mammary gland progesterone response and cell number. *Mol Endocrinol* 25, 1915–1923.

Bernardo BD, Brandt JZ, Grassi TF, Silveira LT, Scarano WR & Barbisan LF. (2015) Genistein reduces the noxious effects of in utero bisphenol A exposure on the rat prostate gland at weaning and in adulthood. *Food Chem Toxicol* 84, 64–73.

Boberg J, Johansson HK, Hadrup N, Dreisig K, Berthelsen L, Almstrup K, Vinggaard AM & Hass U. (2015) Perinatal exposure to mixtures of anti-androgenic chemicals causes proliferative lesions in rat prostate. *Prostate* 75, 126–140.

Bonefeld-Jorgensen EC, Long M, Hofmeister MV & Vinggaard AM. (2007) Endocrine-disrupting potential of bisphenol A, bisphenol A Dimethacrylate, 4-n-Nonylphenol, and 4-n-Octylphenol in vitro: new data and a brief review. *Environ Health Perspect* 115, 69–76.

Chitra KC, Latchoumycandane C & Mathur PP. (2003) Induction of oxidative stress by bisphenol A in the epididymal sperm of rats. *Toxicology* 185, 119–127.

Christiansen S, Axelstad M, Boberg J, Vinggaard AM, Pedersen GA & Hass U. (2014) Low-dose effects of bisphenol A on early sexual development in male and female rats. *Reproduction* 147, 477–487.

Creasy DM. (2001) Pathogenesis of male reproductive toxicity. *Toxicol Pathol* 29, 64–76.

Delclos KB, Camacho L, Lewis SM, Vanlandingham MM, Latendresse JR, Olson GR, Davis KJ, Patton RE, Gamboa da Costa G, Woodling KA, Bryant MS, Chidambaram M, Trbojevich R, Juliar BE, Felton RP & Thorn BT. (2014) Toxicity evaluation of bisphenol A administered by gavage to Sprague Dawley rats from gestation day 6 through postnatal day 90. *Toxicol Sci* 139, 174–197.

Della Seta D, Minder I, Dessi-Fulgheri F & Farabollini F. (2005) Bisphenol-A exposure during pregnancy and lactation affects maternal behavior in rats. *Brain Res Bull* 65, 255–260.

EFSA (2015) Scientific opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs: PART II – toxicological assessment and risk characterisation. *EFSA J* 13, 3978.

Ferguson SA, Law CD & Abshire JS. (2012) Developmental treatment with bisphenol A causes few alterations on measures of postweaning activity and learning. *Neurotoxicol Teratol* 34, 598–606.

Gioiosa L, Fissore E, Ghirardelli G, Parmigiani S & Palanza P. (2007) Developmental exposure to low-dose estrogenic endocrine disruptors alters sex differences in exploration and emotional responses in mice. *Horm Behav* 52, 307–316.

Gioiosa L, Parmigiani S, Vom Saal FS & Palanza P. (2013) The effects of bisphenol A on emotional behavior depend upon the timing of exposure, age and gender in mice. *Horm Behav* 63, 598–605.

Gonçalves CR, Cunha RW, Barros DM & Martinez PE. (2010) Effects of prenatal and postnatal exposure to a low dose of bisphenol A on behavior and memory in rats. *Environ Toxicol Pharmacol* 30, 195–201.

Hass U, Lund SP, Simonsen L & Fries AS. (1995) Effects of prenatal exposure to xylene on postnatal development and behavior in rats. *Neurotoxicol Teratol* 17, 341–349.

Hass U, Lund SP, Hougaard KS & Simonsen L. (1999) Developmental neurotoxicity after toluene inhalation exposure in rats. *Neurotoxicol Teratol* 21, 349–357.

Ho SM, Tang WY, Belmonte de Frausto J & Prins GS. (2006) Developmental exposure to estradiol and bisphenol A increases susceptibility to prostate carcinogenesis and epigenetically

regulates phosphodiesterase type 4 variant 4. *Cancer Res* 66, 5624–5632.

Howdeshell KL, Furr J, Lambright CR, Wilson VS, Ryan BC & Gray LE. (2008) Gestational and lactational exposure to ethinyl estradiol, but not bisphenol A, decreases androgen-dependent reproductive organ weights and epididymal sperm abundance in the male Long Evans hooded rat. *Toxicol Sci* 102, 371–382.

Isling LK, Boberg J, Jacobsen PR, Mandrup KR, Axelstad M, Christiansen S, Vinggaard AM, Taxvig C, Kortenkamp A & Hass U. (2014) Late life effects on rat reproductive system after developmental exposure to mixtures of endocrine disrupters. *Reproduction* 147, 465–476.

Jarfelt K, Dalgaard M, Hass U, Borch J, Jacobsen H & Ladefoged O. (2005) Antiandrogenic effects in male rats perinatally exposed to a mixture of di(2-ethylhexyl) phthalate and di(2-ethylhexyl) adipate. *Reprod Toxicol* 19, 505–515.

Jasarevic E, Williams SA, Vandas GM, Ellersieck MR, Liao C, Kannan K, Roberts RM, Geary DC & Rosenfeld CS. (2013) Sex and dose-dependent effects of developmental exposure to bisphenol A on anxiety and spatial learning in deer mice (*Peromyscus maniculatus bairdii*) offspring. *Horm Behav* 63, 180–189.

Johnson SA, Javurek AB, Painter MS, Ellersieck MR, Welsh TH Jr, Camacho L, Lewis SM, Vanlandingham MM, Ferguson SA & Rosenfeld CS. (2015) Effects of developmental exposure to bisphenol A on spatial navigational learning and memory in rats: ACLARITY-BPA study. *Horm Behav*. doi:10.1016/j.yhbeh.2015.09.005.

Jones BA & Watson NV. (2012) Perinatal BPA exposure demasculinizes males in measures of affect but has no effect on water maze learning in adulthood. *Horm Behav* 61, 605–610.

Kato H, Furuhashi T, Tanaka M, Katsu Y, Watanabe H, Ohta Y & Iguchi T. (2006) Effects of bisphenol A given neonatally on reproductive functions of male rats. *Reprod Toxicol* 22, 20–29.

Kuwahara R, Kawaguchi S, Kohara Y, Cui H & Yamashita K. (2013) Perinatal exposure to low-dose bisphenol A impairs spatial learning and memory in male rats. *J Pharmacol Sci* 123, 132–139.

Liao C & Kannan K. (2011) Widespread occurrence of bisphenol A in paper and paper products: implications for human exposure. *Environ Sci Technol* 45, 9372–9379.

Maamar MB, Lesné L, Desdoits-Lethimonier C, Coiffec I, Lassurguère J, Lavoué V, Deceuninck Y, Antignac JP, LeBizec B, Perdu E, Zalko D, Pineau C, Chevrier C, Dejucq-Rainsford N, Mazaud-Guittot S & Jégou B. (2015) An investigation of the endocrine-disruptive effects of bisphenol A in human and rat fetal testes. *PLoS One* 10, e0117226.

Mandrup K, Boberg J, Isling LK, Christiansen S & Hass U. (2016) Low-dose effects of bisphenol A on mammary gland development in rats. *Andrology* 4, 673–683.

Miyawaki J, Sakayama K, Kato H, Yamamoto H & Masuno H. (2007) Perinatal and postnatal exposure to bisphenol a increases adipose tissue mass and serum cholesterol level in mice. *J Atheroscler Thromb* 14, 245–252.

Munoz-de-Toro M, Markey CM, Wadia PR, Luque EH, Rubin BS, Sonnenschein C & Soto AM. (2005) Perinatal exposure to bisphenol-A alters peripubertal mammary gland development in mice. *Endocrinology* 146, 4138–4147.

Paris F, Balaguer P, Térouanne B, Servant N, Lacoste C, Cravedi JP, Nicolas JC & Sultan C. (2002) Phenylphenols, biphenols, bisphenol-A and 4-tert-octylphenol exhibit a and B estrogen activities and antiandrogen activity in reporter cell lines. *Mol Cell Endocrinol* 193, 43–49.

Patisaul HB, Sullivan AW, Radford ME, Walker DM, Adewale HB, Winnik B, Coughlin JL, Buckley B & Gore AC. (2012) Anxiogenic effects of developmental bisphenol A exposure are associated with gene expression changes in the juvenile rat amygdala and mitigated by soy. *PLoS One* 7, e43890.

Prins GS & Korach KS. (2008) The role of estrogens and estrogen receptors in normal prostate growth and disease. *Steroids* 73, 233–244.

Prins GS, Ye SH, Birch L, Sm H & Kannan K. (2011) Serum bisphenol A pharmacokinetics and prostate neoplastic responses following oral and subcutaneous exposures in neonatal Sprague-Dawley rats. *Reprod Toxicol* 31, 1–9.

Rebuli ME, Camacho L, Adonay ME, Reif DM, Aylor DL & Patisaul HB. (2015) Impact of low dose oral exposure to bisphenol A (BPA) on juvenile and adult rat exploratory and anxiety behavior: a CLARITY-BPA Consortium Study. *Toxicol Sci* 148, 341–354.

Reif DM, Martin MT, Tan SW, Houck KA, Judson RS, Richard AM, Knudsen TB, Dix DJ & Kavlock RJ. (2010) Endocrine profiling and prioritization of environmental chemicals using ToxCast data. *Environ Health Perspect* 118, 1714–1720.

Rubin BS, Murray MK, Damassa DA, King JC & Soto AM. (2001) Perinatal exposure to low doses of bisphenol A affects body weight, patterns of oestrous cyclicity, and plasma LH levels. *Environ Health Perspect* 109, 675–680.

Ryan BC & Vandenbergh JG. (2006) Developmental exposure to environmental estrogens alters anxiety and spatial memory in female mice. *Horm Behav* 50, 85–93.

Ryan BC, Hotchkiss AK, Crofton KM & Gray LE. (2010) In utero and lactational exposure to bisphenol A, in contrast to ethinyl estradiol, does not alter sexually dimorphic behavior, puberty, fertility, and anatomy of female LE rats. *Toxicol Sci* 114, 133–148.

Salian S, Doshi T & Vanage G. (2009) Perinatal exposure of rats to Bisphenol A affects the fertility of male offspring. *Life Sci* 85, 742–752.

Salian S, Doshi T & Vanage G. (2011) Perinatal exposure of rats to Bisphenol A affects fertility of male offspring an overview. *Reprod Toxicol* 31, 359–362.

Shelby MD. (2008) *NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A. NTP CERHR.* NTP CERHR MON, Sep;(22):v, vii–ix, 1–64 passim. http://ntp.niehs.nih.gov/ntp/ohat/bisphenol/bisphenol.pdf

Sik Kim H, Seok Kang T, Hyun Kang I, Sung Kim T, Ju Moon H, Young Kim I, Ki H, Lea Park K, Mu Lee B, Dong Yoo S & Han SY. (2005) Validation study of OECD rodent uterotrophic assay for the assessment of estrogenic activity in Sprague-Dawley immature female rats. *J Toxicol Environ Health A* 68, 2249–2262.

Somm E, Schwitzgebel VM, Toulotte A, Cederroth CR, Combescure C, Nef S, Aubert ML & Hüppi PS. (2009) Perinatal exposure to bisphenol a alters early adipogenesis in the rat. *Environ Health Perspect* 117, 1549–1555.

Stump DG, Beck MJ, Radovsky A, Garman RH, Freshwater LL, Sheets LP, Marty MS, Waechter JM, Dimond SS, Van Miller JP, Shiotsuka RN, Beyer D, Chappelle AH & Hentges SG. (2010) Developmental neurotoxicity study of dietary bisphenol A in Sprague-Dawley rats. *Toxicol Sci* 115, 167–182.

Timms BG, Howdeshell KL, Barton L, Bradley S, Richter CA & vom Saal FS. (2005) Estrogenic chemicals in plastic and oral contraceptives disrupt development of the fetal mouse prostate and urethra. *Proc Natl Acad Sci USA* 102, 7014–7019.

Tinwell H, Haseman J, Lefevre PA, Wallis N & Ashby J. (2002) Normal sexual development of two strains of rat exposed in utero to low doses of bisphenol A. *Toxicol Sci* 68, 339–348.

Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Lee DH, Shioda T, Soto AM, vom Saal FS, Welshons WV, Zoeller RT & Myers JP. (2012) Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. *Endocr Rev* 33, 378–455.

Viberg H, Fredriksson A, Buratovic S & Eriksson P. (2011) Dose-dependent behavioral disturbances after a single neonatal bisphenol A dose. *Toxicology* 290, 187–194.

Vinggaard AM, Korner W, Lund KH, Bolz U & Petersen JH. (2000) Identification and quantification of estrogenic compounds in recycled and virgin paper for household use as determined by an in vitro yeast

estrogen screen and chemical analysis. *Chem Res Toxicol* 13, 1214–1222.

vom Saal FS, Cooke PS, Buchanan DL, Palanza P, Thayer KA, Nagel SC, Parmigiani S & Welshons WV. (1998) A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. *Toxicol Ind Health* 14, 239–260.

Wei J, Lin Y, Li Y, Ying C, Chen J, Song L, Zhou Z, Lv Z, Xia W, Chen X & Xu S. (2011) Perinatal exposure to bisphenol A at reference dose predisposes offspring to metabolic syndrome in adult rats on a high-fat diet. *Endocrinology* 152, 3049–3061.

Xu XH, Zhang J, Wang YM, Ye YP & Luo QQ. (2010) Perinatal exposure to bisphenol-A impairs learning-memory by concomitant down-regulation of N-methyl-D-aspartate receptors of hippocampus in male offspring mice. *Horm Behav* 58, 326–333.

Xu X, Tian D, Hong X, Chen L & Xie L. (2011a) Sex-specific influence of exposure to bisphenol-A between adolescence and young adulthood on mouse behaviors. *Neuropharmacology* 61, 565–573.

Xu X, Tan L, Himi T, Sadamatsu M, Tsutsumi S, Akaike M & Kato N. (2011b) Changed preference for sweet taste in adulthood induced by perinatal exposure to bisphenol A-A probable link to overweight and obesity. *Neurotoxicol Teratol* 33, 458–463.

Yang Y, Hong Y & Chae SA. (2015) Reduction in semen quality after mixed exposure to bisphenol A and isobutylparaben in utero and during lactation periods. *Hum Exp Toxicol*. doi:10.1177/0960327115608927.

Zhang X, Chang H, Wiseman S, He Y, Higley E, Jones P, Wong CKC, Al-Khedhairy A, Giesy JP & Hecker M. (2011) Bisphenol A disrupts steroidogenesis in human H295R cells. *Toxicol Sci* 121, 320–327.