Multiple endocrine disrupting effects in rats perinatally exposed to butylparaben

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Abstract

Parabens comprise a group of preservatives commonly added to cosmetics, lotions and other consumer products. Butylparaben has estrogenic and anti-androgenic properties and is known to reduce sperm counts in rats following perinatal exposure. Whether butylparaben exposure can affect other endocrine sensitive endpoints, however, remains largely unknown. In this study, time-mated Wistar rats (n=18) were orally exposed to 0, 10, 100 or 500 mg/kg bw/day of butylparaben from gestation day 7 to pup day 22. Several endocrine-sensitive endpoints were adversely affected. In the two highest dose groups, the anogenital distance of newborn male and female offspring was significantly reduced, and in prepubertal females, ovary weights were reduced and mammary gland outgrowth was increased. In male offspring, sperm count was significantly reduced at all doses from 10 mg/kg bw/day. Testicular CYP19a1 (aromatase) expression was reduced in prepubertal, but not adult animals exposed to butylparaben. In adult testes, Nr5a1 expression was reduced at all doses, indicating persistent disruption of steroidogenesis. Prostate histology was altered at prepuberty and adult prostate weights were reduced in the high dose group. Thus, butylparaben exerted endocrine disrupting effects on both male and female offspring. The observed adverse developmental effect on sperm count at the lowest dose is highly relevant to risk assessment, as this is the lowest observed adverse effect level in a study on perinatal exposure to butylparaben.

3 to 6 keywords: Reproduction; paraben; testis; breast; prostate, sexual development, endocrine disruption
Introduction

Parabens are preservatives commonly used in cosmetics and lotions, and some also in foods. Dermally applied parabens are absorbed by the skin and metabolized by esterases, conjugated and excreted in urine and bile (reviewed by Soni et al., 2005). Although metabolism following dermal and oral exposure is rapid, toxic effects have been reported in experimental studies on oral exposure to some parabens, including butylparaben.

Some parabens have estrogenic properties both in vitro and in vivo, and butylparaben has been shown to affect endocrine-sensitive organ systems (Boberg et al., 2010). Studies on juvenile rats exposed to butylparaben have shown conflicting results, as reduced sperm count and testosterone level reported in one study at doses from 10 mg/kg bw/day (Oishi et al., 2001) was not corroborated by subsequent studies investigating similar dose levels (Hoberman et al., 2008, Gazin et al., 2013).

Stronger evidence is found regarding effects on sperm count after developmental exposure to parabens (Kang et al., 2002; Zhang et al., 2014; Yang et al., 2015). Reduced epididymal sperm count and motility were seen in rat offspring of dams exposed subcutaneously to 100 and 200 mg butylparaben/kg bw/day during gestation and lactation (Kang et al., 2002). In a recent study, perinatal exposure of rats to 2.5 mg/kg bw/day of isobutylparaben reduced epididymal sperm count and motility compared to control (Yang et al., 2015). In another rat study, oral exposure to n-butylparaben at doses of 0, 64, 160, 400 and 1000 mg/kg/day from gestation day (GD) 7 to postnatal day (PND) 21 led to reduced male anogenital distance at PND 1 and 21, delayed preputial separation, reduced reproductive organ weights at several ages, and reduced epididymal sperm count at PND 90 at the two highest dose levels (Zhang et al., 2014). These effects were associated with reduced testosterone and LH levels, and elevated estradiol and progesterone levels in serum from prepubertal male rats, supporting the view that butylparaben can exert both anti-androgenic and estrogenic effects on the developing organism. The underlying mechanisms for the effect on sperm count remain unknown, but in addition to estrogenic properties (Byford et al., 2002; Routledge et al., 1998), in vitro studies have shown inhibition of androgen receptor activation by butylparaben (Satoh et al., 2005; Chen et al., 2007).

Little is known about effects of butylparaben on other endocrine sensitive endpoints such as prostate and mammary gland development. Estrogenic chemicals have been shown to affect early mammary gland development in female rodents and to accelerate mammary gland growth. For instance, an increase in outgrowth and number of terminal end buds (TEBs) in prepubertal female rat mammary glands have been shown after exposure to estrogenic compounds such
as ethinyl estradiol and genistein (Mandrup et al., 2012; Mandrup et al., 2015; Cotroneo et al., 2002). These findings may predict adverse effects on human breast development and possibly increased risk of breast cancer following early exposure to estrogenic chemicals (Soto et al., 2013). Animal studies have shown that developmental exposure to endocrine disrupting compounds, or elevated perinatal levels of estrogens, can induce atypical hyperplasia of the prostatic epithelium, as well as morphological changes resembling those of precancerous lesions in humans (PIN lesions) (Prins et al., 2007).

Due to the endocrine disrupting activity of butylparaben, a possible influence on prostate and mammary development can be hypothesized, and hence this study aimed to investigate developmental effects of butylparaben on these organs in prepubertal and adult offspring. We also intended to improve risk assessment of butylparaben by investigating endocrine sensitive endpoints in perinatally exposed rats at doses from 10 mg/kg bw/day, and to elucidate the mechanisms underpinning the influence of butylparaben on sperm count.

**Materials and methods**

**Test compounds**

Butylparaben (purity >99.0 %, CAS no. 94-26-8) was purchased from Sigma-Aldrich (Brøndby, Denmark). Corn oil was used both as a control compound and vehicle and purchased from Sigma (Brøndby, Denmark).

**Animals and exposure**

72 time-mated nulliparous, young adult Wistar rats (HanTac:WH, SPF, Taconic Europe, Ejby, Denmark) were supplied at day 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 2 blocks of 36 dams (separated by one week), and all dose groups were equally represented in both blocks. The animal experiment was carried out at the DTU National Food Institute (Mørkhøj, Denmark). Ethical approval was obtained from the Danish Animal Experiments Inspectorate: authorization number, 2012-15-2934-00089 C4. The experiments were overseen by the National Food Institutes in-house Animal Welfare Committee for animal care and use.

The dams were housed in pairs until GD 17 and alone thereafter under standard conditions in semi-transparent polysulfone (PSU) cages (PSU 80-1291HOOSU Type III, Tecniplast) (15 x 27 x 43cm) with aspen wood chip bedding.
The day after arrival (GD 4), time-mated dams were distributed into 4 groups of 18 rats with similar body weight (bw) distributions. The dams received vehicle (controls), or 10, 100 or 500 mg/kg bw/day of butylparaben. Test compounds and vehicle were administered by oral gavage with a stainless steel probe 1.2 x 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 per day. Dams that did not give birth were omitted from the study. The exposure period was chosen to cover the most sensitive windows of reproductive development in rat offspring. 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.

**In vivo measurements**

The day after delivery the pups were counted, sexed, weighed, and checked for anomalies. Dead pups were investigated macroscopically for pathological changes when possible. Anogenital distance (AGD) was measured in all offspring using a stereomicroscope with unit markings on the ocular. 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. The same skilled technician, blinded with respect to exposure groups, recorded both AGD measurements and NR counts. After weaning of the offspring at PD 22, the dams were killed and the number of implantation sites was registered to calculate post-implantation and perinatal loss.
Pubertal onset was assessed by determining day of vaginal opening or the day of balano-preputial separation in weaned female and male offspring, respectively. Registrations were performed daily in females from PD 27 until vaginal opening was detected in all animals. Males were examined daily from PD 39 until the last male was positive. Age and body weight of the rats were recorded on the day in which vaginal opening and balano-preputial separation was first observed. Onset of puberty was assessed blinded with respect to exposure groups by skilled technicians.

Necropsy of male and female offspring PD 16/17 and 22

Reproductive organ weights and histological and gene expression changes in reproductive organ weights were examined at PD 16 (males) and 17 (females), as this age has proved sensitive for detection of particularly anti-androgenic effects of chemicals (Metzdorff et al., 2007; Christiansen et al., 2009). On PD 16 and 17, one male and one female pup from each litter (n=12-18) were weighed and decapitated, and blood was collected in heparinized tubes for hormone analysis. From males the following organs were excised and weighed: Testes, ventral prostate, epididymis, seminal vesicle, levator ani/bulbocavernous muscle (LABC), bulbourethral gland, liver, adrenal, and retroperitoneal fat pad. From females the following organs were excised and weighed: Ovary, liver, thyroid, and retroperitoneal fat pad. One testis per pup was placed in RNAlater for gene expression studies as described below).

On PD 22, one female and one male pup per litter (n=12-14) were weighed and decapitated, and blood was collected in heparinized tubes for hormone analysis. From females, ovaries were excised and weighed, and from males testes and ventral prostate were excised and weighed. Testes were fixed in Bouin’s fixative overnight and ventral prostates were fixed in formalin for histological examination. Mammary glands were dissected from female pups for whole mounting. The tissue was dissected in order to include the abdominal (4th) mammary gland with the adjacent lymph nodes and part of the inguinal (5th) mammary gland. At PD 22 the number of terminal end buds (TEBs) in rat mammary glands is peaking, and this age is considered sensitive to detection of changes in early mammary development (Russo et al., 1979; Mandrup et al., 2012).

Necropsy of adults and epididymal sperm count

Approximately one female and one male pup per litter were sacrificed at PD 80 to 90 and body weights were determined. From males the following organs were excised and weighed: Testes, epididymis, seminal vesicle with prostate, ventral prostate (separated from dorsolateral prostate and seminal vesicle) levator ani/bulbocavernous muscle, bulbourethral gland, liver, thyroid, 4th mammary gland, and retroperitoneal fat pad. One testis per animal was fixed in
Bouin’s fixative overnight, and the contralateral was frozen for gene expression studies. Epididymides, ventral prostates and mammary glands were fixed in formalin for histological examination.

For sperm count analysis, samples were analyzed using computer assisted sperm analysis (CASA) system (HTM-IVOS, Hamilton Thorne Research, MA, USA). From 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 analyzed using a 10 × UV fluorescent objective and IDENT OPTIONS on the CASA. Ten fields were analyzed for each sample and three counts were performed for each suspension. Counts were averaged and data are presented as number of sperm per gram cauda.

Sperm cell motility and morphology was not investigated.

Females were sacrificed on the day of estrous as judged from a vaginal smear in the morning. The following organs were excised and weighed: Ovaries, uterus, liver, thyroid, mammary glands (4th gland), and retroperitoneal fat pad. Abdominal (4th) mammary glands, ovaries and uteri were fixed in formalin for histological examination.

Hormone analysis and gene expression

Serum estradiol was measured in 8 to 10 plasma samples per dose group in PD 16 males and PD 22 females using Enzyme Linked Assay (Cayman kit no. 582251, Bertinpharma, Montigny-le-Bretonneux, France).

Gene expression analyses were performed on testes PD 16 (n=9-10) and PD 90 (n=4) by RT-qPCR. Protocols and verification of suitable reference genes for data normalization were as previously described (Svingen et al., 2015). Briefly, total RNA was extracted from the entire testis at PD16 or approximately 120 mg tissue (median cross-section) at PD90 using an RNA Mini kit (Qiagen), including on-column DNase I treatment. RNA was subsequently quantified and purity-verified on a nano-drop spectrophotometer and 500 ng total RNA used to synthesize cDNA (Omiscript kit; Qiagen) in the presence of 6µM Random Primer mix (New England Biolabs). cDNA was diluted 1:20 and used at 3 µl per RT-qPCR reactions. TaqMan Gene Expression Assays (Life Technologies) were: Ddx4 (Rn01489814_m1), Fshr (Rn01648507_m1), Hsd3b1 (Rn01774741_m1), Lhr (Rn00564309_m1), Nr5a1 (Rn00584298_m1), Sox9 (Rn01751069_mH), Cyp19a1 (Taxvig et al., 2008) and Ar, Cyp11a1, Cyp17a1 (Laier et al., 2006). RT-qPCR assays were run on a 7900HT Fast Real-Time PCR System (Applied Biosystems) over 45 cycles using a two-step cycling protocol with annealing temp at 60 °C. Relative transcript abundance was calculated by the comparative Ct-method.
using the reference genes *Rpl13a* (Rn00821946_g1) and *Sdha* (Rn00590475_m1). Intra-assay variability of technical replicates was <0.5 cycles.

**Mammary whole mounts**

Mammary glands from PD 22 female offspring were placed on a glass slide and stained with alum carmine as described in Mandrup *et al.*, 2015. Briefly, the whole mounts were scanned on a flatbed scanner (4800 dpi) and mammary development was assessed on the digital images. Measurements were performed in Image Pro Plus 7.0 software (Media Cybernetics, Bethesda, MD, USA). The glands were evaluated for outgrowth and extent of mammary development. For each dose group, 12 to 14 samples were evaluated. Outgrowth was assessed by measuring the outer area, longitudinal growth, transverse growth, distance to the lymph node (shortest distance from the 4th gland to the adjacent lymph node) and distance to the 5th gland (shortest distance from the 4th to the 5th abdominal mammary gland). The number of terminal end buds was counted (TEBs, defined as tear-drop shaped buds in zone C with a diameter of 100 µm or more, as defined by Russo and Russo, 1996a).

**Histological examination**

Female mammary glands PD 80-90 were sectioned and stained with haematoxylin and eosin by standard procedures. Histologic evaluation included ductular changes, lobular changes and functional changes in females confirmed to be in oestrous. Ductular changes included the evaluation and distribution of intraductal hyperplasia (defined as duct epithelium with 3 or more layers of epithelial cells as described by Singh *et al.*, 2000). Evaluation of lobular changes included lobule types (as single alveoli/ lobules smaller than type 1, lobules type 1 or lobules type 2 as defined by Russo *et al.*, 1996a for humans) and lobuloalveolar structure of the lobules (loss of the typical tubuloalveolar architecture of alveoli). Finally, the distribution of secretory material in the ducts was evaluated as a sign of secretory activity.

Ovary histology was examined in one section from all adult females from the control and high dose groups and focused only on health status and cyclic activity. No quantitative measures were made. Uterine histology was examined only to confirm estrous cycle stage to aid the histological examination of mammary glands.
Histological examination of one section per organ (testes, epididymides and ventral prostate) was performed in all adult males from the high dose and control groups. A detailed qualitative examination of the testes was performed taking into account the tubular stages of the spermatogenic cycle to identify effects such as missing germ cell layers or types, retained spermatids, multinucleate or apoptotic germ cells and sloughing of spermatogenic cells into the lumen. Epididymides were evaluated for ductal atrophy, changes in sperm content and presence of sloughed testicular germ cells and cell debris in the epididymal lumen. Ventral prostates of adult males were evaluated with regard to degree of inflammation, epithelial atrophy, and atypical hyperplasia. Additionally, morphometric examination was made in ventral prostate from adult and prepubertal (PD 22) males to identify possible changes in the relative areas of different compartments. Each area was applied a grid with 15 points that were manually assigned as epithelium, stroma, lumen or outside tissue section/undefined. This was done in 10 randomly selected areas per tissue section under 20x or 10x magnification (PD 22 and 90, respectively) using Image-Pro1 Plus ver. 7.0 (Media Cybernetics, Inc., USA). The percentage of areas occupied by each compartment (epithelium, stroma, and lumen) was compared between exposed groups and controls, and the ratio between epithelium and lumen was calculated and compared between groups. Grid points outside the tissue or undefined were not included in the statistical analysis.

Statistics

GraphPad Prism 5 was used for analysis of gene expression data and morphometrical data, whereas SAS Enterprise Guide 4.3 was used for all other data.

Data from continuous endpoints were examined for normal distribution and homogeneity of variance, and if relevant, log transformed. Data with normal distribution and homogeneity of variance were analyzed using analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. Organ weights were analyzed using body weight as a covariate. When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor in ANOVA or litter means were used. In cases where normal distribution and homogeneity of variance could not be obtained by data transformation (i.e. whole mount measurements), a non-parametric Kruskall-Wallis test was applied followed by Dunn’s multiple comparison test comparing exposed groups with controls.
AGD and organ weights were analyzed using body weight as a covariate. The number of nipple/areolas was assumed to follow a binomial distribution with a response range between 0 and θmax, with θmax being equal to the biologically possible maximal number of nipples in rats, either 12 or 13. The choice of θmax was decided on considering the global fit (information criterion of Schwarz). To account for litter effects on nipple retention, correlation structures between number of nipple/areolas and litter were modelled by the Generalized Estimating Equations method using the SAS procedure PROC GENMOD.

Histological scoring data were evaluated using the 2x2 Fisher's Exact Test without correction for multiple comparisons.

Results

Dams and delivery

Exposure to butylparaben did not affect maternal body weight gain, gestation length, litter size, pre- or perinatal offspring survival, or body weights either at birth or in the postnatal period (Table 1).

In vivo markers of endocrine disrupting effects

Endocrine-disrupting effects of butylparaben were seen in neonatal pups, as AGD (analyzed with body weight as a covariate) was reduced dose-dependently in both males and females at PD 1 following perinatal exposure to 100 or 500 mg/kg bw/day (Fig. 1 and Table 1). No significant effects were seen for male nipple retention (Table 1).

Sexual maturation, measured as day of vaginal opening (VO) in females and day of preputial separation (PPS) in males occurred slightly later in both male and female offspring exposed to butylparaben, but this was not statistically significant (Table 1).

Organ weights, epididymal sperm counts and histology

No effects of butylparaben on the body weight of offspring were seen on PD 16, 17, 22 or in adulthood. In male offspring, no changes in reproductive organ weights were detected at PD 16 or 22 (Table 2). In female offspring, ovary weights were reduced dose-dependently at PD 17, and the effect was statistically significant at 100 and 500 mg/kg bw/day (Fig. 1; Table 2). Ovary weights were slightly higher compared to controls at PD 22, but this was not statistically significant (p=0.086 at high dose) (Table 2).
In adult males (PD 90), the weight of prostate and seminal vesicle (together) and the weight of ventral prostate (separated from seminal vesicle) were reduced in the highest dose group compared to controls (p=0.015 and p=0.05, respectively) (Fig. 1; Table 2). Epididymal weight was significantly increased in the middle dose group only (p=0.01) (Table 2), and epididymal sperm count was significantly decreased in all dosed groups compared to the controls (p < 0.0001) (Fig. 3). The histological examination of testes and epididymides from adult controls and high dose males showed no differences between groups.

No other effects were seen on adult male or female organ weights (Table 2).

**Testicular gene expression**

At PD 16, *Cyp19a1* expression was significantly lower in testes from all exposure groups compared to controls (Fig. 2a). There was a tendency towards highest levels of exposure showing lowest level of expression; relative mRNA levels were 33%, 30% and 23% that of control group for 10, 100 and 500 mg/kg bw/day groups, respectively. Expression level of several cell-specific marker genes was unchanged at PD16 following exposure to butylparaben. The germ cell marker *Ddx4* was unchanged in all groups, suggesting that germ cell numbers were unchanged (Fig. 2a). The Sertoli cell markers *Sox9* and *Fshr* showed stable expression across groups, indicating that, at least Sertoli cell numbers were unaffected by exposure (Fig. 2a). So too, the Sertoli/Leydig cell marker *Nr5a1* and *Ar* were unchanged, as were all the Leydig cell markers *Lhr*, *Cyp11a1*, *Cyp17a1* and *Hsd3b1* (Fig. 2a).

In adult testes, *Cyp19a1* expression was similar in exposed and control animals. All other marker genes were also unchanged except *Nr5a1*, which was lower than controls in all exposed groups (Fig. 2b).

**Histological examination of prostate**

In ventral prostates of prepubertal males, morphometric analysis showed that the epithelial area (Fig. 4A) and the ratio between epithelium and lumen (Fig. 4B) were decreased in the middle dose group compared to controls. At this age, most prostates had small acini with columnar epithelium and an abundant amount of secretory material in the apical part of the cells (Fig. 4C), whereas animals with a low ratio between epithelium and lumen had an increased incidence of large acini with cuboidal epithelium (Fig. 4D).

Adult ventral prostate histology differed markedly between individuals, but this was not dose related. Several animals showed variable degree of inflammation in the ventral prostate, consisting of focal or diffuse accumulation of
mononuclear cells. We observed no difference between groups in total numbers of animals with interstitial inflammation (5 out of 16 controls and 7 out of 18 high dose animals), nor between groups in scores for degree of inflammation (data not shown). In some cases, the epithelium adjacent to inflammatory foci showed minor reactive hyperplasia with cellular atypia (4 out of 16 controls and 6 out of 18 high dose animals). There was no difference between groups in scores for epithelial height, and morphometric analysis of the ratio between luminal area and epithelial area showed no differences between dose groups at PD 90 (data not shown).

Dorsolateral prostate showed a much lower incidence of inflammatory foci than ventral prostate, with small inflammatory foci only seen in 2 controls and 1 high dose animal. Dark granules in epithelial cell cytoplasm or nuclei were seen in several areas of dorsolateral prostate for some animals, but no difference in incidence was seen between dose groups (7 out of 16 controls and 5 out of 18 high dose animals affected).

Mammary gland development and histology in female offspring

Prepubertal mammary glands were affected by perinatal exposure to butylparaben. At PD 22, female mammary glands showed a significantly higher number of terminal end buds (TEBs) in the two highest dose groups compared to controls (Fig. 4F-H). Indications of increased outgrowth towards the lymph node was seen, as the distance between mammary tissue and lymph node was significantly reduced in females exposed to 100 mg/kg bw/day of butylparaben compared to control females (Fig. 4E).

No clear effect of butylparaben was seen on mammary glands of adult female offspring. However, more females displayed delayed differentiation of the adult mammary glands and increased proliferation of duct epithelium in the lowest dose group compared to controls, but this was not statistically significant. Five out of 13 females from the low dose group had less developed lobules (lobules smaller than type 1) compared to 1 of 14 females from the control group (p=0.08 in 2x2 Fisher). Three out of 13 females had intraductal hyperplasia (>30% of tissue) in the low dose group, whereas no controls had intraductal hyperplasia (p=0.1 in 2x2 Fisher) (Fig. 4I-J). No sign of secretory activity or changes in tubuloalveolar morphology of the lobules were observed.

Histological examination of ovaries

The histological examination of ovaries from adult controls and high dose females showed apparently healthy ovaries with presence of all stages of follicles and with an abundant number of corpora lutea (data not shown).
Serum hormone levels

Serum estradiol levels were examined at PD 16 in males and at PD 22 in females, i.e. time points when we observed effects that may be related to altered estradiol production (reduced aromatase gene expression in testes and increased female mammary outgrowth). No effects of treatment were observed at these time points.

Discussion

Butylparaben is known to affect sperm count in rat offspring, but until now knowledge on other reproductive endpoints has been lacking. In this study we found endocrine disrupting effects in both male and female offspring after perinatal exposure of rats, including shortened AGD, reduced sperm count and reproductive organ weight, disrupted gene expression in the testes, and abnormal mammary gland development. Of particular importance for risk assessment is the observation that the sperm count was lower at all doses, starting at 10 mg/kg.

Mechanisms of actions for effects on the male reproductive system

We observed a reduction in epididymal sperm count, which corroborates previous findings in rat offspring following subcutaneous exposure of dams to 100 and 200 mg/kg bw/day of butylparaben (Kang et al., 2002), or oral exposure to 400 and 1000 mg/kg bw/day (Zhang et al., 2014). Table 3 summarizes findings in rat studies with perinatal exposure to butylparaben or isobutylparaben. Zhang et al., 2014, did not see any changes in sperm counts at the dose levels applied in the current study, although rat strain and study design were comparable. To some extent, our findings of effects at relatively low oral doses are supported by a study showing reduced sperm count and motility in rats perinatally exposed to 2.5 mg/kg bw/day of the structurally related isobutylparaben (Yang et al., 2015).

In the current study, a shortened neonatal male AGD was seen from 100 mg/kg bw/day, but the same was only seen at doses from 400 mg/kg bw/day of butylparaben in the study by Zhang et al., 2014, and not in the study by Kang et al., 2002 (see Table 3). In a previous study we showed that subcutaneous exposure of pregnant dams to 200 or 400 mg/kg bw/day of butylparaben did not affect AGD or testicular testosterone synthesis in fetal male rats (Taxvig et al., 2008).

Shortened male AGD is often considered an indicator of anti-androgenicity, fitting with the known anti-androgenic effects of butylparaben in vitro (Satoh et al., 2005; Chen et al., 2007). Notably, the present study showed a shallow dose-response relationship between butylparaben exposure and male AGD with a maximum decrease of only 7%. A comparably shallow dose-response curve was seen in a recent study on bisphenol A (Christiansen et al., 2014).
contrast, no change in male AGD was seen after perinatal exposure to the potent estrogen ethinyl estradiol (Mandrup et al., 2013). These findings may suggest that some chemicals with a common profile of estrogenic and weakly anti-androgenic effects only induce subtle effects on AGD.

To gain further insight into potential mechanisms behind the effects on male reproductive development, we analyzed the expression of several genes in prepubertal and adult testes. Several cell-specific marker genes were unchanged both at PD16 and adulthood, suggesting that the general testis cellularity was maintained. However, at PD16 the expression of Cyp19a1 (aromatase) was lower in all dose groups compared to controls. CYP19 catalyzes the conversion of androgens into estrogens (Simpson et al., 2002), and at this stage aromatase is expressed by immature rat Sertoli cells and is stimulated by FSH to elevate serum estradiol for a few days (Picut et al., 2015). The role of estradiol at this age is not clear, but the increase in estradiol coincides with the first wave of spermatogenesis and establishment of the blood-testis barrier. Therefore, it may be speculated that the reduction of aromatase levels at this stage is related to the observed low sperm count later in life. Alternatively, our findings may reflect a slight delay in development of the exposed rats. Although butylparaben did not appear to affect estradiol synthesis or secretion at this age, it would be interesting to further scrutinize possible effects of butylparaben on testicular aromatase levels and estradiol production.

In adult testes we observed a reduction in Nr5a1 expression in exposed animals. Nr5a1 encodes a nuclear receptor commonly called Steroidogenic Factor-1 (SF-1), which is involved in the regulation of numerous genes, including several steroidogenic factors (Buaas et al., 2012). Thus, reduced NR5A1 could help explain alterations in hormone levels observed in adulthood in the study by Zhang et al. (2014). Actually, albeit not statistically significant, Cyp17a1 showed a slightly lower expression in the same testes displaying reduced Nr5a1 levels, which is in line with the known involvement of NR5A1 in the transcriptional regulation of several CYP genes, including Cyp17. Therefore, butylparaben exposure could be suggested to compromise reproductive function by interfering with steroidogenesis. However, butylparaben had no or limited effects on estradiol or testosterone levels adrenal H295R cells (Taxvig et al., 2008).

The observed reduction in prostate and seminal vesicle weight in adult animals may also be a consequence of altered hormone production early or late in life. Indications of early changes in prostate development were seen as the relative epithelial area in PD 16 prostates was reduced at 100 mg/kg bw/day compared to controls, but we found no histological changes in prostates of young adults after butylparaben exposure. It is well known that developmental exposure to other estrogenic chemicals or elevated perinatal levels of estrogens in rodents can induce atypical hyperplasia of the prostatic
epithelium (PIN lesions) and morphological changes resembling those of precancerous lesions in humans (reviewed by Prins et al., 2007). Focal proliferative effects are, however, unlikely to be reflected by prostate weights. But neonatal exposure to high doses of estradiol benzoate in rats was found to induce prostatic PIN lesions and reduced dorsal prostate weight later in life (Ho et al., 2006). Therefore, despite being indicative, our data on prostate weights alone does not allow us to conclude whether proliferative effects were induced by butylparaben or not, nor does it lend enough insight to elucidate possible effects of parabens on prostate cancer susceptibility. For this, further examination in older animals or in a cancer induction model is necessary.

Altered female reproductive development

The fact that prepubertal female rats exposed to butylparaben displayed increased outgrowth of mammary glands and a greater number of terminal end buds (TEBs) supports an estrogenic mode of action. Similarly, an increase in mammary gland outgrowth in female rats has been seen with perinatal exposure to ethinyl estradiol or a mixture of estrogenic chemicals (Mandrup et al., 2012; Mandrup et al., 2015), and with prepubertal exposure to the phytoestrogen genistein (Murill et al., 1996). Early postnatal exposure to phytoestrogens or bisphenol A can increase the number of TEBs in prepubertal rats (Tan et al., 2004, Moral et al., 2008). In our previous study on ethinyl estradiol, we observed no change in TEB numbers in females, but an increase in male TEB numbers (Mandrup et al., 2012). TEBs are the site of origin of mammary carcinomas and a target structure of carcinogens (Russo et al., 1979; Russo and Russo, 1996b), and a link between chemically induced increases in TEB numbers and an increased risk of mammary cancer has been proposed (Fenton, 2006). In adult mammary gland, possible effects on intraductal hyperplasia and reduced lobular development were observed at 10 mg/kg bw/day, but the low incidence of intraductal hyperplasia and the lack of effect at higher doses complicate the interpretation of these findings. Interestingly, intraductal hyperplasia has also been observed at low doses of bisphenol A (Murray et al., 2007, Durando et al., 2007), emphasizing that some endocrine disruptors may induce proliferative changes in the mammary ducts at low, but not high doses (Murray et al., 2007, Vandenberg et al., 2013, Mandrup et al. submitted). It may be speculated that the observed changes in early mammary development may be related to adverse effects later in life, but further studies are needed to clarify this.

The observed reduction in prepubertal ovary weight is in agreement with a study on neonatal rats showing delayed follicular recruitment at PD 8 after 7 days of subcutaneous exposure to propyl- and butylparaben (Ahn et al., 2012). In our study, adult ovaries appeared normal, but it may be speculated that a possible interference with early follicle recruitment may have long-term adverse effects manifesting as altered reproductive function later in life. Currently, few
studies have targeted the influence of chemicals on early ovarian development. One example of altered early ovary
development with late-life consequences following exposure to a weak estrogen is the finding of inhibited germ cell
nest breakdown on PND 4 and an early decline in fertility in mice prenatally exposed to low doses of bisphenol a
(Wang et al., 2014).

Few studies have reported changes in female AGD at birth, but we observed a slight reduction in female pup AGD
(corrected for body weight changes) following butylparaben exposure in this study and with bisphenol A exposure in a
previous study (Christiansen et al., 2014). In contrast, ethinyl estradiol increased the AGD and the number of retained
nipples of female offspring (Mandrup et al., 2013). Moreover, prochloraz exposure in utero increased female AGD in
several studies (Laier et al., 2006; Melching-Kollmuss et al., 2016). Different effect patterns thus appear for weak and
strong estrogens, but little is known regarding the relevance of these findings and whether such disruptions to endocrine
homeostasis can predict any late life reproductive dysgenesis in females.

Implications for risk assessment

We found that both prepubertal aromatase expression and adult sperm count were affected at the lowest administered
dose of butylparaben. This corresponds to the dose level previously shown to affect sperm count following juvenile
dietary exposure in rats (Oishi 2001), but disagreeing with two other studies on butyl- and propylparaben, respectively
(Hoberman et al., 2008, Gazin et al., 2013). Due to these discrepancies, it has been difficult to determine a robust no-
adverse effect level (NOAEL) for risk assessment. The Scientific Committee of Consumer Safety applied a no-effect
level (NOEL) of 2 mg/kg bw/day in their risk assessment of butylparaben (SCCS 2013). This was based on the lack of
effect on epididymis following juvenile subcutaneous exposure (Fisher et al. 1999). In their opinion, SCCS considered
that this starting point for risk assessment was a “conservative choice”. However, the current data suggest that a
NOAEL of 2 mg/kg bw/day is not conservative, as the observed reductions of epididymal sperm count and testicular
aromatase expression at 10 mg/kg bw/day points to a NOAEL at or possibly below 2 mg/kg bw/day. This is further
corroborated by the recent finding of reduced sperm count and motility in rats perinatally exposed to 2.5 mg/kg bw/day
of the structurally related isobutylparaben (Yang et al., 2015).

The SCCS discussed that rats may be a poor model for assessing and extrapolating potential harmful effects of
butylparaben in humans, as rats have a much greater metabolic rate. For instance, internal exposure levels of free
parabens measured in human males exposed dermally to 10 mg/kg bw/day were similar to internal exposure of rats
exposed orally to 1000 mg/kg bw/day (Appendix 2 of SCCS 2013). Therefore, the SCCS noted that when using toxicokinetic data, it is uncertain whether the desired margin of safety of 25 can be achieved and thus, that uncertainties with risk assessment remain which presently cannot be resolved (SCCS 2013). Notably, blood levels of parabens do not directly reflect levels in solid tissues. Examination of tissue distribution of C14-labelled butylparaben and metabolites in rats showed higher tissue concentrations than in blood for oral, intravenous, as well as dermal exposure routes (Mathews et al., 2013). Furthermore, fetal exposure levels may exceed maternal blood levels (Frederiksen et al., 2008). Therefore, a concern remains that the developing human may not be sufficiently protected against some of the multiple endocrine disrupting effects of butylparaben, either alone or in combination with other endocrine disrupting chemicals.

In summary, butylparaben altered AGD, ovary and prostate weights, sperm counts and mammary development, and thus exerted endocrine disrupting effects on both male and female offspring. Interestingly, gene expression profiling of testis indicated that some of the male reproductive effects may be related to changes in steroidogenesis.

Acknowledgements:

This study was part of a large project funded by the Centre on Endocrine Disrupters in Denmark and the Danish Environmental Protection 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 Sarah Grundt Simonsen, Ulla El-Baroudy, Vibeke Kjær, Heidi Letting, Lillian Sztuk, Dorte Lykkegaard Korsbech, Birgitte Plesning, Bo Oscar Herbst, Gülcan Geyik, and Anne Ørngreen & Co-workers from the animal facility.
Reference List


Table 1. Pregnancy and litter data of dams and offspring exposed to 0, 10, 100 or 500 mg butylparaben/kg bw/day from GD 7 to PD 22. Data represent group means based on litter means ± SD.

<table>
<thead>
<tr>
<th>Dams and litters</th>
<th>Control</th>
<th>10 mg BP</th>
<th>100 mg BP</th>
<th>500 mg BP</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of viable litters</td>
<td>n=16</td>
<td>n=13</td>
<td>n=15</td>
<td>n=17</td>
</tr>
<tr>
<td>Dam BW gain GD 7-21 (g)</td>
<td>78.73 ±16.6</td>
<td>81.04 ± 17.0</td>
<td>86.13 ± 11.2</td>
<td>75.34 ± 19.2</td>
</tr>
<tr>
<td>Dam BW gain GD 7-PND 1 (g)</td>
<td>15.67 ± 7.6</td>
<td>13.46 ± 7.9</td>
<td>13.83 ± 10.7</td>
<td>9.50 ± 10.0</td>
</tr>
<tr>
<td>Dam BW gain PND 1-17 (g)</td>
<td>31.56 ± 12.3</td>
<td>39.08 ± 1.3</td>
<td>37.33 ± 11.5</td>
<td>36.65 ± 12.7</td>
</tr>
<tr>
<td>Gestation length (days)</td>
<td>22.94 ± 0.3</td>
<td>23.00 ± 0.0</td>
<td>23.0 ± 0.0</td>
<td>23.03 ± 0.3</td>
</tr>
<tr>
<td>% postimplantation loss</td>
<td>8.98 ± 11.2</td>
<td>6.83 ± 6.0</td>
<td>8.9 ± 11.7</td>
<td>5.92 ± 10.6</td>
</tr>
<tr>
<td>% perinatal loss</td>
<td>13.48 ± 17.3</td>
<td>7.37 ± 7.0</td>
<td>8.90 ± 11.7</td>
<td>7.39 ± 12.2</td>
</tr>
<tr>
<td>Litter size</td>
<td>10.00 ± 3.5</td>
<td>11.92 ± 1.9</td>
<td>10.6 ± 3.1</td>
<td>10.47 ± 2.9</td>
</tr>
<tr>
<td>% perinatal deaths</td>
<td>4.50 ± 16.1</td>
<td>0.64 ± 2.3</td>
<td>0.0 ± 0.0</td>
<td>1.67 ± 5.1</td>
</tr>
<tr>
<td>% males</td>
<td>47.45 ± 23.8</td>
<td>49.97 ± 11.5</td>
<td>56.45 ± 12.8</td>
<td>50.45 ± 17.8</td>
</tr>
</tbody>
</table>

Offspring

| Mean male birth weight (g) | 6.41 ± 0.4 | 6.45 ± 0.4 | 6.62 ± 0.3 | 6.31 ± 0.6 |
| AGD males (mm)             | 3.96 ± 0.1 | 3.98 ± 0.1 | 3.77 ± 0.2** | 3.69 ± 0.3*** |
| AGDI males (mm/ g$^3$)     | 2.13 ± 0.1 | 2.14 ± 0.1 | 2.01 ± 0.1*** | 2.00 ± 0.1*** |
| Mean female birth weight (g)| 6.13 ± 0.3 | 6.04 ± 0.4 | 6.29 ± 0.3 | 5.98 ± 0.6 |
| AGD females (mm)           | 2.22 ± 0.1 | 2.16 ± 0.1 | 2.07 ± 0.1** | 2.01 ± 0.2*** |
| AGDI females (mm/g$^3$)    | 1.22 ± 0.0 | 1.19 ± 0.1 | 1.12 ± 0.1** | 1.11 ± 0.1*** |
| Nipples (areolas) males    | 0.27 ± 0.35 | 0.15 ± 0.28 | 0.33 ± 0.39 | 0.54 ± 0.72 |
| Nipples (areolas) females  | 12.3 ± 0.78 | 12.3 ± 0.3 | 12.3 ± 0.28 | 12.3 ± 0.25 |
| Mean body weight PD 6 (g)  | 13.2 ± 1.6 | 12.4 ± 1.1 | 12.8 ± 1.4 | 12.2 ± 1.4 |
| Mean body weight PD14 (g)  | 28.6 ± 4.0 | 26.1 ± 2.5 | 26.1 ± 4.2 | 26.6 ± 3.6 |
| Mean body weight PD 24 (g) | 48.3 ± 6.7 | 44.0 ± 4.5 | 46.2 ± 6.6 | 45.8 ± 6.1 |
| Age at vaginal opening (VO)| 31.3 ± 2.8 | 33.1 ± 3.1 | 32.5 ± 2.4 | 33.2 ± 2.9 |
| Body weight at VO          | 78.1 ± 13  | 83.7 ± 14  | 80.8 ± 15  | 84.3 ± 13  |
| Age at preputial separation (PPS)| 43.6 ± 1.4 | 44.1 ± 1.7 | 44.3 ± 1.9 | 44.7 ± 2.0 |
| Body weight at PPS         | 144 ± 12   | 148 ± 11   | 153 ± 13   | 155 ± 16   |
Table 2. Body and organ weights in rat offspring following perinatal exposure to butylparaben. Asterisks indicate statistically significant difference from control group. *: p<0.05, **: p<0.01, ***: p<0.001.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Butylparaben 10</th>
<th>Butylparaben 100</th>
<th>Butylparaben 500</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PD 16 males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>12</td>
<td>13</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>32 ± 5.0</td>
<td>30 ± 2.6</td>
<td>29 ± 4.4</td>
<td>30 ± 5.1</td>
</tr>
<tr>
<td>Liver (mg)</td>
<td>848 ± 167</td>
<td>796 ± 104</td>
<td>748 ± 142</td>
<td>805 ± 154</td>
</tr>
<tr>
<td>Retroperitoneal fat pad (mg)</td>
<td>49.7 ± 21.5</td>
<td>45.6 ± 10.9</td>
<td>42.5 ± 14.8</td>
<td>47.9 ± 18.4</td>
</tr>
<tr>
<td>Right testis (mg)</td>
<td>54.0 ± 9.3</td>
<td>56.4 ± 8.1</td>
<td>52.3 ± 8.6</td>
<td>54.0 ± 9.6</td>
</tr>
<tr>
<td>Left testis (mg)</td>
<td>55.6 ± 8.4</td>
<td>57.9 ± 7.9</td>
<td>52.9 ± 9.5</td>
<td>54.4 ± 9.5</td>
</tr>
<tr>
<td>Ventral prostate (mg)</td>
<td>12.5 ± 2.7</td>
<td>12.0 ± 2.4</td>
<td>12.2 ± 3.7</td>
<td>12.4 ± 2.8</td>
</tr>
<tr>
<td>Seminal vesicle (mg)</td>
<td>7.93 ± 2.79</td>
<td>8.60 ± 2.16</td>
<td>9.32 ± 2.96</td>
<td>8.36 ± 1.87</td>
</tr>
<tr>
<td>Epididymis (mg)</td>
<td>24.7 ± 3.9</td>
<td>24.8 ± 3.3</td>
<td>24.4 ± 4.2</td>
<td>25.2 ± 3.8</td>
</tr>
<tr>
<td>LABC (mg)</td>
<td>27.7 ± 3.6</td>
<td>30.1 ± 5.7</td>
<td>29.8 ± 7.9</td>
<td>32.5 ± 8.8</td>
</tr>
<tr>
<td>Bulbourethral gland (mg)</td>
<td>1.67 ± 0.37</td>
<td>2.02 ± 0.65</td>
<td>1.99 ± 0.48</td>
<td>1.95 ± 0.37</td>
</tr>
<tr>
<td>Adrenal (mg)</td>
<td>10.1 ± 2.9</td>
<td>9.5 ± 0.9</td>
<td>9.0 ± 2.2</td>
<td>9.8 ± 2.0</td>
</tr>
<tr>
<td><strong>PD 17 females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>14</td>
<td>13</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>34 ± 4.6</td>
<td>30 ± 3.9</td>
<td>31 ± 4.2</td>
<td>32 ± 5.0</td>
</tr>
<tr>
<td>Liver (mg)</td>
<td>931 ± 143</td>
<td>819 ± 112</td>
<td>843 ± 128</td>
<td>867 ± 173</td>
</tr>
<tr>
<td>Retroperitoneal fat pad (mg)</td>
<td>38.1 ± 12.0</td>
<td>29.5 ± 13.1</td>
<td>31.7 ± 13.8</td>
<td>37.0 ± 14.8</td>
</tr>
<tr>
<td>Right ovary (mg)</td>
<td>3.58 ± 0.68</td>
<td>3.29 ± 0.63</td>
<td>2.91 ± 0.72</td>
<td>2.72 ± 0.65**</td>
</tr>
<tr>
<td>Left ovary (mg)</td>
<td>3.93 ± 0.61</td>
<td>3.45 ± 0.54</td>
<td>3.21 ± 0.66*</td>
<td>3.08 ± 0.60***</td>
</tr>
<tr>
<td>Pooled ovary weight (mg)</td>
<td>7.51 ± 1.19</td>
<td>6.74 ± 1.02</td>
<td>6.13 ± 1.27*</td>
<td>5.80 ± 1.21***</td>
</tr>
<tr>
<td>Thyroid (mg)</td>
<td>5.14 ± 1.01</td>
<td>5.34 ± 1.42</td>
<td>5.71 ± 1.70</td>
<td>5.45 ± 1.94</td>
</tr>
<tr>
<td><strong>PD 22 males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>11</td>
<td>12</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>45 ± 6.7</td>
<td>46 ± 4.6</td>
<td>46 ± 4.0</td>
<td>47 ± 6.0</td>
</tr>
<tr>
<td>Right testis (mg)</td>
<td>116 ± 14</td>
<td>125 ± 17</td>
<td>124 ± 11</td>
<td>124 ± 20</td>
</tr>
<tr>
<td>Left testis (mg)</td>
<td>116 ± 15</td>
<td>125 ± 16</td>
<td>125 ± 12</td>
<td>124 ± 19</td>
</tr>
<tr>
<td>Prostate (mg)</td>
<td>30.8 ± 3.9</td>
<td>31.5 ± 3.5</td>
<td>34.3 ± 5.7</td>
<td>34.5 ± 5.0</td>
</tr>
<tr>
<td><strong>PD 22 females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>14</td>
<td>13</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>45 ± 5.5</td>
<td>44 ± 4.4</td>
<td>46 ± 4.9</td>
<td>43 ± 3.8</td>
</tr>
<tr>
<td>Right ovary (mg)</td>
<td>7.59 ± 0.95</td>
<td>7.90 ± 1.20</td>
<td>8.28 ± 0.81</td>
<td>8.48 ± 1.30</td>
</tr>
<tr>
<td>Left ovary (mg)</td>
<td>7.76 ± 1.37</td>
<td>7.70 ± 0.99</td>
<td>8.01 ± 0.86</td>
<td>8.37 ± 1.24</td>
</tr>
<tr>
<td><strong>PD 80-90 males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>16</td>
<td>14</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>339 ± 26.8</td>
<td>327 ± 23.5</td>
<td>333 ± 27.8</td>
<td>336 ± 33.7</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>10.9 ± 1.1</td>
<td>10.7 ± 1.1</td>
<td>10.8 ± 1.5</td>
<td>10.6 ± 1.2</td>
</tr>
<tr>
<td>Retroperitoneal fat pad (g)</td>
<td>2.09 ± 0.6</td>
<td>1.69 ± 0.60</td>
<td>1.87 ± 0.48</td>
<td>2.17 ± 0.93</td>
</tr>
<tr>
<td>Right testis (g)</td>
<td>1.75 ± 0.17</td>
<td>1.70 ± 0.11</td>
<td>1.81 ± 0.20</td>
<td>1.77 ± 0.15</td>
</tr>
<tr>
<td>Left testis (g)</td>
<td>1.79 ± 0.15</td>
<td>1.76 ± 0.15</td>
<td>1.86 ± 0.19</td>
<td>1.82 ± 0.15</td>
</tr>
<tr>
<td>Ventral prostate (g)</td>
<td>0.51 ± 0.08</td>
<td>0.47 ± 0.10</td>
<td>0.48 ± 0.10</td>
<td>0.44 ± 0.08*</td>
</tr>
<tr>
<td>Seminal vesicle with prostate (g)</td>
<td>2.29 ± 0.31</td>
<td>2.12 ± 0.31</td>
<td>2.21 ± 0.32</td>
<td>2.00 ± 0.20*</td>
</tr>
<tr>
<td>LABC (g)</td>
<td>0.965 ± 0.161</td>
<td>0.945 ± 0.097</td>
<td>0.939 ± 0.120</td>
<td>0.892 ± 0.082</td>
</tr>
<tr>
<td>Tissue</td>
<td>15</td>
<td>14</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>----------------------------</td>
<td>----------</td>
<td>----------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td>Bulbourethral gland (g)</td>
<td>0.107 ± 0.020</td>
<td>0.113 ± 0.017</td>
<td>0.098 ± 0.024</td>
<td>0.097 ± 0.020</td>
</tr>
<tr>
<td>Adrenal (g)</td>
<td>0.508 ± 0.040</td>
<td>0.513 ± 0.043</td>
<td>0.547 ± 0.050</td>
<td>0.509 ± 0.045</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>6.57 ± 0.68</td>
<td>6.31 ± 0.59</td>
<td>6.60 ± 0.65</td>
<td>6.75 ± 0.68</td>
</tr>
<tr>
<td>Retroperitoneal fat pad (g)</td>
<td>1.14 ± 0.27</td>
<td>0.92 ± 0.27</td>
<td>1.00 ± 0.25</td>
<td>1.00 ± 0.25</td>
</tr>
<tr>
<td>Right ovary(g)</td>
<td>0.048 ± 0.009</td>
<td>0.043 ± 0.004</td>
<td>0.045 ± 0.004</td>
<td>0.046 ± 0.006</td>
</tr>
<tr>
<td>Left ovary (g)</td>
<td>0.045 ± 0.007</td>
<td>0.042 ± 0.004</td>
<td>0.049 ± 0.008</td>
<td>0.045 ± 0.007</td>
</tr>
<tr>
<td>Uterus (g)</td>
<td>0.396 ± 0.061</td>
<td>0.382 ± 0.059</td>
<td>0.404 ± 0.046</td>
<td>0.394 ± 0.061</td>
</tr>
<tr>
<td>Thyroid (g)</td>
<td>0.023 ± 0.009</td>
<td>0.018 ± 0.004</td>
<td>0.018 ± 0.003</td>
<td>0.018 ± 0.007</td>
</tr>
</tbody>
</table>
Table 3. Comparison of studies examining effects of perinatal exposure to butyl- or isobutyl paraben in rats. AGD: anogenital distance, GD: gestation day; PND: postnatal day; NOAEL: no-observed adverse effect level.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Study design</th>
<th>Effects on body weight, AGD, sperm count, sperm motility, pubertal onset</th>
<th>Remarks and other effects.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butylparaben</td>
<td>Subcutaneous exposure of Sprague Dawley rats from GD 6 to PND 20. 0, 100 or 200 mg/kg bw/day. Vehicle: DMSO. N=6 control litters, 8 BP litters. 5-7 per sex per litter killed at PND 21, 49, 70 and 90.</td>
<td>From 100 mg/kg: Reduced female pup body weight at PND 49, 70 and 90. Reduced epididymal sperm count and sperm motility at PND 90 (n=5). No effect on male or female AGD at PND 1 (0.05 mm accuracy). Early vaginal opening at 100 mg/kg only. No data on age at preputial separation. No NOAEL was obtained.</td>
<td>Reduction of sperm count to ≈50% of controls; same effect size at both doses. Reduced number of live pups from 100 mg/kg. Reduced pup survival to weaning at 200 mg/kg. Decreased weight of testes, seminal vesicles and prostate in 100 mg/kg dose group at some ages only.</td>
<td>Kang et al., 2002</td>
</tr>
<tr>
<td>Butylparaben</td>
<td>Oral exposure of Wistar rats from GD 7 to PND 21. 0, 64, 160, 400 or 1000 mg/kg bw/day. Vehicle: Corn oil. N=7-8 litters. 7-8 males per dose group were killed at PND 21, 35, 49, 90 and 180.</td>
<td>From 400 mg/kg: Reduced male pup body weight at several ages. Shortened male AGD on PND 1 and 21. Delayed preputial separation. Reduced epididymal sperm count and daily sperm production. No examination of sperm motility. NOAEL 160 mg/kg.</td>
<td>Slight reduction in sperm count at 160 mg/kg, but same effect size (reduction to 64% of controls) at 160 and 400 mg/kg. Male serum hormone levels: decreased testosterone, increased estradiol and progesterone, and increased/decreased LH and FSH depending on age and dose level starting from 400 mg/kg.</td>
<td>Zhang et al., 2014</td>
</tr>
<tr>
<td>Isobutylparaben</td>
<td>Oral exposure of Sprague Dawley rats from GD 6 to PND 21. 0 or 2.5 mg/kg bw/day (in a study including also bisphenol A). Vehicle: corn oil. N=3 litters. 8 males per dose groups (representing 3 litters) were killed at PND 70.</td>
<td>At 2.5 mg/kg: Reduced epididymal sperm count and sperm motility (n=5). No change in male pup body weight, male AGD or age at preputial separation. No NOAEL was obtained.</td>
<td>Small study, one dose of isobutylparaben only. Reduction of sperm count to ≈40% of controls. Reduced male serum estradiol at PND 70. No changes in testis or epididymis weight at PND 70.</td>
<td>Yang et al., 2015</td>
</tr>
<tr>
<td>Butylparaben</td>
<td>Oral exposure of Wistar rats from GD 7 to PND 21. 0, 10, 100 or 500 mg/kg bw/day. Vehicle: corn oil. N=18 litters. One male and one female pup per litter killed at each of three ages: PND 16-17, 22, and 80-90.</td>
<td>From 10 mg/kg: Reduced epididymal sperm count. From 100 mg/kg: Shortened male and female AGD. No effect on pup body weight, age at preputial separation or age at vaginal opening. No examination of sperm motility. No NOAEL was obtained.</td>
<td>Reduction of sperm count to 76-78% of controls; same effect size at all doses. Reduced ovary weight at PND 17 from 100 mg/kg, reduced prostate weight at PND 90 at 500 mg/kg, altered mammary gland development from 100 mg/kg. No effect on nipple retention of males.</td>
<td>Current study</td>
</tr>
</tbody>
</table>
Figure 1. Morphological effects in butylparaben-exposed rats. A, B) Perinatal exposure to 100
or 500 mg/kg bw/day of butylparaben caused shortened AGD in both male and female offspring
relative to control animals. C, D) Ovary weights were reduced at PD 17, but not at PD 22, following
perinatal exposure to 100 or 500 mg/kg bw/day of butylparaben. E, F) The weights of ventral
prostate, total seminal vesicle and prostate were reduced at PND 90 following perinatal exposure
to 500 mg/kg bw/day of butylparaben. Mean+SEM. *: p<0.05, **: p<0.01, ***: p<0.001. AGD was
analyzed with body weight as a covariate and litter as a random factor; organ weights were
analyzed with body weight as a covariate. n=13-17 litters per dose group.

Figure 2. Relative gene expression levels in PD16 and PD 90 testes from control and
butylparabene-exposed rats. A) At PD 16, relative expression levels of the germ cell marker
Ddx4, Sertoli cell markers Sox9 and Fshr, the Sertoli/Leydig expressed genes Ar and Nr5a1, and
Leydig cell markers Lhr, Cyp11a1, Cyp17a1 and Hsd3b11 were unchanged following exposure to
10, 100 or 500 mg/kg bw/day butylparaben. Expression of Cyp19a1 (aromatase) was significantly
lower in testes from all exposure groups at PD 16. B) In adult testes, the expression of the same
genes was unchanged except for Nr5a1, which was lower in testes from all exposed groups
compared to controls. Relative expression levels are shown with mean value of control testes set
to 1, with data normalized using the geometric mean of Rpl13a and Sdha (prepubertal, n=9-10;
adult, n=4; Mean ± SEM; *p<0.05).

Figure 3. Number of sperm per gram cauda in male rats exposed to 0; 10; 100 and 500 mg
butylparaben/kg bw/day from GD 7 to PD 22. Group mean values ± SD are shown, n = 13-17.
Sperm count is significantly lower, indicated by *** with p < 0.0001, in all dosed groups compared
to the control group.

Figure 4. Effects of perinatal butylparaben exposure of rats on early prostate and mammary
gland development. A, B) Morphometric analysis revealed a reduced epithelial area (as a
percentage of total prostate area) and reduced epithelium to lumen ratio in PD 22 male offspring
from the middle dose group. Data represents group means +SEM, n=11-14. Asterisks indicate
statistically significant differences from controls analyzed in ANOVA followed by Dunnett’s test, *:
p<0.05; **: p<0.01. C, D) Ventral prostate histology at PD 22, bars indicate 50 µm. Control animals
(C) had columnar epithelium in ventral prostate, whereas enlarged acini with reduced epithelial
height were seen mainly in the middle dose group (D). E) Outgrowth of female mammary glands,
measured as the distance from the mammary gland to nearest lymph node, was increased in
animals exposed to 100 mg/kg bw/day butylparaben. F) The number of terminal end buds in
females was increased at 100 and 500 mg/kg bw/day. Data points indicate measurements from
individual animals with lines representing group means. ** indicate a statistically significant
difference from controls with p<0.01 analyzed by Kruskal-Wallis’ nonparametric test followed by
image of a PD 22 control female mammary gland. H) Representative mammary whole mount
image of a PD 22 high dose female mammary gland with an increased number of Terminal End
Buds (TEB, black arrows). I) Mammary duct from a PD 90 control female. J) Intraductal
hyperplasia (double-headed arrow) in mammary gland from a PD 90 female from the low-dose
group.
Figure 1. Morphological effects in butylparaben-exposed rats. A, B) Perinatal exposure to 100 or 500 mg/kg bw/day of butylparaben caused shortened AGD in both male and female offspring relative to control animals. C, D) Ovary weights were reduced at PD 17, but not at PD 22, following perinatal exposure to 100 or 500 mg/kg bw/day of butylparaben. E, F) The weights of ventral prostate, total seminal vesicle and prostate were reduced at PND 90 following perinatal exposure to 500 mg/kg bw/day of butylparaben. Mean+SEM. *: p<0.05, **: p<0.01, ***: p<0.001. AGD was analyzed with body weight as a covariate and litter as a random factor; organ weights were analyzed with body weight as a covariate. n=13-17 litters per dose group.
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254x190mm (96 x 96 DPI)
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PD 22 control female mammary gland. H) Representative mammary whole mount image of a PD 22 high dose female mammary gland with an increased number of Terminal End Buds (TEB, black arrows). I) Mammary duct from a PD 90 control female. J) Intraductal hyperplasia (double-headed arrow) in mammary gland from a PD 90 female from the low-dose group.