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Grouping of endocrine disrupting chemicals for mixture risk assessment – Evidence from a rat study

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ABSTRACT

Exposure to mixtures of endocrine disrupting chemicals may contribute to the rising incidence of hormone-related diseases in humans. Real-life mixtures are complex, comprised of chemicals with mixed modes of action, and essential knowledge is often lacking on how to group such chemicals into cumulative assessment groups, which is an essential prerequisite to conduct a chemical mixture risk assessment.

We investigated if mixtures of chemicals with diverse endocrine modes of action can cause mixture effects on hormone sensitive endpoints in developing and adult rat offspring after perinatal exposure. Wistar rats were exposed during pregnancy and lactation simultaneously to either bisphenol A and butylparaben (Emix), diethylhexyl phthalate and procymidone (Amix), or a mixture of all four substances (Totalmix). In male offspring, the anogenital distance was significantly reduced and nipple retention increased in animals exposed to Amix and Totalmix, and the mixture effects were well approximated by the dose addition model. The combination of Amix and Emix responded with more marked changes on these and other endocrine-sensitive endpoints than each binary mixture on its own. Sperm counts were reduced by all exposures. These experimental outcomes suggest that the grouping of chemicals for mixture risk assessment should be based on common health outcomes rather than only similar modes or mechanisms of action. Mechanistic-based approaches such as the concept of Adverse Outcome Pathway (AOP) can provide important guidance if both the information on shared target tissues and the information on shared mode/mechanism of action are taken into account.

1. Introduction

Chemical exposure is suspected to contribute to a high prevalence of endocrine disorders in the Western world (WHO/UNEP, 2013). This calls for an improved understanding of how exposure to complex chemical mixtures in our everyday life may lead to adverse health effects such as hormone-dependent cancers, fertility problems and congenital malformations. Risk assessment of chemicals with mixed modes and mechanisms of action presents an unsolved challenge.

Over the last decades, our understanding of combined exposures to environmental chemicals and how to assess the humans and the environment has improved substantially (Kortenkamp and Faust, 2018). Consequently, scientific and regulatory focus has shifted from only performing risk assessments for an individual substance towards performing Mixture Risk Assessment (MRA), where the risk of combined exposures to multiple chemicals is assessed (Boberg et al., 2019; Bopp et al., 2018; EC European Commision, 2012; EFSA, 2019; Howdeshell et al., 2017).

There is clear experimental evidence that mixture effects can be anticipated for chemicals with similar mechanisms of action even at doses where each chemical is ineffective (Christiansen et al., 2012, 2009; Conley et al., 2018, 2016; Hass et al., 2012, 2007; Metzdorff et al., 2007; Silva et al., 2002). Experiments show that cumulative effects of chemicals with the same molecular initiating events (e.g. AR antagonism) follow the dose-addition principles (Christiansen et al., 2008; Hannas et al., 2011; Hass et al., 2007; Howdeshell et al., 2015, 2008b, 2007; Metzdorff et al., 2007; Rider et al., 2009). Additionally, dose-additive effects on androgen sensitive endpoints have been

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demonstrated after exposure to anti-androgenic compounds with different mechanisms of action (Christiansen et al., 2009; Conley et al., 2018) and even for multi-causal effects such as reduced birth weight (Hass et al., 2017). This evidence for mixture effects of chemicals with different mechanisms of action implies that mixture risk assessment should go beyond grouping based on shared mechanisms of action.

In the present paper, we propose to group chemicals based on a common target tissue rather than common mechanisms of action. In line with this, EFSA and ECHA propose tiered strategies for component-based mixture risk assessment, where at the first tiers chemicals are grouped on basis of shared effects on target organs, and at more refined tiers chemicals are grouped based on mode or mechanism of action (ECHA, 2017; EFSA, 2019, 2013).

In developmental and reproductive toxicity studies, commonly used predictive endpoints for male reproductive disorders are the anogenital distance (AGD) and nipple retention (NR). Both are considered androgen-sensitive and are expected to be primarily affected by anti-androgens (Christiansen et al., 2008; Schwartz et al., 2019). Other endpoints such as sperm count and development of testis, mammary gland and prostate can be influenced via both androgen and estrogen sensitive pathways.

Importantly, numerous EDCs act via more than one endocrine mechanism of action, thus the focus on common outcomes at first tiers avoids the complicated task to “classify” chemicals by molecular mechanisms.

We propose that four EDCs with mixed modes/mechanisms of action will act additively on endocrine sensitive endpoints. Two anti-androgens, di-(2-ethylhexyl) phthalate (DEHP) and procymidone, are well known to reduce both male anogenital distance (AGD) and sperm count, and our recent studies showed similar effects of BPA and butylparaben (Fig. 1, Boberg et al., 2016; Christiansen et al., 2014; Hass et al., 2016). Additive effects on these and other endocrine sensitive target organs may occur via a shared network of adverse outcome pathways (AOP) as illustrated in Fig. 2. In a recent paper, (Kortenkamp, 2020) also suggests to apply information on adverse outcome pathways for grouping of chemicals affecting the male reproductive system.

In a proof of principle study, we, therefore, examined male reproductive toxicity targets in rats perinatally exposed to mixtures of Bisphenol A (BPA), butylparaben, di-(2-ethylhexyl) phthalate (DEHP) and procymidone. These four compounds have been reported to act via different endocrine modes of action, as DEHP reduces testosterone production during critical stages of sex differentiation in male rat fetuses (Foster et al., 2006; Parks et al., 2000; Wilson et al., 2004), procymidone is an AR antagonist binding to mammalian androgen receptors (Hass et al., 2007; Ostby et al., 1999; Wolf et al., 1999), and BPA and butylparaben are best known for their estrogenic properties (Byford et al., 2002; Routledge et al., 1998; Wetherill et al., 2007).

A straightforward approach for the MRA of these four compounds would be to allocate them into two distinct assessment groups, with BPA and butylparaben as “estrogens” and BPA and DEHP as “anti-androgens”. In addition to the fact that both estrogens and androgens can affect male reproductive tissues, experimental evidence suggests that these compounds have more than one endocrine mechanism of action. For butylparaben, our finding of reduced male AGD and sperm count (Boberg et al., 2016) is in line with results from several other studies showing reduced sperm counts in developmentally exposed male rats (KANG et al., 2002; Yang et al., 2016; Zhang et al., 2014). However, there are also studies reporting no effects on AGD for butylparaben (Boberg et al., 2008; Guerra et al., 2016; KANG et al., 2002; Taxvig et al., 2008) Reductions in male AGD and sperm count in our BPA study (Christiansen et al., 2014; Hass et al., 2016) is not corroborated by other studies using similar doses (Ferguson et al., 2011; Howdeshell et al., 2008a; Takagi et al., 2004; Tinwell et al., 2002; Tyl et al., 2008)

At high doses, BPA is well-known to induce male reproductive toxicity in studies involving developmental exposure, however, at lower doses, the evidence is controversial, with considerable inter-study variation in quality and outcomes (EFSA, 2015). Moreover, numerous *in vitro* studies have demonstrated a weak anti-androgenic activity for BPA and butylparaben (Chen et al., 2007; Kjærstad et al., 2010; Reif et al., 2010; Rosenmai et al., 2014; Satoh et al., 2005).

In the current study we exposed pregnant and lactating rats to a binary mixture of DEHP and Procymidone (Amix), a binary mixture of BPA and butylparaben (Emix), and a combination of all four compounds (Totalmix) and we evaluated both early androgen-sensitive endpoints (AGD, nipple retention, reproductive organ weights) and late-life effects such as sperm count. Doses were selected based on our previous rat studies showing effects on a common adverse endpoint, male anogenital distance (AGD) (Boberg et al., 2016; Christiansen et al., 2010; Christiansen et al., 2014; Hass et al., 2012, 2007), see Fig. 1. The lowest dose of each compound corresponded to around 5% reduction in AGD in previous studies (Emix-low, Amix-low, Totalmix-low). The highest doses were twice the lowest dose.

Acknowledging that androgens play a key role not only in early life sexual development but also during puberty (Monosson et al., 1999), we wanted to investigate if exposure to the same mixtures of EDs both perinatally and during puberty would lead to an increased sensitivity for adverse effects later in life. This part of the study design was inspired by a study showing how adverse effects of perinatal BPA exposure in mice were exacerbated by additional pubertal BPA exposure (Rubin et al., 2017).

Overall, with this developmental study, we aimed to investigate:

1. Can estrogenic compounds enhance the effects of anti-androgenic substances?

2. Can the concept of dose-addition be used to describe the observed responses from the four-compound mixture, and for which endpoints?

3. Can historical single-compound data be used to predict dose-additive mixture responses?

4. Can a re-dosing of the animals during puberty influence adult health outcomes?

2. Materials and methods

2.1. Chemicals and mixtures

BPA (Bisphenol A) (purity > 99.5%, CAS no. 80–05-7), Butylparaben, (purity 99%, CAS no. 94–26-8, (no. W220302-1 kg)) and Procymidone (purity 99.9%, CAS no. 32809–16-8, from Fluka no. 36640–250 mg-R) were purchased from Sigma-Aldrich (Brøndby, Denmark). DEHP (purity 99%, CAS no. 117–81-7, from Merck no. 8.2174.1000) was purchased from VWR- Bie & Berntsen, (Herlev, Denmark). Corn oil purchased in glass bottles from Sigma-Aldrich (Brøndby, Denmark) was used as both negative control and vehicle. The mixture solutions used for dosing were stored in glass bottles at room temperature, protected from light, and continuously stirred during the dosing period.

For the chemical analysis of Procymidone, we obtained Acetonitrile, methanol, formic acid and 25% ammonium hydroxide, all of LC-MS grade, and ethyl acetate from Sigma Aldrich, Schneldorf, Germany. Isolute bulk C-18 sorbent was obtained from Biotage, Sweden, the sorbent was washed with acetonitrile and ethyl acetate and dried prior to use. Water was purified on a Milli Q system, Millipore Corporation, US. Stock solutions of Procymidon of 1 mg/ml were prepared in DMSO.

2.2. Animals and exposure

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

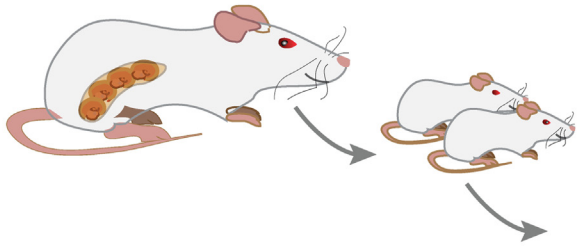
126 time-mated nulliparous, young adult Wistar rats (HanTac:WH,

a) In vivo studies on single compounds BPA, butylparaben, procymidone and DEHP were carried out using perinatal exposure.
All compounds induced reductions of male anogenital distance (AGD). Procymidone and DEHP induced nipple retention.
Data on individual substances were applied for dose selection in mixture study and for prediction modelling.

b) Three mixtures (Emix, Amix and Totalmix) were composed for testing at two doses (low, high) in Wistar rats:
- Low mixture dose: doses of individual compounds that were shown in previous studies to reduce male anogenital distance (AGD) by ca. 5%.
- High mixture dose: twice as large as the low mixture dose.

Mixture composition					
Dose group	BPA (mg/kg bw/day)	Butylparaben (mg/kg bw/day)	Procymidone (mg/kg bw/day)	DEHP (mg/kg bw/day)	Sum in mixture (mg/kg bw/day)
Control	-	-	-	-	**0**
Emix-low	0.25	100	-	-	**100.25**
Emix-high	0.5	200	-	-	**200.5**
Amix-low	-	-	10	30	**40**
Amix-high	-	-	20	60	**80**
Totalmix-low	0.25	100	10	30	**140.25**
Totalmix-high	0.5	200	20	60	**280.5**

c) Wistar rats were exposed to mixtures by oral gavage from gestation day 7 to pup day (PD) 22 (N=18 litters per dose group).



d) Re-dosing of a subset of pups from Control, Totalmix-low and Totalmix-high groups from PD 27-36 (females) or PD 38-47 (males), n=17-18 pups.

Examined endpoints

Dams: chemical analysis at gestation day 15.

Perinatally exposed offspring:
Examination of anogenital distance PD 1,
nipple retention PD 14, prostate gene expression PD 16,
reproductive organ weights PD 16, 22 and 80,
mammary development PD 22, puberty timing PD 38-50,
sperm count, testis and prostate histology PD 80.

Re-dosed offspring:
Examination of puberty timing PD 38-50,
reproductive organ weights,
sperm count, testis and prostate histology PD 80.

e) Dose-additive mixture effects on AGD and nipple retention were modelled for the 4-compound mixture from
(1) individual dose-response data of the compounds, and (2) mixture outcomes of Emix and Amix

Fig. 1. Overview of study. a) Individual compounds were examined in developing rats (see references in methods section), b) Mixtures were composed for the current study, c) Design of the current study examining effects of the specified mixtures, d) A subset of animals was re-dosed during puberty and e) Data on AGD and nipple retention were used for mixture modelling.

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 independently of the actual day of delivery, the expected day of delivery GD 23 was designated as pup day (PD) 1. The animals were housed in pairs until GD 17, and thereafter alone, under standard conditions in semi-transparent polysulfone (PSU) type III cages (PSU 80-1291HOOSU Type III, Tecniplast) (15 × 27 × 43 cm) with Aspen wood chip bedding (Tapvei, Gentofte, Denmark), Enviro Dri nesting material (Brogaarden, Lynge, Denmark) and Tapvei Arcade 17 (Aspen wood) shelters (Brogaarden, Lynge, Denmark). Environmental conditions were controlled with a 12-hour light–dark cycle with light intensity 500 lx starting at 9 pm, humidity 55% ± 5, temperature at 21 °C ± 1 °C and ventilation changing air 10 times per hr. All animals were fed a standard diet with ALTROMIN 1314 (soy- and alfalfa-free, ALTROMIN GmbH, Lage, Germany). Acidified tap water (to prevent microbial growth) in polysulfone bottles (84-ACBTO702SU Tecniplast) was provided *ad libitum*. The polysulfone bottles and cages, as well as the aspen wood shelters (instead of plastic), were used to reduce the risk of migration of BPA or other plasticisers that potentially could confound the study results.

The study was performed in three blocks (separated by one week), and all dose groups were equally represented in the blocks (n = 18). On the day after arrival (GD 4), dams were assigned to one of the seven exposure groups (see table in Fig. 1) by a stratified randomization scheme designed to achieve similar group mean body weights. A binary mixture of DEHP and Procymidone (Amix), a binary mixture of BPA and butylparaben (Emix), and a combination of all four compounds (Totalmix) were tested in the same study. Details about the exact mixture composition are given in a table in Fig. 1. The control group was exposed to vehicle (corn oil). Each of the three mixtures was investigated in two dose groups (low and high).

Low and high doses of the mixtures were selected based on AGD results in previous studies on single chemicals (Boberg et al., 2016; Christiansen et al., 2014, 2010; Hass et al., 2012; Hass et al., 2007). Low doses of each compound were found to induce reduced AGD by around 5% in these studies and high doses were twice the low doses. The two Total-mix doses equalled the sum of the low and high doses of Amix and Emix, respectively. This experimental design was considered as optimal for identifying potential effect modulations between both binary mixtures.

Exposure was conducted once daily via gavage with a stainless-steel probe 1.2 × 80 mm (Scanbur, Karlslunde, Denmark) at a constant volume of 2 ml/kg bw/day (the individual doses were based animal body weight on the day of dosing). Dams were dosed from GD 7 to PD 22 (day of delivery excluded). This exposure period was chosen to cover sensitive periods of reproductive development. On GD 15 dams were dosed first and then anesthetized with Hypnorm® (fentanyl citrate/flunisone)/Dormicum® (midazolam), and blood was drawn from the tail vein. Maternal plasma were stored at − 80 °C until analysis for levels of DEHP, Procymidon, Bisphenol A and butylparaben. Dams not giving birth were eliminated from the experiment.

At weaning of the offspring on PD 22, exposure to the test chemicals was stopped for most of the examined offspring. One to two males and females from each litter were kept for the examination of adult

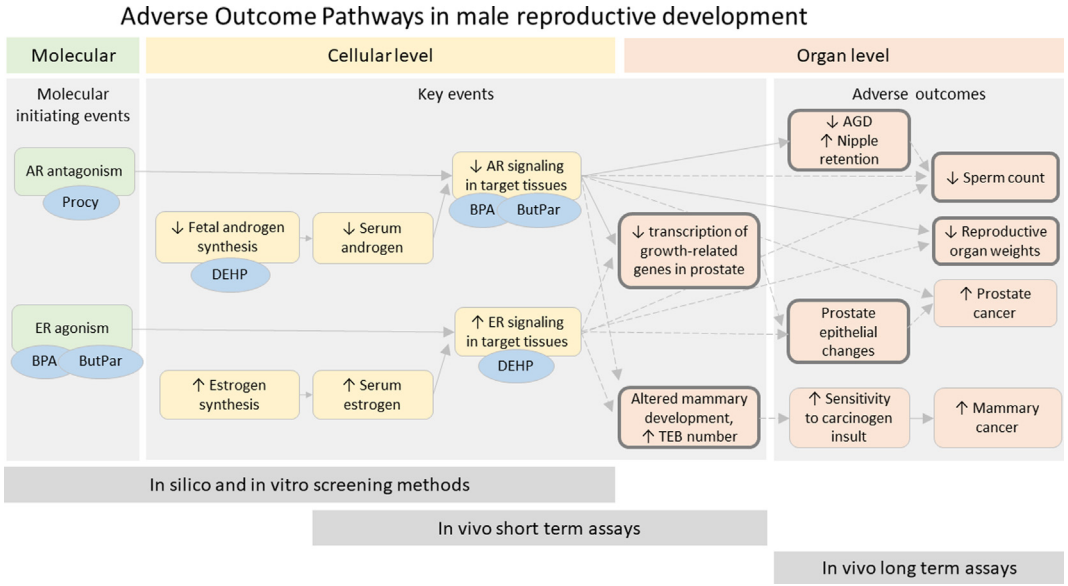


Fig. 2. Adverse outcome pathways in male reproductive toxicity. We propose that mixture risk assessment is based on information from all parts of the adverse outcome pathways so that substances for which only *in vitro* data is available are grouped with substances for which only *in vivo* effect data are available, when these data indicate effects on the same mode of action. This figure illustrates adverse outcome pathways leading to impaired fertility and increased risk of endocrine-sensitive cancers. Diverse classes of chemicals can influence these pathways, and the current study shows how four chemicals acting via different modes of action. The four chemicals are included in ovals indicating which effects are known to be exerted by each chemical. Combination of these four compounds leads to exaggerated effects on adverse outcomes. Boxes lined by fat lines indicate effects observed *in vivo* in the current study. The most marked effects were seen with combined exposure to all four compounds. ButPar = butylparaben, Procy = Procymidon.

reproductive endpoints (N = 15–17). An additional sub-cohort of weaned offspring from the control and both Totalmix groups (N = 15–17, 1–2 males and females from each litter) were re-dosed during *peri*-puberty, with females from PD 27–36 and males from PD 38–47, based on the timing of puberty in the two sexes. These groups are designated as Re-Control, Re-Totalmix-high, and Re-Totalmix-low.

2.3. In vivo examination

He 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.

On the day after delivery PD1, all pups were counted, sexed, weighed, checked for anomalies, and anogenital distance (AGD) was measured. AGD was measured as the distance between the genital papilla and the anus by a stereomicroscope with a micrometer eyepiece. The AGD index (AGDi) was calculated by dividing AGD by the cube root of the body weight. All offspring were weighed on PD 6. On PD 14, all male and female pups were weighed and examined for the 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. Normally, female rats have 12–13 nipples whereas male rats have none. All *in vivo* examinations were performed blinded with respect to the exposure group.

The onset of puberty was registered in all weaned male and female offspring. In female offspring sexual maturity was assessed by determining the day of vaginal opening (VO) as described by Goldman et al., (2000), starting examination from PD 27. In male offspring, the onset of puberty was assessed as the time of preputial separation (PPS) starting examination from PD 38. On the day of VO or PPS the age and weight of the animals were recorded.

2.4. Pup day 16–17 dissection

On PD 16, one male pup from each litter (N = 11–16) was weighed and decapitated. Male offspring were examined for testicular descent and reproductive organs were examined macroscopically for anomalies. The testes, epididymides, ventral prostate, seminal vesicles, levator ani/bulbo-cavernosus muscles (LABC), and bulbourethral glands were excised and weighed. Ventral prostate was placed in RNA later and frozen for gene expression analysis. On PD 17, one female pup per litter (N = 12–16) was weighed, decapitated and livers, ovaries, thyroid and retroperitoneal fat pad was excised and weighed.

2.5. Pup day 22 dissection

Dams and PD 22 pups were weighed and decapitated in CO2/O2 anaesthesia. In dams, the number of uterine implantations was counted. Testes and prostate were dissected and weighed from one male pup per litter. From one female pup per litter, ovaries were dissected and weighed, and mammary glands were collected for whole mount preparation.

From one male per litter testes and ventral prostate was excised and weighed, and mammary glands were collected for whole mount preparation. Mammary glands were excised, placed on a glass slide, stained with alum carmine. Whole mounts scanned on a flatbed scanner (4800 dpi) were evaluated for mammary outgrowth and extent of development on the digital images using Image Pro Plus 7.0 software (Media Cybernetics, Bethesda, Maryland) as described in Mandrup et al. (2015). The number of terminal end buds (TEBs) was counted (defined as tear-drop shaped buds in zone C with a diameter of 100 mm or more (Russo and Russo, 1996). Additionally, a mean of scores (1 to 5) given for each of the parameters area, number of buds, and number of branches, branch generations, and TEBs were calculated.

2.6. Dissection of adult offspring

PD 83–87, the adult male offspring were weighed and decapitated in CO2/O2 anaesthesia. Reproductive organs were examined macroscopically for anomalies. Weights of testes, liver, retroperitoneal adipose tissue, epididymis (alternately left and right), and seminal vesicle including coagulating glands and prostate were determined. In addition, the ventral prostate was weighed separately. Ventral and dorsolateral prostate were fixed in formalin before processing for paraffin

embedding. Tissue was sectioned (3 μm), stained with hematoxylin and eosin and examined by light microscopy (blinded with respect to treatment group). Histological examination of the prostate was performed on animals from the control, Re-Control and Re-Totalmix-high group, as well as from all high dose mixture groups (Amix-high, Emix-high and Totalmix-high). One section per animal was examined. The dorsolateral prostate was examined with an emphasis on inflammation and interstitial, intraluminal and total inflammation was scored 0–3, 0–4 and 0–4, respectively relative to the severity of inflammation. Ventral prostate was evaluated with a focus on inflammation, epithelial hyperplasia, and epithelial atrophy as described by Isling et al. (Isling et al., 2014).

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 previously (Jarfelt et al., 2005) using computer-assisted sperm analysis (CASA). A DNA-specific stain and fluorescence illumination was used to identify sperm cells. The average value of the three counts is presented as number of sperm per gram cauda.

At PD 80–90, all the females were sectioned when in estrous and examined macroscopically for external anomalies in the reproductive organs. Uterus, ovaries, retroperitoneal fat pads and liver were dissected and weighed.

2.7. Gene expression

On PD 16, prostates from 13 to 15 males/exposure group were isolated and stored in RNAlater (Qiagen, Hilden, Germany) at −80 °C. Total RNA was isolated using RNeasy MiniKit (Qiagen, Hilden, Germany), quantified on a NanoDrop 1000 Spectrophotometer, and cDNA synthesized with the Omniscript RT kit (Life Technologies Europe BV, Naerum, Denmark). The gene text is provided in the supplementary file (S-1).

2.8. Chemical analysis in serum from dams

Chemical content was analysed in plasma samples from dams at GD 15 (N = 6, pooled from 1 to 3 dams). The dams were dosed first and anesthetized and blood was drawn from the tail vein approximately an hour after dosing. The description of the chemical analysis is given in Supplementary (S-1).

2.9. Mixture modelling

The effects of the mixtures were estimated according to the principle of dose addition (DA) under the assumption of non-interaction between the compounds (Greco et al., 1995): the dose of the n-compound mixture causing *x*% effect (EDx_{mix}) is calculated from the *EDx* for each individual compound leading to the same response X and the relative dose contribution *pi* of each compound *i* to the mixture (Faust et al., 2003) as

$$EDx_{mix} = \left[\sum_{i=1}^{n}\left(\frac{p_i}{EDx_i}\right)\right]^{-1} \quad (1)$$

Accordingly, dose-additive mixture doses were calculated for all three mixtures on the basis of the individual compounds. The EDx for the four compounds were estimated from historical data obtained under similar testing conditions (Boberg et al., 2016; Christiansen et al., 2014, 2010; Hass et al., 2012, 2007) (Table S6). For a non-effective compound, the term p_i/EDx_i in Eq. (1) was set to zero If a compound did not reach an x% effect level the estimation of its corresponding effect dose EDx is not possible, and, consequently, an EDx_{mix} cannot be calculated for the mixture according to Eq1. In this case, we followed the dose extrapolation approach for DA predictions (Scholze et al., 2014) which provides a range of worst-case predictions for the mixture dose EDx_{mix}: the compound's p_i/EDx_i in Eq. (1) is assumed to be between zero (no contribution) and the highest value of p_i/EDx_i that can be derived according to Eq (1) at any of the lower mixture concentrations (maximal contribution). Consequently, the lower mixture response prediction corresponds to the "zero" contribution of the compound(s), and the higher response to the "maximal" contribution. In addition, we estimated from the dose–response data of the two binary mixtures (Amix, Emix) their effect doses which then were used as input data to calculate the expected effect dose for the four-compound mixture (Totalmix), i.e. the two binary mixtures were treated as two individual compounds in Eq. (1). It should be noted, that the binary mixtures were tested only at two doses, and consequently, the outcomes of their regression modelling should be considered with caution at doses outside the tested dose range.

To account for the statistical uncertainty in the DA prediction, a combined Monte-Carlo (MC) and nonlinear regression bootstrap simulation was conducted to establish approximate 95% confidence limits around the predicted mean mixture response: for each compound a distribution of resampled model fits was simulated by parametric bootstrap with resamples drawn from the fitted nonlinear (mixed) effect model (Efron and Tibshirani, 1993) which then was used as input for MC to generate a distribution of mixture prediction. Differences between predicted and observed mixture effects were deemed statistically significant when the 95% confidence belts of the prediction did not overlap with those of the experimentally observed mixture effects.

2.10. Statistical analysis

For all analyses, litter was the statistical unit. When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor. AGD and organ weights were analysed using body weight as covariate, and birth weights were analysed using the number of offspring per litter as covariate. Body weight in all animals PD 6, 14 and 22 were analysed in a mixed linear model including dose group and sex. All dose-related effect changes on continuous endpoints were analysed by ANOVA methods (mixed effect modelling), and statistical significance was assessed using multiple contrast tests (Bretz et al., 2005). Robustness of data analysis was supported by outlier and influence analysis, and if necessary data analysis was repeated on basis of winsorized means, a mean estimation method that is relatively insensitive to outliers: 2.5% of the lowest and 97.5% of the highest observations per dose group are replaced by their group-specific 2.5% and 97.55 percentiles, which minimizes the impact of extreme values. The statistical significance that was achieved only after data winsorization is reported accordingly. The quantal distribution of sperm was considered as sufficiently close to the continuous Gaussian distribution, and therefore this endpoint was analysed by the same ANOVA methods as mentioned before. In the case of animals with abnormally low sperm counts (not exposure related), data analysis was repeated without their counts. The number of nipple/areolas (NR) was assumed to follow a binomial-distribution with a response range between 0 and 12, with the latter assumed to reflect the biologically possible maximal number of nipples in rats. Litter effects on NR and over-dispersion in the data were accounted for by using Generalized Estimating Equations (GEE) as reported in (Christiansen et al., 2012). Fisher's exact test or chi-square test was used for statistical evaluation of prostate histopathology. Time-to-Event data endpoints (day of sexual maturation) were analysed by Kaplan-Meier methods, with general dose-related pattern identified by a trend test and paired mean differences between controls and dose group by the nonparametric Wilcoxon test under the control of p-value adjustments according to Dunnett-Hsu (Hsu, 1992). All remaining non-continuous endpoints (e.g. percentage post-implantation loss) were analysed by the non-parametric Dunn's Multiple Comparison Test. These analyses were performed in GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). All pairwise statistical testing referred to the Null hypothesis "no

difference between control and exposure mean" which was rejected if the p-value was below the false-positive rate of 5%. The experimental design ensured the statistical detection of at least a 10% change for the endpoints AGD and NR (assuming error rates of $\alpha = 5\%$ and $\beta = 20\%$). Direct comparison of effect means between two exposure groups was analysed by the same models and methods as described before. Dose-response regression analysis was performed by a best-fit approach (Scholze et al., 2001) where various nonlinear regression models were fitted to the same data set and the model that describes data best was selected. All statistical analyses were conducted in SAS (SAS Enterprise Guide 4.3).

3. Results

Overall, this study showed that developmental exposure to a combination of anti-androgens and estrogens can lead to effects on endocrine-sensitive endpoints that cannot be explained by the binary mixtures of anti-androgens or estrogens alone and therefore should be considered as combination effects. Specifically, male AGD and seminal vesicle weight were significantly more reduced in the four-compound mixture group (Totalmix) than in each of the binary mixture groups (Emix and Amix). The same was seen for nipple retention, which was significantly higher in the Totalmix group than in the binary mixture groups. Also, effects on other endpoints including sperm count appeared most marked in the Totalmix group, indicating the presence of combination effects between estrogens and anti-androgens.

3.1. Pregnancy data, postnatal growth and general toxicity

There were no statistically significant effects of dosing on maternal body weight gain in gestation or lactation, no change in gestation length, post implantation-loss and litter size. Pup body weights were unaffected at the examined ages (Table S-2). No macroscopic anomalies or undescended testes were seen at any of the examined ages in the males and no genital malformations were seen in the female offspring.

3.2. Anogenital distance (AGD) and nipple retention

Both high and low mixture doses of Totalmix and Amix significantly decreased AGD at birth in male offspring ($p < 0.05$), with the AGD reduction at Totalmix-high being slightly more marked than at Amix-high ($p < 0.05$, Fig. 3A and Table S-2). AGD reductions were also observed in female offspring, with both doses of Amix and the high dose of Totalmix having significantly reduced AGD (Table S-2).

he observed AGD responses of Totalmix agreed well with the dose-additivity curve that was derived from the AGD data observed for Amix and Emix, with both binary mixtures expressed as regression curves for the additivity calculation (Fig. 3C, Table S5, with AGD always expressed as relative AGDI). In Fig. 3C we have added the regression curves for both binary mixtures, and for a better comparison to the additivity curve both were scaled to the mixture concentration: the additivity curve is nearly identical to the Amix curve, and from this comparison it is unclear whether the Emix compounds contributed to the observed Totalmix responses. If we used historical dose–response AGDI data of each compound to generate a prediction curve for additivity (Table S6), we achieved a good agreement between observed and predicted mixture responses (Fig. 3E). As we observed in previous studies on BPA and Butylparaben only AGDI reductions up to 15%, the DA prediction curve can be generated according to Eq. (1) only to this effect level. Therefore mixture responses beyond a 15% AGD reduction were predicted by the toxic unit extrapolation method (Scholze et al., 2014) which makes worst-case assumptions for the expected contribution of these two compounds to the overall mixture response, and as consequence generates a range of possible prediction values.

Nipple retention in male offspring was significantly increased both low and high dose mixture groups of Amix and Totalmix (Fig. 3B). When the Amix was tested in combination with the Emix, Totalmix produced significantly higher numbers of nipples and areolas than observed for Amix alone (Fig. 3B). This suggests that the Emix enhanced the effect of the Amix.

An additivity expectation derived from Emix and Amix was hampered, as the Emix did not induce nipple retention within the dose range tested. Assuming that at higher doses of Emix we would have seen also no effects, we expected the observed mixture responses from the four compounds to equal the responses from the Amix. However, the observed number of nipples/areolas at Totalmix exposure were near twice the responses observed at Amix (Fig. 3D). In contrast, if the prediction curve for additivity was based on single substance dose–response data from previous studies, we got an excellent agreement between predicted and observed mixture responses (Fig. 3D). Similar to the additivity curve in Fig. 3D, we did not expect a contribution from the estrogenic compounds to the overall mixture response, as only procymidone and DEHP were active (Christiansen et al., 2010; Hass et al., 2012, 2007) but not BPA and butylparaben at these doses (Boberg et al., 2016; Christiansen et al., 2014). Therefore, the agreement between observed and expected dose additivity seems to depend on whether data of the single substances or their binary mixture outcomes were used. As only Amix was tested in parallel to Totalmix, but not the individual compounds, we cannot rule out that either procymidone or DEHP (or both) were less active than expected from previous studies, and predictions based on historical dose–response data for procymidone and DEHP, therefore, overestimated their additive response (Fig. 3D). Alternatively, the combined action of the two anti-androgenic compounds on NR did not follow dose addition, which would contradict previous studies on these compounds (Christiansen et al., 2008; Hass et al., 2007; Rider et al., 2009). From an additivity point of view, it is thus likely that either BPA or butylparaben (or both) must have enhanced the mixture response from Amix.

3.3. Offspring body weights and puberty timing

Offspring body weights on PD 6, 14 and 22 showed no difference with respect to dose groups or sex when analysing all pups per litter (data not shown).

In the subset of females sectioned at PD22, female body weights were decreased by both doses of Totalmix (Table S-4).

In males, sexual maturation was slightly delayed, indicating hormone-dependent effects. Re-dosing during puberty did not affect the time of sexual maturation, and therefore further analyses comprised all animals, i.e. both those exposed only perinatally and those re-dosed during puberty. In males, the mean day of sexual maturation was significantly delayed by 1.5 days at both Emix-low and the high dose of the Totalmix compared to controls (control: PD = 44.6, Emix-low: PD = 46.0, Totalmix-high: PD = 46.1). When analysing this time-course data (day of sexual maturation in males) as fractions, statistical significance was obtained in both in Emix-low compared to controls ($p = 0.0115$) and Totalmix-high compared to controls ($p = 0.022$) (Table S-2 and Fig. 5A). In females, the mean day of sexual maturation appeared to be one day delayed in the groups exposed to the two doses of the Totalmix compared to controls (control PD31.8, Totalmix-low PD 32.8 and Totalmix-high PD 33.0), but the differences were not statistically significant (Table S-2). There were no significant effects on body weight on the day of sexual maturation of males or females.

Male body weights at PD 80 were unaffected by the exposure in animals exposed only perinatally (Table S-5). Males re-dosed with the Totalmix during puberty had lower body weights at PD 80 than controls, though the effect was more marked in the low than the high dose of the Totalmix (Table S-5). In female offspring exposed perinatally, body weights were decreased on PD 80 by all doses except Amix-high (Table S-4). In contrast, females re-dosed with the Totalmix showed no significant changes in body weights at PD 80 (Table S-5). For both males and females there were no statistically significant differences

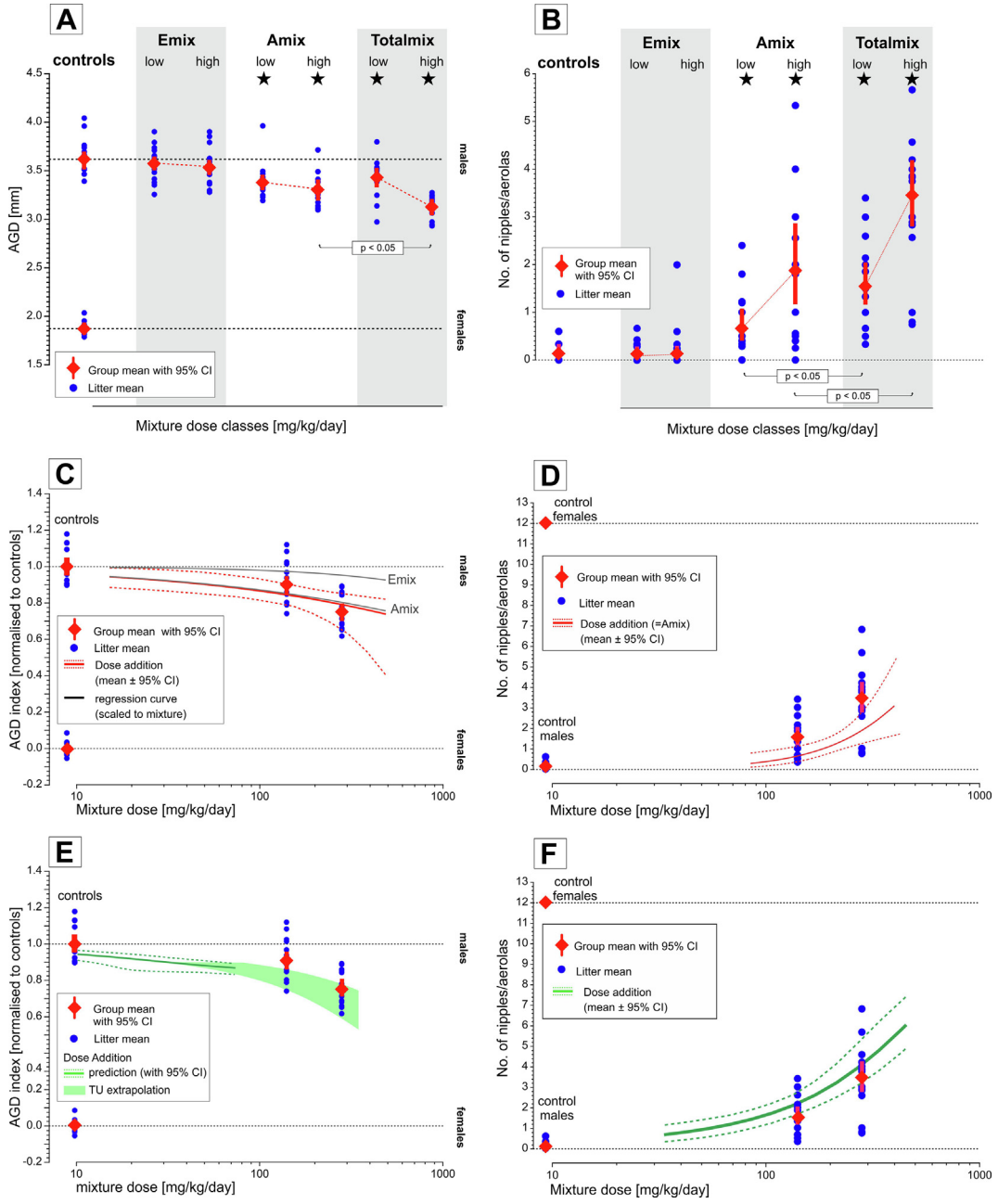


Fig. 3. Anogenital distance (AGD) and nipple retention in male rats exposed perinatally to mixtures of endocrine disrupters. Blue dots are litter means and red diamonds are group means. A mixture of DEHP and procymidone (Amix) reduced anogenital distance (A) and increased the number of nipples/areolas (B). No effects were seen for a mixture of bisphenol A and butylparaben (Emix), but marked effects were seen when all four substances were combined to a Totalmix (A, B). Asterisks indicate statistically significant differences from controls, and horizontal bars illustrate that the effects of the Totalmix were significantly different from the effects of the Amix. Significance level $p < 0.05$. Observations on AGD index showed good agreement with dose-additivity expectations calculated on the basis of data from the Amix and Emix groups (internal study data, green line, C) and predicted on the basis from historical data (external study data, E). In contrast, the observations for nipple retention were underestimated when dose-additivity was derived from Amix (D), but in good agreement when predicted from historical data (F). To achieve comparability to the dose scale of Totalmix, doses of Amix and Emix were rescaled by 1.4 and 3.5, respectively (C, for mixture composition, see Fig. 1). All data shown in C-F refer to Totalmix, and the additivity prediction in panel D equals the regression curve of Amix. Dotted horizontal lines indicate values for control males and females respectively. Dose additive AGD responses were estimated at high doses according to the toxic unit (TU) extrapolation method. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

between groups exposed only perinatally and groups re-dosed in puberty, indicating that re-dosing did not have a major influence on body weights.

3.4. Mixture effects on reproductive organ weights

Organ weights were examined at PD 16, 22 and 80. In prepubertal males, the Totalmix and some sub-mixtures reduced the weights of most male reproductive organs (Fig. 4B-F, Table S-3). In contrast, the high doses of Amix and Totalmix increased testis weights (Fig. 4A). On PD 16, when dosing was ongoing, the Amix decreased the weight of epididymides, LABC, bulbourethral glands and the ventral prostate. The Emix decreased the weight of LABC and bulbourethral and adrenal glands, and the Totalmix decreased the weight of epididymides, seminal

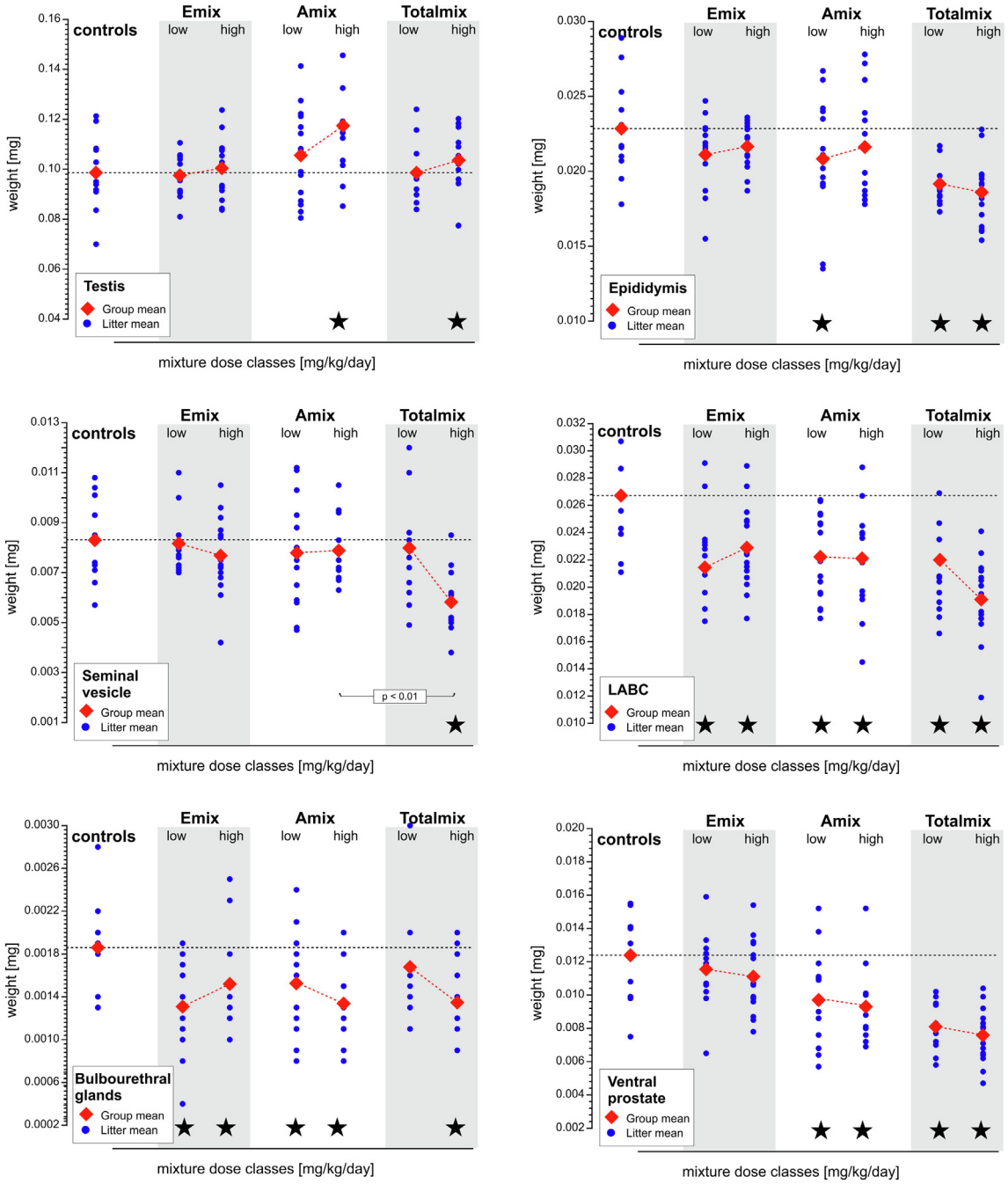


Fig. 4. Male reproductive organ weights at PD 16 were affected by perinatal exposure of rats to a mixture of DEHP and procymidone (Amix), a mixture of bisphenol A and butylparaben (Emix), and a Totalmix of all four substances. Blue dots are litter means and red diamonds are group means. Asterisks indicate statistically significant differences from controls, and horizontal bars illustrate that the effect of the Totalmix on seminal vesicle weight was significantly different from the effects of the Amix. For mixture composition, see Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vesicle, LABC, bulbourethral glands and the ventral prostate (Fig. 4B-F, Table S-3). At this age, the effect of the Totalmix was significantly more marked than the effect of the Amix or the Emix on seminal vesicle (Fig. 4C, Table S-3).

In adulthood (at PD 80), a reduction in epididymis weight was still present in Amix and Emix groups (Table S-3), and also in the Totalmix-low group when data from perinatally exposed animals and re-dosed animals were combined (Table S-5). A reduction in ventral prostate weight persisted in the Totalmix group but was only statistically significant in the re-dosed animals (Table S-5).

Interestingly, the high dose Amix group increased prepubertal testis weight more markedly than the high dose Totalmix group at 16, 22 and 80 days of age (Table S-3), and together with the significantly lower reduction observed at the Emix-high group on PND80, this indicates opposing effects of Emix and Amix on this endpoint.

3.5. *Sperm counts reduced by all mixtures*

Epididymal sperm counts were significantly reduced in all mixture groups (Fig. 5E, Table S-3 and S-5, $p < 0.01$). As no statistically significant effect of re-dosing was seen, the analyses of sperm count were performed on all animals, i.e. combining animals exposed only

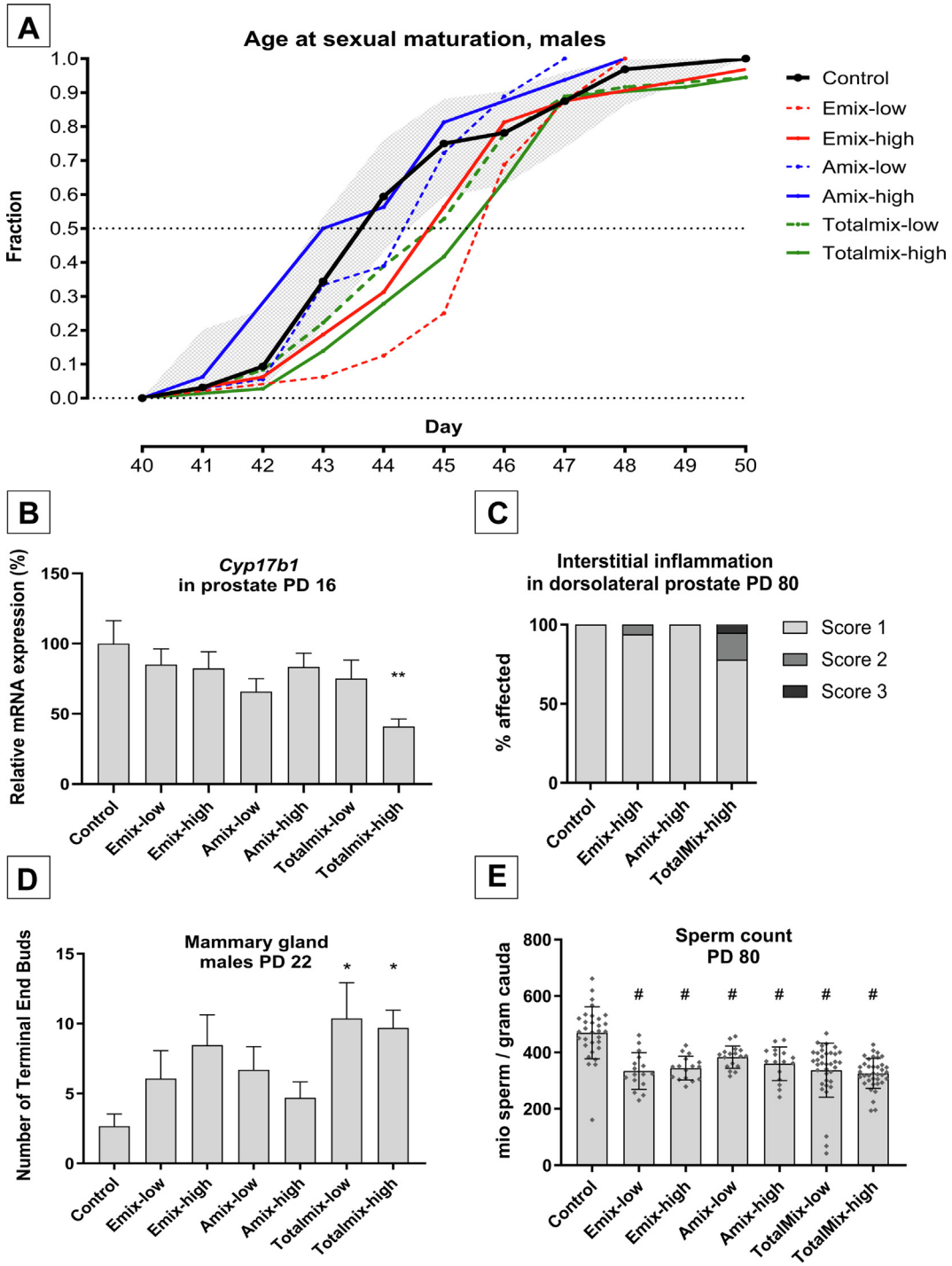


Fig. 5. Endocrine-sensitive processes and tissues in rats after perinatal exposure to a mixture of DEHP and procymidone (Amix), a mixture of bisphenol A and butylparaben (Emix), and a combination of all four substances (Totalmix). In offspring, the Totalmix delayed preputial separation (A, evaluated in two males per litter, N = 15–17 litters per dose group) see also Table S-2 for mean values. Fraction means the number of animals displaying preputial separation out of the total number of animals. At PD 16, expression of androgen-regulated gene CYP17b1 was reduced in ventral prostate (B, N = 13–15, mean + SEM). At PD 22, hormone-sensitive development of mammary glands was identified as an increase in the number of Terminal End Buds (D, N = 11–13, mean + SEM). In adult offspring, Totalmix-exposed animals displayed histological changes in prostate (C, N = 15–17), and all mixture groups displayed significantly reduced sperm counts (E, evaluated in two males per litter, n = 15–17 litters per dose group, mean + SD). Asterisks * indicate statistically significant differences from controls, *:$p < 0.05$, **: $p < 0.01$. Hashtags # indicate statistically significant differences from controls, $p < 0.01$, when combining animals exposed only perinatally and those re-dosed during puberty. For mixture composition, see Fig. 1.

perinatally and those re-dosed during puberty. The most marked reductions in sperm count were seen in the Totalmix-high group but this effect was not significantly different from effects in Emix-high or Amix-high groups. One animal in the control group and three animals in the Totalmix-low group had very few sperm cells compared to all other animals in the study. This pattern with a few animals showing much lower sperm counts has been seen in previous studies from our laboratory and has been reported irrespective of dose. Statistical analysis was performed both including and excluding these four outliers, and the same pattern of effect was seen in both analyses (data excluding

outliers not shown).

3.6. Early markers of late effects on prostate

Early changes in prostate development were evidenced by reduced organ weights and reduced expression of the AR regulated gene *Cyp7b1*. Both Totalmix groups showed significantly lower ventral prostate weights at PD 16 and 22 compared to controls and also significantly lower than prostate weights at corresponding doses of Amix or Emix individually. This indicates combination effects of Amix and Emix (Fig. 4F). The expression of the AR-regulated gene *Cyp7b1* was significantly downregulated in the Totalmix-high group on PD 16 ($p = 0.01$, Fig. 5B). No effect was seen on the AR-regulated genes *Pbpc3, Odc, Trpm2*, nor on *Esr2*. Gene expression was not affected by any of the binary mixture groups, which may reflect a combined effect of the four compounds of the Totalmix.

Histological examination of the dorsolateral prostate of adults generally revealed large variations in prostate morphology within all dose groups. Some indications of long-term effects were seen, as the highest scores (2–3) for interstitial inflammation in dorsolateral prostate were observed in the Totalmix-high group, but not in controls (Fig. 5C). Examination of ventral prostate histopathology showed no statistically significant differences between exposed high dose mixture groups and controls or between the Totalmix-high and Re-Totalmix-high groups (data not shown). Variable degree of interstitial and intraluminal infiltration, epithelial atrophy, and epithelial hyperplasia were observed in all groups. Reactive epithelial hyperplasia with the presence of variable degree of cellular atypia was observed in 14 males with moderate total inflammation (score 3) and in 13/36 males with moderate total inflammation (score 2) from different dose groups.

3.7. Mixture effects on mammary development

Prepubertal mammary glands were affected in both males (Fig. 5D) and females (data not shown), as a significantly higher number of TEBs was seen in the Totalmix compared with controls. No significant effects on these endpoints were seen for either the Amix or the Emix groups, indicating the mixture effects of anti-androgens and estrogens. No effects were seen in males or females on outgrowth measures (area, growth towards the lymph node, longitudinal growth) or in a score combining information on branching, duct number, area and TEB number.

3.8. Chemical analysis of maternal plasma

Chemical analysis confirmed the presence of the parent compounds or main metabolites in the dosed animals. The plasma concentrations of procymidone and the DEHP metabolites from the dams were around twofold higher in the high concentration mixture groups than in the corresponding low concentration mixtures (Fig. 6) and thus in proportional agreement with the intake doses. Their concentrations were roughly the same in the Amix and Totalmix, but slightly lowered in the Totalmix-high groups when compared to the Amix-high group. These patterns were not confirmed for BPA or butylparaben, as all groups showed comparable concentrations.

4. Discussion

We show that developmental exposure to combinations of chemicals with different endocrine modes of action can lead to enhanced adverse effects on the developing and adult reproductive system in rats. We found no experimental evidence against describing the mixture effects on AGD and nipple retention as dose-additive. As illustrated in an AOP framework (Fig. 2), different molecular initiating events can lead to the same adverse outcomes, and we propose that substances with mixed endocrine modes of action should be grouped together in mixture risk assessment.

4.1. Early exposure to EDCs with mixed modes of action leads to late-life adverse health effects

We demonstrated that a mixture of DEHP and procymidone (Amix) induced nipple retention and shortened AGD in developing male rats, whereas a mixture of BPA and butylparaben (Emix) did not. All four compounds combined (Totalmix) showed more marked effects than seen for the Amix alone. The same pattern was seen for prepubertal male reproductive organ weights, which for seminal vesicles was significantly lower in the Totalmix group than in the Amix and the Emix groups. These early markers of endocrine disruption can be considered predictors of late-life adverse effects, as in adulthood the Totalmix showed the most marked effects on sperm count and prostate histopathology. Overall, we suggest that the Emix exposure potentiated the effects of the Amix by direct endocrine action on target tissues (Fig. 2). Even though the Emix alone did not affect AGD or nipple retention, the endocrine activity of this mixture was evident from the lower sperm counts and reduced weights of LABC and bulbourethral gland. BPA was present in the mixture at a dose that caused an AGD reduction in our previous study (Christiansen et al., 2014) and has been reported for its reproductive toxicity in males (EFSA, 2015). Nevertheless, many other studies have reported first signs of male reproductive toxicity at much higher dose ranges (EFSA, 2015; Tinwell et al., 2002; Tyl et al., 2008, 2002). We speculate that a similar study with higher doses of BPA or using another estrogenic model compound - would show even clearer evidence of additive effects on male reproductive development.

4.2. Lack of toxicokinetic interference

An alternative explanation to the potentiation of Amix responses by Emix could be toxicokinetic interference. It could be speculated that the presence of Emix would reduce or delay the elimination of Amix components and thus increase their bioavailability and effect responses. However, chemical concentrations in dam plasma at GD 15 did not support this explanation, as concentrations of procymidone, MEHP (the main and active metabolite of DEHP in serum and plasma (Martino-Andrade and Chahoud, 2010) and other DEHP metabolites were slightly lower in the Totalmix-high group than in the Amix-high group. Considering the common endocrine targets of these substances, the marked effects in the Totalmix-high group was thus attributed to the combined effects of all four compounds.

BPA and butylparaben in the serum were present at similar concentrations in low and high dose Emix-groups. The identical concentrations of butylparaben in maternal plasma in all dose groups echo our earlier rat studies showing similar concentrations of butylparaben at different doses in maternal plasma, but increasing concentrations with higher doses in amniotic fluid (Frederiksen et al., 2008). In addition, butylparaben levels in amniotic fluid were significantly higher than in maternal plasma (Frederiksen et al., 2008). In this study, no fetal blood or amniotic fluid samples were available for further analysis, but an investigation of fetal internal exposures and time-course data would be necessary to investigate further the relationship between administered doses and internal exposures to these chemicals. Finally, the detailed metabolism and excretion pattern in rats for all four chemicals remains to be studied for different mixture concentrations.

4.3. AOP-based grouping for mixture risk assessment

The current findings of combination effects of chemicals with different mechanisms of action highlight the importance of performing mixture risk assessment by grouping compounds with effects on the same targets and not only by grouping compounds with similar modes or mechanisms of action (Boberg et al., 2019). We suggest using knowledge on AOPs to define broader groups of chemicals for inclusion

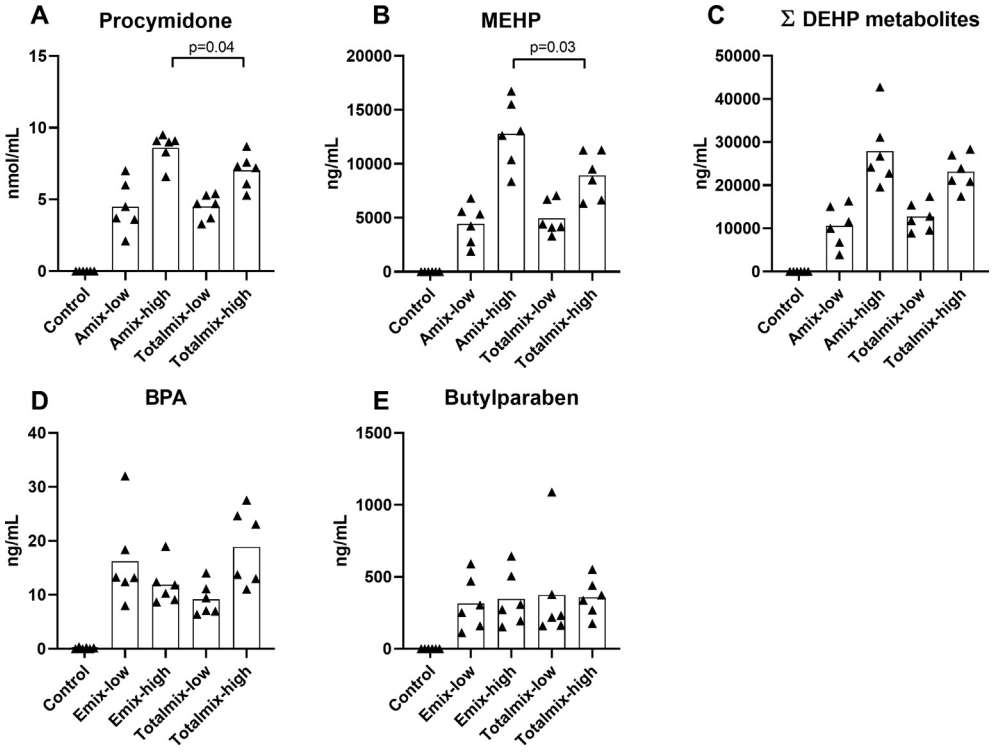


Fig. 6. Chemical concentrations were analysed in plasma samples from dams at GD 15 (N = 6, pooled from 1 to 3 dams) see Fig. 1. Higher concentrations were seen of procymidone, nmol/mL (A) and the DEHP metabolite MEHP, ng/mL (B) in the Amix-high group than in the Amix-low group. Plasma concentrations of Amix chemicals were lower in the Totalmix-high group than in the Amix-high group ($p = 0.03$ and $p = 0.04$ for MEHP and procymidone, respectively). The sum of five DEHP metabolites, ng/mL (C) did not show significant difference between Totalmix-high and Amix-high groups. For BPA (D) and butylparaben, ng/mL (E) no significant differences between Emix and Totalmix groups were seen. Data is shown as mean values.

in the mixture risk assessment than groups limited to either chemicals with "shared target organs" or with "shared mode of action". AOP based grouping will thus enable mixture risk assessment of more chemicals including those for which data are scarce and only either *in vitro* mechanistic data or *in vivo* effect data are available. We thus provide evidence for suggestions by EFSA (2019) to use the knowledge on AOP for mixture risk assessment. Specifically, we propose that substances acting on male reproductive target tissues should be grouped with substances that have been shown to be estrogenic and/or anti-androgenic *in vitro*. Generally, the same approach could be taken for substances with other endocrine or non-endocrine targets: substances known to act on molecular targets at one end of an AOP should be grouped with substances acting on endpoints at the other end of the same AOP. For example, a substance acting as an AR antagonist for which no *in vivo* data are available should be grouped with a substance reducing male AGD for which no information on mechanism of action is known.

4.4. Predicting mixture effects from internal study data or historical data

Dose-additivity predictions of the four-compound mixture (Totalmix) were calculated on basis of (i) binary mixture outcomes from the current study and (ii) single compound dose–response data from studies conducted earlier in our lab (Boberg et al., 2016; Christiansen et al., 2014, 2010; Hass et al., 2007), and their predictive power then assessed by comparing them to the observed mixture responses.

For AGD we found no clear experimental evidence against additivity. However, only weak AGD reductions were observed in previous studies for BPA and Butylparaben, which limited a priori the assessment range for AGD responses. According to the principles of dose addition, we furthermore expected a high contribution of procymidone and DEHP to the overall mixture additivity, which prevented clear discrimination between the binary mixture responses and their expected overall additivity. Therefore, it remains unclear whether the combined action of both anti-androgenic and estrogenic compounds on AGD can be described always by dose addition.

Mixture effects on nipple retention provided a better basis for an assessment as BPA and Butylparaben showed neither individually nor in combination effects. Therefore, the suitability of the dose addition model was reduced to the question of whether procymidone and DEHP were able to describe the observed mixture effect of Amix in the presence of Emix. The effects in the Totalmix were accurately predicted on the basis of historical single compound data, but slightly higher than the responses observed by Amix.

These experimental findings cannot readily point out whether mixtures of chemicals with diverse endocrine modes of action can cause mixture effects on hormone-sensitive endpoints that can be described accurately by dose addition. However, we found no experimental evidence against using dose addition as a regulatory worst-case reference as all predictions derived from historical data either agreed well or slightly overestimated the observed mixture responses but did not underestimate them. When aiming to protect humans against adverse effects of chemical exposures, the overestimation of predicted mixture effects may lead to a higher level of protection. From a public health viewpoint, such conservative estimates may be acceptable.

4.5. Multiple modes of action leading to adverse reproductive effects

A long-term adverse effect on sperm count was seen in all dose groups, most markedly in the high dose Totalmix. This highlights that chemicals with mixed modes of action can target the same organ even long after the last gavage exposure.

Male reproductive effects were alerted already in pre-puberty when mainly the Totalmix reduced weights of accessory reproductive organs (epididymis, prostate, seminal vesicle). These early marker tissues are known to be affected by anti-androgenic substances(Christiansen et al., 2010; Jacobsen et al., 2012). Combination effects of anti-androgenic substances *in vivo* have also been shown by other research groups (Conley et al., 2018; Howdeshell et al., 2008b; Rider et al., 2008). Interestingly, Emix decreased the weight of androgen-dependent tissues LABC, bulbourethral glands and epididymides. This may support an anti-androgenic effect of these two compounds when administered together, however, no such effects were seen in historical data from our

laboratory on the single compounds BPA and butylparaben (Boberg et al., 2016; Christiansen et al., 2014). In line with this, a study on the structurally related propylparaben showed clear evidence of an anti-androgenic effect in the Hershberger assay (Özdemir et al., 2018), however, no data are available from Hershberger studies on butylparaben.

Early fetal testis development is largely androgen-independent while the accessory reproductive organs and especially spermatogenesis is completely dependent on androgen signalling (Sharpe, 2006). Interestingly, prepubertal testis weights were increased rather than reduced by the Amix, and the Emix appeared to counteract the effect of Amix on testis weights when combined in the Totalmix. Increased testis weight was also seen with exposure to procymidone alone at similar doses (Jacobsen et al., 2012; Metzdorff et al., 2007). Possibly, procymidone reduces the normal negative feedback on follicle-stimulating hormone (FSH), a key regulator of Sertoli cell proliferation and in turn testis size (Atanassova et al., 2005). The resulting increase in testis weight appears to be normalized by Emix, and this corresponds with the knowledge that neonatal estrogen exposure delays testis development and reduces testis weight (Atanassova et al., 2000).

Prostate development is sensitive to estrogenic as well as anti-androgenic chemicals that may induce ventral prostate inflammation (Cowin et al., 2008; Stoker et al., 1999). In the current study, Totalmix exposure affected the androgen-sensitive gene *Cyp7b1* in developing prostate (Metzdorff et al., 2007; Tang and Norlin, 2006). *Cyp7b1* is considered a key regulator maintaining the balance between AR and ER signalling in the prostate (Tang and Norlin, 2006), and the current results emphasize a possible role of this factor as an early marker of changes through both ER and AR related pathways. In adult offspring, signs of increased incidence of epithelial inflammation were seen after Totalmix exposure. An increased incidence of prostatic lesions has previously been seen in young adult rats after perinatal or neonatal exposure to estrogenic compounds (Howdeshell et al., 2008a; Prins et al., 2007) and may be related to pre-cancerous lesions in humans. Examination of the prostate from older rats could further elaborate on whether the present mixtures would induce atypical hyperplasia later in life.

Early mammary gland development of males was affected by the Totalmix, as the number of TEBs was increased. This indicates an accelerated development of mammary glands, possibly due to an altered androgen/estrogen balance, and was also seen in females. Previously, we have seen an increased TEB number in males with exposure to the potent estrogen ethinyl estradiol (Mandrup et al., 2012). TEBs are the site of origin of mammary carcinomas and a target structure of carcinogens (Russo and Russo, 1996). An increased number of TEB for a longer period of time may be related to an increased sensitivity to carcinogenic insults and thus a higher cancer risk later in life, as seen in female rodents (Fenton, 2006). It should be noted that also men get breast cancer, and that increased breast development in men (gynecomastia) is frequently observed (Sansone et al., 2017). Examination of early mammary gland development in males may be useful as a sensitive marker of long-term adverse effects on the mammary glands, as already proposed in an OECD test guideline for reproductive toxicity (OECD, 2018; Osborne et al., 2015).

4.6. Re-dosing during puberty

Puberty is a sensitive developmental window and pharmaceutical and environmental compounds may alter pubertal development (Stoker et al., 2000). The re-dosing of males and females during puberty provided no indications for changes in the puberty timing or sperm counts. However, the period of re-dosing applied was initiated later than suggested by protocols for pubertal assays of the US Environmental Protection Agency's Endocrine Disrupter Screening Program (Stump et al., 2014), which recommends the re-dosing starts at weaning (PD 22). In this study, the re-dosing started at PD 27 and 38 in females and males respectively and this could have affected the results. Nevertheless, weights of seminal vesicle and prostate from male animals that were re-dosed with the Totalmix doses during puberty were slightly more decreased than in only perinatally exposed animals. This finding reached statistical significance when related to the controls (Supplementary Table S-5). Therefore, we cannot rule out that additional peripubertal exposure can have an additional impact on reproductive endpoints. Less clear were the findings for other endpoints, but the statistical significance detected for epididymal weight changes when data from both the re-dosed and non re-dosed animals were pooled (using litter as the statistical unit) may indicate that higher sample sizes would have revealed a clearer picture about subtle effects on reproductive organ weights.

5. Conclusions

Combined exposure to both anti-androgens and estrogens leads to effects on anogenital distance, sperm count and several other reproduction endpoints. Generally, the presence of estrogenic compounds enhanced the effects observed for the anti-androgens, suggesting that this potentiation was caused by direct endocrine action on target tissues. This is supported by internal concentration measurements which provided no evidence for toxicokinetic interaction as a possible explanation.

Dose-additivity predictions derived from historical data for anogenital distance and nipple retention agreed relatively well with the observed mixture effects, and the deviations observed for these mixtures can be considered as acceptable from a regulatory point of view. However, the experimental evidence from this study is too limited for general conclusions and mixture studies with other compounds are necessary to explore how well the combined action between anti-androgens and estrogens can be described by dose-addition. The lower sperm counts observed at all mixture exposures and the reduced adult male reproductive organ weights identified after exposure to both anti-androgens and estrogens (particularly in animals re-dosed during puberty) lead us to conclude that the exposures produced long-term adverse health effects in rats.

Our proof-of-concept study demonstrated that combination effects between anti-androgens and estrogens are possible, and if ignored may lead to an underestimation of risks associated with exposures to chemicals that disrupt male sexual differentiation. Since unhindered endocrine action is essential for human development, these findings are highly relevant to human risk assessment.

6. Perspectives

Our study provides an example of a mixture scenario where several modes of action can converge into a common adverse health outcome. This supports the view that cumulative assessment groups for the mixture risk assessment should be defined along all shared AOPs that can affect the adverse health outcome (Boberg et al., 2019; Kortenkamp, 2020). We propose that mixture risk assessment should be conducted even for compounds with sparse data as long as sufficient information is available that indicates a potential interruption to key events of at least one of the relevant AOPs. For some compounds, we may only have information on early events from *in vitro/in silico* studies while for others we only have observations on an adverse outcome *in vivo*, and all should be grouped for mixture risk assessment (Fig. 2). Beyond endocrine disruption, this has a major impact on regulatory decision-making and is an important contribution to current work by regulatory authorities worldwide concerning enforcing mixture risk assessment as an essential step towards human chemical safety.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105870.

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