Perfluorohexane Sulfonate (PFHxS) and a Mixture of Endocrine Disrupters Reduce Thyroxine Levels and Cause Anti-Androgenic Effects in Rats

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Abstract

The developmental toxicity of perfluorohexane sulfonate (PFHxS) is largely unknown despite widespread environmental contamination and presence in human serum, tissues and milk.

To thoroughly investigate PFHxS toxicity in developing rats and to mimic a realistic human exposure situation, we examined a low dose close to human relevant PFHxS exposure, and combined the dose-response studies of PFHxS with a fixed dose of twelve environmentally relevant endocrine disrupting chemicals (EDmix).

Two reproductive toxicity studies in time-mated Wistar rats exposed throughout gestation and lactation were performed. Study 1 included control, two doses of PFHxS and two doses of PFHxS+EDmix (n=5-7). Study 2 included control, 0.05, 5 or 25 mg/kg body weight/day PFHxS, EDmix-only, 0.05, 5 or 25 mg PFHxS/kg plus EDmix (n=13-20).

PFHxS caused no overt toxicity in dams and offspring but decreased male pup birth weight and slightly increased liver weights at high doses and in combination with the EDmix. A marked effect on T4 levels was seen in both dams and offspring, with significant reductions from 5 mg/kg/day.

The EDmix caused anti-androgenic effects in male offspring, manifested as slight decreases in anogenital distance, increased nipple retention and reductions of the weight of epididymides, ventral prostrate and vesicular seminalis.

PFHxS can induce developmental toxicity and in addition results of the co-exposure studies indicated that PFHxS and the EDmix potentiate the effect of each other on various endpoints, despite their different modes of action. Hence, risk assessment may underestimate toxicity when mixture toxicity and background exposures are not taken into account.

Introduction

Pre- and postnatal development is under the control of a complex network of tightly regulated processes that ensures health at later adult stages, and disruption of these processes may result in irreversible changes or increased disease susceptibility in adult life. Some of the changes may arise from endocrine disrupting chemicals (EDC), i.e. chemicals that can interfere with the hormonal systems and consequently adversely affect numerous organs and organ systems (Bergman *et al.*, 2013). Since human EDC exposure is likely to occur as combined exposures, mixture investigations are relevant to include, when performing animal studies on endocrine disruption (Christiansen *et al.*, 2012; Hass *et al.*, 2012).

Poly- and perfluoroalkyl substances (PFAS) are a diverse group of chemicals that have been used in industrial applications and consumer products, e.g. as surfactants since the 1950s (Lindstrom *et al.*, 2011). They are persistent, resist degradation in the environment, and bioaccumulate in humans (Blum *et al.*, 2015; Scheringer *et al.*, 2014). PFASs can act on various endpoints within the mammalian body, including the metabolic-, immune- and endocrine systems (DeWitt, 2015; USDHHS, 2016). There are indications from the industry and biomonitoring studies that the use of the three most commonly found PFAS, perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and perfluorohexane sulfonate (PFHxS), has decreased (Kato *et al.*, 2011; Schröter-Kermani *et al.*, 2013; US EPA, 2006). Nonetheless substantial human and environmental exposure to these compounds will continue for some time, due to their long half-lives of 3-7 years (Olsen *et al.*, 2007). *In vivo* studies have shown that PFOS can cause pup death and thyroid hormone disruption (Lau *et al.*, 2003; Yu *et al.*, 2009a), leading to a classification as a reproductive toxicant category 1B (ECHA). Epidemiologic studies show inconsistent associations between serum T4 levels and PFOS exposures (Ballesteros *et al.*, 2017; Jain, 2013). PFHxS has recently been classified as a substance of very high concern, due to its very persistent and very bioaccumulative

(vPvB) properties, and it is proposed for listing as persistent organic pollutant (POP) according to the Stockholm Convention (ECHA, 2017; UNEP). In spite of this, surprisingly little is known about the potential toxicity of PFHxS (Danish Ministry of the Environment, 2015; FSANZ, 2016). *In vitro* studies with PFHxS have shown that it can bind to the serum thyroid hormone transport protein transthyretin releasing the hormones to the circulation for hepatic metabolism (Ren *et al.*, 2016; Weiss *et al.*, 2009). As with PFOS, epidemiologic studies investigating PFHxS exposure and human T4 levels find inconsistent associations (Jain, 2013; Wang *et al.*, 2014; Webster *et al.*, 2016).

To our knowledge only one developmental toxicity study with PFHxS has been published (Butenhoff *et al.*, 2009). Toxicity was observed in parental males, with minimal to moderate hypertrophy in liver and thyroid gland appearing at the two highest doses (3 and 10 mg/kg body weight/day). No adverse effects were observed in dams and offspring, however, due to sex differences in elimination of this compound, serum PFHxS were approximately 75% lower in dams than in the parental males receiving the same dose. Additionally dams only showed a minimal PFHxS accumulation in the liver. Hence, we hypothesized lower internal exposures to be the cause of the lack of effects in the dams and offspring.

Due to the evidence of toxicity of PFAS and especially PFOS, the continued human exposure to these compounds and the limited knowledge of developmental toxicity of PFHxS, we decided to conduct a developmental toxicity study of PFHxS in Wistar rats. We focused especially on assessment of endpoints sensitive to endocrine disruption; anogenital distance (AGD), nipple retention (NR), male reproductive organ weights and thyroid hormone levels. Moreover, to investigate internal exposures, PFHxS levels were measured in dam serum. To mimic a more

realistic human exposure situation a low dose, closer to human relevant PFHxS exposure, was included, and we also combined the dose-response (Christiansen *et al.*, 2012) studies of PFHxS with a fixed dose of a mixture of 12 environmentally relevant EDCs (EDmix). A similar mixture has previously been investigated by our research group (Total mix100 in Axelstad *et al.*, 2014; Johansson *et al.*, 2016; Mandrup *et al.*, 2015), and it was administered at a dose reflecting a hundred fold high-end human exposure.

Methods

Chemicals

Perfluorohexane Sulfonate (PFHxS) purity >98 % was purchased from Sigma-Aldrich (Tridecafluorohexane-1-sulfonic acid potassium salt, CAS-No: 3871-99-6, lot #BCBC3545V). The powder was tested for other PFAS impurities by LC-MS, but none were identified (data not shown). Corn oil (Sigma-Aldrich) was used as vehicle for all treatments and dosing in the control group. The mixture of 12 endocrine disrupting chemicals (EDmix) (chemicals and doses are shown in table 1) and the rationale behind choosing these compounds and exposure levels has previously been described in detail (Christiansen *et al.*, 2012). Briefly, the mixture consisted of 12 environmental endocrine disruptors, given at a dose reflecting 100 times high end human intakes. For some of the chemicals, the intake estimates were adjusted to reflect the joint exposure to several chemicals of the same class, e.g. the dose of epoxiconazole was increased to reflect the exposure to all anti-androgenic azole fungicides. Together the DEHP and DBP doses represented exposure to all anti-androgenic phthalates, while for BP and 4-MBC the dose was decreased to adjust for a high intake in specific population groups. However, the total mix 100 described previously also included acetaminophen (paracetamol) which in the studies presented herein was omitted from the EDmix, resulting in a total mixture dose of 32.11 mg/kg body weight (bw)/day (Table 1).

Animals and dosing

Study design of the two studies is presented graphically in figure 1A. In both studies, time-mated nulliparous, young adult Wistar rats (HanTac:WH, Taconic Europe, Ejby, Denmark) were received on gestation day 3 (GD) of pregnancy (the day of plug-detection designated as GD 1) and randomly distributed for pairwise housing. On the day after arrival (GD 4) animals were pseudo-randomly allocated into groups with similar distributions in body weight. The expected day of delivery (GD 23) was termed PND 1 for the pups. Hence, the age of the pups was related to time of mating rather than day of birth, but the two time-points were rather similar as only 5 out of 30 births in Study 1 and 13 out of 128 births in Study 2 did not occur on GD 23.

Dosing of the dams was performed daily (between 8 and 10 am) by oral gavage at a constant volume of 2 ml/kg bw/day from GD 7 through postnatal day 22 (PND), except the day of delivery.

In the range-finding study (Study 1), five groups of 8 animals were exposed to vehicle or 25 or 45 mg/kg bw/day PFHxS both with and without a background EDmix exposure (32.11mg/kg bw/day)(Table 2). The main study (Study 2) consisted of 8 groups of 16-20 animals (Table 2) dosed with lower doses of PFHxS; 0.05, 5 and 25 mg/kg bw/day, both with and without a background EDmix exposure. Besides a vehicle control group, a group receiving only the EDmix was included in study 2.

Study 1 was carried out in a single block, i.e. dosing started for all animals at the same day, whereas Study 2 was subdivided into 4 blocks. Each block in study 2 involved all treatment groups.

The animals were housed in semi-transparent polysulfone (PSU) type III cages (PSU 80-1291HOOSU Type III, Tecniplast, Buguggiate, Italy) (15 x 27 x 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). The animals were kept pairwise from GD 3 to GD 17 and alone thereafter. From the day of arrival the housing standard controlled environmental conditions were: Reversed light/dark cycles of 12 hours (light from 9 pm-9 am, dark from 9 am-9 pm), humidity 55 ± 5 %, temperature at 21 ± 1°C, and ventilation changing air ten times per hour. All animals were fed ad libitum on a standard diet with Altromin 1314 (soy and alfalfa-free, Altromin GmbH, Lage, Germany) and were provided ad libitum acidified tap water (to prevent microbial growth) in PSU bottles (84-ACBTO702SU Tecniplast). The animals were inspected twice a day for overt toxicity. The body weights were recorded on GD 4 and daily during dosing from GD 7 onwards to adjust the dose, to follow changes in weight gain, and to follow pregnancy status.

The animal experiments were carried out at the DTU National Food Institute facilities (Mørkhøj, Denmark). Ethical approval was given by the Danish Animal Experiments Inspectorate. The authorization number given is 2015-15-0201-00553 C3. The experiments were overseen by the National Food Institute's in-house Animal Welfare Committee for animal care and use.

AGD and nipple retention

On the morning after delivery the pups were counted, weighed, sexed, and checked for anomalies. Pups found dead were macroscopically checked for anomalies when possible. Anogenital distance (AGD) was measured on all live pups with an ocular stereomicroscope. All offspring were counted and weighed again on PND 6 and PND 14. The number of nipples/areolas of the pups was counted on PND 14. The nipple retention (NR) in male pups was defined as the number of areolas/nipples (a dark focal area with or without a nipple bud), present where nipples are usually located in female

offspring. AGD and NR retention measurements were performed by the same experienced technician blinded to exposure group.

Autopsy

Offspring

Autopsy in Study 1 took place on PND 16 where up to one male and one female pup from each litter were sacrificed, trunk blood collected for plasma, and liver and adrenal glands excised and weighed. In Study 2, sacrifice was performed on PND 16 and PND 17 for male and female pups, respectively. If available, one pup per gender and litter was sacrificed for macroscopic examination, organ removal and blood collection. Liver, adrenal glands and one retroperitoneal fat pad was excised and weighed from each pup, so was ovaries from female pups and sex organs from male pups (testis, ventral prostate, epidydimis, vesicular seminalis, musculus levator ani and glandula bulbourethralis). On PND 22, up to one male and female pup per litter was chosen for macroscopic examination and organ removal. Liver and adrenal glands were excised and weighed.

Dams

In both studies dams with viable litters were sacrificed at PND 22. Dams were weighed, anesthetized with CO2/O2, decapitated, and trunk blood collected for thyroid hormone analysis (see below). Uteri of the dams were excised and the number of implantation scars was registered. Livers were excised and weighed. All dams not giving birth were sacrificed, as described above, on PND 3 and implantation scars registered to exclude non-pregnant from the study and include dams with full-litter resorption in the determination of postimplantation loss and perinatal loss.

Thyroxine

In both studies trunk blood was collected from dams on PND 22 and from male and female pups on PND 16/17 in, respectively, 10 and 4 ml vacutainer tubes with sodium heparin. Additionally, on GD 15 in Study 2 tongue blood was drawn from the dams without anesthesia in heparinized Eppendorf tubes. All samples were stored on ice until centrifugation for 10 minutes at 4°C and 4000 rpm (rounds per minute). The plasma was collected and stored at -80°C until analysis for T4 levels by electrochemiluminescence-immunoassay (ECLIA) – photoncount at the Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark using a Cobas 8000 E-modul.

All dams in Study 1, and 15 dams from each exposure group in Study 2 (20 controls) were sampled randomly for analysis. In Study 1 pup T4, PND 16, was determined based on litter means (from up to one male and one female pup per litter) and measured in control and PFHxS-only groups. In Study 2 each litter was represented by either a male or female pup (the different sampling times for male and female pups impedes conclusions on sex-specific effects) and was determined in the control, all PFHxS-only groups and the 25-Px+ED group.

Chemical Analysis

Internal PFHxS levels in the serum from dams were determined at the end of the dosing period (Study 1, sacrifice on PND 22), at Environmental Medicine, University of Southern Denmark (SDU), by online solid-phase extraction and analysis, using high-pressure liquid chromatography with tandem mass spectrometry. The analysis was performed by a slightly modified version of the method described by Haug et al. (2009) (Haug *et al.*, 2009). The serum samples were diluted 20-40.000 times prior to extraction to quantify within the calibration range of the method, normally used for human serum samples (Grandjean *et al.*, 2012).

Statistical Analysis

Data from continuous endpoints were checked for normal distribution and homogeneity of variance and then analyzed by analysis of variance (ANOVA). If assumptions were not fulfilled, data was transformed accordingly. Body weight and litter size was included as covariates in data analyses when considered as relevant (e.g. organ weights, AGD, and birthweight). If data from more than one pup per litter were available, litter was included as an independent, random and nested factor in ANOVA or analysis was based on litter means.

The number of nipples/areolas (NR) was assumed to follow a binomial distribution and analyzed according to Hass et al., 2007 (Hass *et al.*, 2007). Statistical significance were assessed using multiple contrast tests (global error rate $\alpha = 5\%$, two-sided) (Bretz *et al.*, 2005). Here we used Dunnett contrasts to detect any dose-related effect and trend contrasts (linear, Helmert, reverse Helmert) assuming different dose-response shapes.

In Study 2, effect differences between the PFHxS-only and the control group and between PFHxS+ED and the EDmix group were tested by pairwise hypothesis testing, with the type I error controlled by the two-tailed Dunnett test (continuous endpoints) and Dunnett contrast (NR). Outcomes from this pairwise hypothesis testing were further investigated by integrating all exposures and control groups into one full linear model (2 x 4 design matrix, Fig 1B), with PFHxS and EDmix exposures including their interactions parameterized as indicator variables (i,e, magnitude of exposure is not considered). The advantage is full model is not only a better control of false-positive decisions, but by assuming an identical data variability across all groups (which we consider fulfilled), it can result in more robust statistical outcomes. The chosen model parameterization allows explicitly making a statistical decision whether (and to which magnitude) EDmix changes in average the PFHxS responses across the different exposure groups. Furthermore, as a larger number of samples are considered in this full model, the increased sample size can identify much smaller effect changes than a simple pairwise comparison. The model was tested by a

main factor for EDmix and dose-dependent interaction terms to account for non-parallelity between the PFHxS and PFHxS+EDmix dose-response pattern.

Outcomes from the full model analysis are only reported if they lead to different conclusions than the pairwise hypothesis testing.

SAS Enterprise Guide 4.3 (2010), SAS Institute Inc, Cary, NC 27513, USA, was used for statistical analyses.

Results

Serum PFHxS

In Study 1, PFHxS was quantified in dam serum on PND 22 (n = 5-7, Fig. 2). The average PFHxS concentration in the control group was <0.1µg/ml whereas 139 and 174 µg/ml PFHxS were measured in animals exposed at 25 and 45 mg/kg PFHxS, respectively. In the groups co-exposed to EDmix and PFHxS the PFHxS levels were slightly increased to 156 and 182 ug/ml, corresponding to a 12.7% and 4.9% increase, respectively (not statistically significant).

Pregnancy data, postnatal growth and general toxicity

PFHxS and EDmix alone or in combination did not affect maternal weight gain during pregnancy, post-implantation loss, perinatal loss, litter size and sex-ratio (Table 3A & B). In Study 2, the EDmix significantly increased postnatal maternal weight gain on PND 1-14 ($p = 0.0140$), whereas a reduced weight gain was indicated by Study 1. Male pup birth weight was slightly decreased by 25 mg/kg PFHxS in Study 2; in the full statistical model, exposure to a dose of 25 mg/kg decreased male pup birth weight by 3.5% ($p = 0.0351$) and this effect was also significant when comparing 25-Px+ED with the background EDmix group ($p = 0.0070$). In Study 1, birth weight was slightly

reduced (6%, not statistically significant) in both the 25-Px+ED and 45-Px+ED groups compared to control (adjusted for litter size). There was no statistically significant effect on female pup birth weight in any of the studies.

Pup growth and weights on PND 6, 14 and 22 were unaffected in Study 1. In Study 2, slight significant decreases in body weight were seen in female offspring on day 6 and 14 at 5 mg/kg in the full statistical model ($p = 0.0209$ and $p = 0.0274$, respectively) and in the 5-Px+ED group compared to EDmix alone on day 6 ($p = 0.0121$) . As decreased body weights were not seen at higher doses (25-Px+ED, 45-Px+ED), we evaluate these body weight changes as chance findings.

One pup from the EDmix group in Study 2 had a genital tubercle with features in between male and female and it had 9 nipples on PND 14. Upon sacrifice on PND 16 it had an underdeveloped testicle and an underdeveloped ovary. Having characteristics of an intersex pup (the first ever to be identified in our group), unrelated to exposure, it was excluded from the study.

Serum thyroxine, T4

PFHxS clearly reduced total T4 levels in both dams and offspring. After only 7 days of gavage dosing (GD 15) the T4 levels in dams were significantly reduced to about 80% of controls at 5 mg/kg PFHxS ($p<0.0001$), and to about 60% of controls at the highest dose (Fig. 3A). These T4 reductions were similarly strong with the EDmix co-exposure. At later stages (PND 22), the T4 reductions were more pronounced, as they decreased to ca.70 and 30% of control levels at 5 and 25 mg/kg PFHxS, respectively ($p<0.0001$). Furthermore, on PND 22 EDmix decreased significantly T4 levels by approximately 10% if data were analyzed in the full model ($p = 0.0242$) (Fig. 3C). In Study 1, dam T4 levels were only measured on PND 22. Here the effects followed the same trend as

in Study 2, however less pronounced, as the serum T4 levels were only reduced to app. 40% of the controls in all 4 exposure groups (Fig. 3B).

T4 levels in the offspring were decreased after PFHxS exposure (Fig. 3D & E). In Study 1, pup serum T4 levels on PND 16 were decreased to app. 60 and 50% of controls in the 25 and 45 mg/kg PFHxS groups, respectively ($p<0.0001$) (Fig. 3D).

In Study 2, pup serum thyroxine was measured at PND16/17 in the control, the three PFHxS-only groups and in the high dose 25-Px+ED mix group. PFHxS significantly decreased serum T4 levels to app. 70% and 55% of control levels, in the 5 and 25 mg/kg PFHxS groups, respectively (p-values<0.001) (Fig. 3E). The EDmix had no additional decreasing effect on the T4 levels in the pups (all groups were compared to control only, a full model analysis was not possible as T4 was measured only in PFHxS-only groups and in the high dose 25-Px+ED mix group).

Anogenital distance and nipple retention

PFHxS and EDmix caused weak effects on anogenital distance (AGD) and nipple retention (NR). In the smaller Study 1 no significant effects on AGD were observed (Table 3A). In Study 2, AGD in male pups was slightly but statistically significant decreased by about 2% after exposure to EDmix ($p = 0.0393$, full model) (Fig. 4A). No significant effects of PFHxS exposure was seen on male and female AGD.

For male pups from Study 1 the average number of nipples on PND 14 was significantly increased at highest PFHxS dose in combination with the background EDmix exposure (45-Px+ED, $p = 0.039$, Dunnett contrast) (Fig. 4B).

In Study 2, PFHxS weakly, but significantly, increased NR at 25 mg/kg PFHxS when using trend analysis ($p = 0.01$ using Helmert contrast, and $p = 0.04$ using linear contrast) and in the full model

(p=0.016). NR was significantly increased when PFHxS exposure took place along with the background EDmix at the two highest doses (5-Px+ED and 25-Px+ED, $p < 0.01$, Dunnett contrast compared to control), and compared to the EDmix group (p=0.02, linear contrast). When data from Study 2 was analyzed by the full model including all dose-dependent interaction terms, a high statistical significance of EDmix was revealed ($p<0.01$, see Figure 1B for model visualization) indicating a strong overall effect of the EDmix on NR. NR in female pups was not significantly affected by exposure to either PFHxS or the EDmix.

Autopsy and organ weights PND 16 and PND 22

In both studies, no significant effects on dam or offspring body weights were seen at sacrifice on PND 16 or PND 22 (Table S1A & B).

Reproductive organs

PFHxS had no effect on the weight of male reproductive organs examined on PND 16 in Study 2. EDmix exposure reduced the weight of the epididymides, ventral prostate and vesicula seminalis in the full statistical model ($p = 0.0023$, $p = 0.0055$ and $p = 0.0288$, respectively) (Fig. 5A-C). The remaining reproductive organ weights were not significantly affected by the EDmix (Fig. 5D-G). In females, no significant effects on ovary weights PND 17 or PND 22 were observed (Table S1B). No reproductive organ weights were assessed in Study 1.

Retroperitoneal fat pad and adrenals

Influences on weights of adrenals and the retroperitoneal fat pat were examined in the pups to evaluate potential influences on fat stores and steroidogenesis. In males, the weight of the retroperitoneal fat pad with body weight as a covariate was increased in male offspring exposed to PFHxS at 25 mg/kg ($p = 0.0309$, full statistical model) (Table S1B). In a simple model the fat pad

and the adrenal gland weight in the 25-Px group was also increased compared to control (p = 0.0154 and 0.0223, respectively). Overall, effects were not judged to be of major importance as the slightly lower body weights in the high dose group may have influenced the statistics, and no effects were seen in females, or in Study 1 males (Table S1A), or at PND 22 (Table S1B).

Liver

In both studies liver weights in offspring on PND 16/17 were increased at high doses of PFHxS (Fig. 6A and B and Table S1A and S1B). In Study 1, 25 and 45 mg/kg bw/day PFHxS caused higher liver weights in male on PND 16 compared to control, (Table S1A). In males from Study 2 the effect of PFHxS was only significant when the full statistical model was applied or when PFHxS exposure took place along with EDmix exposure. This observation emphasizes the increased statistical power of the full model including all 8 groups and may indicate increased sensitivity to PFHxS when exposed to a background of chemicals (EDmix).
In Study 2 females, PFHxS increased liver weights in both the 5-Px and 25-Px groups compared to the control (p = 0.0182 and p = 0.0478, respectively) and in the full model (Table S1B). At PND 22, similar, although less pronounced, increases in liver weights were found in both sexes (Table S1B). Although the EDmix appeared to reduce liver weights in Study 2 using the full statistical model, this may be due to a chance finding of high liver weights in the 5-Px group (p = 0.0279, Table S1B).

No exposure related effects on dam liver weight were observed in any study apart from a reduction in Study 2 by the lowest dose of PFHxS (compared to no PFHxS, full model p = 0.0329) and in the 0.05-Px+ED group compared to EDmix only (p = 0.0156, Table S1B). These effects were judged to be driven by the rather high mean liver weight in the EDmix-only group and do not appear to represent any toxicity of the chemicals.

Discussion

In the present study we have demonstrated that PFHxS can cause endocrine disruption in the thyroid hormone system of Wistar rats. Additionally, our results have shown that co-exposure to PFHxS and a mixture of environmentally relevant endocrine disrupting chemicals can affect nipple retention, anogenital distance and some reproductive organ weights, even if PFHxS or the mixture of EDCs on their own, showed no or only weak effects on these endpoints.

Thyroid hormone disruption

The consequences of decreased thyroid hormone levels or altered action during human brain development can be serious, even in the case of subclinical T4 reductions (Berbel *et al.*, 2009; Morreale de Escobar *et al.*, 2000). Such alterations in neurodevelopment may result in adverse behavioral effects and low IQ, outcomes with large consequences for healthcare expenses and life-time income (Bellanger *et al.*, 2015; Grandjean *et al.*, 2014). To our knowledge this is the first study that demonstrates significantly decreased total serum T4 levels in rat dams and their offspring after PFHxS administration. These significantly lower T4 levels were seen at 5 mg/kg bw/day, after only 7 days of exposure, indicating that PFHxS is an effective thyroid hormone disruptor in rats.

The thyroid effects observed here are consistent with studies showing decreased T4 levels in rats exposed to the structurally similar compound PFOS (Chang *et al.*, 2008; Yu *et al.*, 2009a, 2009b). Furthermore, hypertrophy/hyperplasia of the thyroid follicular epithelium was seen in male rats after 42 days of PFHxS exposure (Butenhoff *et al.*, 2009), supporting our findings of decreased T4 levels in dams. Butenhoff et al. suggested microsomal liver enzyme induction to be the cause of the

observed effects, as marked increase in liver weight was seen in their highest dosed animals (Butenhoff *et al.*, 2009). Such liver effects were not seen in our dams, and another likely mode of action for the observed T4 reductions could be binding of PFHxS to the thyroid hormone transport protein transthyretin (TTR), a common mode of action for perfluorinated compounds (Ren *et al.*, 2016; Weiss *et al.*, 2009).

Dam T4 reductions became more marked with time, with further decrease at weaning (PND 22), compared to GD 15. Data from Study 1 indicated that at higher doses, T4 reductions plateaued around 40% of control levels.

The observed postnatal T4 decreases in offspring, seen at PND 16/17, were likely due to lactational transfer of PFHxS. This is supported by data from Butenhoff et al. showing that at doses of 10 mg/kg, fetuses on GD 21 had serum concentrations 26% lower than dams, whereas pups at later stages (PND 22) had serum PFHxS concentrations 56% higher than dams at GD 21 (Butenhoff *et al.*, 2009). Additionally some studies on human PFHxS exposure find that breast milk is an important route of exposure (Kärrman *et al.*, 2007; Sundström *et al.*, 2011).

The mixture of 12 environmentally relevant endocrine disrupting chemicals (EDmix) contained some chemicals that on their own can affect the thyroid hormone system. However, the doses of the compounds included in the EDmix were all well below the reported No Observed Adverse Effect Levels (NOAELs) for T4 effects (Klammer *et al.*, 2007; Liu *et al.*, 2015; O'Connor *et al.*, 2002; Schneider *et al.*, 2011; Seidlová-Wuttke *et al.*, 2006, 2005; Yamada *et al.*, 2004). Yet, in PND 22 dams the ED mix caused a small, but statistically significant decrease in T4 levels, an effect which may have been caused by combination effects of the single chemicals in the EDmix.

Anti-androgenicity

PFHxS led to a slight but statistically significant positive trend in male nipple retention, indicating

some endocrine activity. However, PFHxS alone did not cause adverse effects on anogenital distance or reproductive organ weights at any dose. Interestingly, co-exposure to PFHxS and EDmix significantly increased nipple retention at 5 mg/kg PFHxS, and led to overall significant effects of EDmix on male anogenital distance and weight of the epididymides, ventral prostrate, and seminal vesicle. Thus, co-exposure of PFHxS to the EDmix seemed to enhance the anti-androgenic action of the EDmix, possibly due to altered toxicokinetics of EDmix as discussed further below.

In rats, alterations in AGD, nipple retention and prepubertal reproductive organ weights are good markers of anti-androgenic action, and predictive of adverse effects on the male reproductive system later in life (Christiansen *et al.*, 2008; van den Driesche *et al.*, 2011), with male nipple retention often being the most sensitive marker of anti-androgenic exposure during fetal sexual differentiation (Christiansen *et al.*, 2009; Laier *et al.*, 2006).

A mixture of EDCs similar to the EDmix in this study has previously been shown to adversely affect reproductive development. In our previous studies, the mixture also included paracetamol, which in itself seems to possess anti-androgenic properties (Axelstad *et al.*, 2014; Kristensen *et al.*, 2011). In those studies, the lowest tested mixture dose contained 32.11 mg/kg per day of environmental chemicals (corresponding to 100 times human high-end exposure) plus 80 mg/kg paracetamol (close to maximally recommended human therapeutic doses) given during two developmental windows sensitive to anti-androgens. This mixture significantly affected the female reproductive system at the lowest dose (Johansson *et al.*, 2016) whereas 2-4.5 fold higher doses affected male AGD, nipple retention and prepubertal reproductive organ weights (Axelstad *et al.*, 2014). In the present study the EDmix consisted of only the 32.11 mg/kg of environmental chemicals and although no statistical significance was detected on male nipple retention, AGD and reproductive organ weights if compared directly to the control, the full statistical model on the basis of all data suggested that these endpoints were weakly affected. In our typical experimental design

the direct effect comparison allows for the statistical detection of an average AGD reduction of at least 5% (Christiansen *et al.*, 2008; Isling *et al.*, 2014), whereas the full statistical model specifically adapted to the experimental design in this study was able to detect an approximately 2% decrease in AGD as statistically significant.

General- and reproductive toxicity

In the range-finding study, doses up to 45 mg/kg PFHxS did not lead to overt systemic toxicity in dams or offspring. However, in male pups doses of 25 and 45 mg/kg PFHxS increased liver weights and induced modest but significant decreases in birth weight. Additionally, we found female pup liver weight to be increased already at 5 mg/kg PFHxS on PND 17 and at 25 mg/kg on PND 22. This indicates a potential for PFHxS-induced developmental toxicity at doses even below the 10 mg/kg that was previously reported as a NOAEL for effects in dams or offspring (Butenhoff *et al.*, 2009). The lack of other systemic toxicities of PFHxS is in contrast to the effects of PFOS, which caused >95% postnatal pup dead at 5 mg/kg (Lau *et al.*, 2003), possibly by acting as a surfactant in the lungs of the newborn rats (Lau, 2012).

Gender differences and toxicokinetics

Interestingly, Butenhoff et al., (2009) observed adverse effects on liver and thyroid gland after repeated PFHxS administration (3 mg/kg and 10 mg/kg) in the parental males, but not in dams, fetuses or pups. They also found strong gender differences in internal PFHxS levels, with serum levels in the males at least 4 times greater than the adult females in the 10 mg/kg group. In addition their results indicate a gender difference in distribution of PFHxS as the liver to serum ratio of PFHxS was greater than 2.5 in male rats and below 0.5 in females and offspring (Butenhoff *et al.*, 2009). In our study, external doses of 25 and 45 mg/kg resulted in serum PFHxS concentrations in pregnant dams similar to those in adult male rats after exposure to 10 mg/kg (Butenhoff *et al.*,

2009). Indeed, the half-life of PFHxS in female rats is lower than in males (Kim *et al.*, 2016; Sundström *et al.*, 2012) with consequently less internal exposure. The major elimination route for PFHxS is through the urine and for PFOA it has been demonstrated that this secretion can be strongly increased via organic anion transporter(s) in the renal tubules that appear in females rats around puberty (Kudo *et al.*, 2002) . If also true for PFHxS we can expect similar kinetics for prepubertal males and females and that might explain why we see no clear gender differences in thyroid and liver effects at PND 16/17.

Based on the observation of more marked effects on T4, anti-androgenic endpoints and liver weight in the groups co-exposed to EDmix and PFHxS, we hypothesize that co-exposure could cause changes to absorption, distribution, and/or excretion properties of either PFHxS or the EDmix (note that PFHxS is not expected to be metabolized). This hypothesis can be examined indirectly via serum PFHxS determinations from Study 1, where PFHxS serum levels appeared higher in groups with concomitant exposure to the mixture. The reason for the non-significant increase in internal serum PFHxS levels when administered in combination with other EDCs is unknown to us and we can only speculate that the EDC co-exposure may have interacted directly with PFHxS-specific kinetic processes such that more PFHxS was absorbed and/or less PFHxS eliminated. Similarly, it is conceivable that the increased chemical load in the animals exposed to both PFHxS and the EDmix may have exacerbated the anti-androgenic effects of the EDmix by decreasing the elimination of the mixture.

Conclusions

We have demonstrated that PFHxS can induce marked reductions in circulating serum T4 in rats, which at critical developmental stages can lead to altered brain morphology and adverse behavior. Additionally, we have shown that a mixture of environmentally relevant EDCs can cause anti-androgenic effects at a lower dose than previously reported. Interestingly, PFHxS and the EDmix

appear to exacerbate the effects of each other despite different modes of action. This illustrates that risk assessment may underestimate the toxicity of a chemical when mixture toxicity and background exposures are not taken into account.

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Figure Legends

Fig. 1. Study designs. A. Study design Study 1 and 2. Two reproductive toxicity studies were performed as shown. The endpoints for dams and offspring are depicted along with time-points. Exposure of dams to vehicle, perfluorohexane sulfonate (PFHxS) and/or a mixture of endocrine disrupting chemicals (EDmix) from gestational day 7 (GD) through postnatal day 22 (PND) except day of delivery. B. The full statistical model in Study 2 was modelled on the 2 x 4 design matrix depicted, this model allowed for comparisons of PFHxS dose against no PFHxS exposure (control and EDmix groups) and for EDmix exposed groups against no EDmix groups (control and PFHxS exposed groups).

AGD = anogenital distance, NR = nipple retention, T4 = thyroxine

n = time mated dams.

Fig. 2. Dam serum PFHxS concentrations on PND 22 (Study 1). Control mean = 0.081 µg/ml, close to detection limit due to dilutions performed in the assay.

Px = Perfluorohexane sulfonate (PFHxS), ED = EDmix. Data are shown as mean + SEM. n = 5-7.

Fig. 3. Serum thyroxine (T4) after exposure to PFHxS alone and in combination with a fixed dose of endocrine disrupting chemicals (EDmix). Dams were affected by both PFHxS and EDmix, pups only by PFHxS. A. Dam serum T4 on GD 15 was reduced from 5 mg/kg bw/day PFHxS. B. Dam serum T4 on PND 22 were reduced in all 4 exposed groups, Study 1. C. Dam serum T4 at PND 22, Study 2. The background EDmix exposure overall had a decreasing effect on T4 compared to no EDmix exposure ($p=0.0242$). D. Pup serum T4 based on litter means of up to 1 male and 1 female pup per litter, Study 1. Doses of 25 and 45 mg/kg body weight reduced serum T4 on PND 16. E.

Pup serum T4 on PND 16/17, Study 2. Doses of 5 and 25 mg PFHxS/kg body weight/day reduced serum T4 on PND 16/17.

Px = Perfluorohexane sulfonate (PFHxS), ED = EDmix, N/A = not available. Data are shown as mean + SEM. A. and C: n = 13-15 (except control with n = 20) for each group. B: n = 5-7. D: n = litter means (from up to one male and one female pup per litter) of 5-7 litters. E: n = 14-18 litters represented by either a male or a female pup.

** $p < 0.01$ compared to control, *** $p < 0.001$ compared to control, # $p < 0.05$ compared to EDmix, +++ $p < 0.0001$ for full model comparison of indicated dose of PFHxS compared to no PFHxS exposure in the control and EDmix group. Comparisons of PFHxS+EDmix groups against EDmix not shown.

Fig. 4. Anogenital distance (AGD, A) and nipple retention (NR, B-C) in male pups following gestational exposure to PFHxS alone and in combination with a fixed dose of endocrine disrupting chemicals (EDmix). EDmix significantly decreased AGD and NR was increased at high doses of PFHxS in the presence of the EDmix..

Px = Perfluorohexane sulfonate (PFHxS), ED = EDmix. A: Data are shown as litter mean + SEM. B and C: Data are shown as litter mean ± SEM A and C: n = 13-20 litters, B: n = 5-7 litters. Anogenital distance is expressed as units (1 unit = 0.1626 mm). Statistical analysis performed on data from all pups in each litter and adjusting for litter effects.

* $p < 0.05$ compared to control, ** $p < 0.01$ compared to control.

Fig. 5. Male reproductive organs in male pups on PND 16 following gestational exposure to PFHxS alone and in combination with a fixed dose of endocrine disrupting chemicals (EDmix). PFHxS

exposure had no effect on organ weights. The EDmix had an overall decreasing effect on the weights of the epididymides (A), ventral prostrate (B), and the vesicular seminalis (C), but no effects on glandula bulbourethralis (D), levator ani (E) or testis weight (F and G).
Px = Perfluorohexane sulfonate (PFHxS), ED = EDmix. Data are shown as mean + SEM. n = 10 – 16.
* $p<0.05$ compared to no EDmix exposure.

Fig. 6. Pup relative liver weights after developmental exposure to PFHxS alone and in combination with a fixed dose of endocrine disrupting chemicals (EDmix). There was a linear relationship between liver weight and body weight (male (A) PND 16: $R^2 = 0.85$ and female (B) PND 17: $R^2 = 0.91$). Therefore, to ease visual inspection, relative weights of the livers are depicted along with the results of the statistical analysis on relative weights, the absolute weights can be found in Table S1A & B). Relative liver weights PND 16/17 were increased by PFHxS in the presence of the background EDmix exposure (not shown) and when analyzed in the full statistical model comparing PFHxS exposure at 3 levels against no PFHxS exposure in the control and EDmix groups.
Px = Perfluorohexane sulfonate (PFHxS), ED = EDmix. Data are shown as mean + SEM. n = 11-16 for each group. + <0.05 and ++ <0.01 for full model comparison of indicated dose of PFHxS compared to no PFHxS exposure in the control and EDmix group.

Tables

Table 1. Composition of the mixture of endocrine disrupting chemicals (EDmix)*

Chemical	**CAS Registry Number**	**Purity**	**Dose (mg/kg bw/day)**
DBP (Dibutyl phthalate)	84-74-2	>99.0%	1.00
DEHP (di-2-ethylhexyl phthalate)	117-81-7	>99.5%	2.00
Vinclozolin	50471-44-8	>99.5%	0.90
Prochloraz	67747-09-5	>98.5%	1.40
Procymidone	32809-16-8	>99.5%	1.50
Linuron	330-55-2	>99.0%	0.06
Epoxyconazole	106325-08-8	>99.0%	1.00
4-MBC (4-Methylbenzylidene camphor)	36861-47-9	>98.0%	6.00
OMC (Octyl methoxycinnamate)	5466-77-3	>98.0%	12.00
p,p'-DDE (Dichlorodiphenyldichloroethylene)	72-55-9	>98.5%	0.10
Bisphenol A	80-05-7	>99.5%	0.15
Butyl paraben	94-26-8	>99.0%	6.00
Total dose			**32.11**

*See Axelstad et al. (2014) for details (Axelstad *et al.*, 2014)
bw = body weight

Table 2. Dosing

Group	PFHxS (mg/kg bw/day)	EDmix (mg/kg bw/day)	Time mated dams	Viable litters
Study 1				
Control	-	-	8	5
25-Px	25	-	8	6
25-Px+ED	25	32.11	8	5
45-Px	45	-	8	7
45-Px+ED	45	32.11	8	7
Study 2				
Control	-	-	20	20
EDmix	-	32.11	16	13
0.05-Px	0.05	-	20	16
0.05-Px+ED	0.05	32.11	16	13
5-Px	5	-	20	19
5-Px+ED	5	32.11	16	15
25-Px	25	-	20	17
25-Px+ED	25	32.11	16	15

Px = Perfluorohexane sulfonate (PFHxS).
ED = EDmix (mixture of endocrine disrupting chemicals, see Table 1)

Table 3A Pregnancy and litter data – Study 1

	Control	25-Px	25-Px+ED	45-Px	45-Px-+ED
Time-mated females (no.)	8	8	8	8	8
Viable litters (no.)	5	6	5	7	7
Maternal bw GD 7 (g)^	231.1 ± 12.5	227.2 ± 12.9	225.4 ± 11.5	229.3 ± 9.5	227.8 ± 11.2
Maternal bw gain GD 7-GD 21 (g)^	80.7 ± 19.6	86.4 ± 18.0	85.2 ± 13.7	77.0 ± 8.4	71.0 ± 17.6
Maternal bw gain GD 7- PND 1 (g)^	15.5 ± 11.6	14.8 ± 6.1	19.0 ± 4.9	13.3 ± 10.9	15.6 ± 6.9
Maternal bw gain PND 1-PND 14 (g)^	40.8 ± 14.5	41.0 ± 17.0	34.4 ± 8.0	43.0 ± 8.9	33.9 ± 15.7
Gestational length (days)	23.2 ± 0.41	22.9 ± 0.45	22.8 ± 0.45	22.8 ± 0.39	23.0 ± 0.58
Litter size. Live pups PND 1 (no.)	10.0 ± 4.1	9.4 ± 4.6	10.8 ± 2.5	10.0 ± 2.3	7.9 ± 3.4
Postimplantation loss (%)	29.6 ± 40.1	13.9 ± 19.4	16.2 ± 6.7	26.9 ± 31.4	20.3 ± 17.1
Perinatal loss (%)	29.6 ± 40.1	26.9 ± 35.7	21.2 ± 11.1	27.8 ± 31.1	21.4 ± 16.9
Birth weight. Male pups (g)	6.7 ± 0.6	6.4 ± 0.5	6.3 ± 0.5	6.7 ± 0.5	6.6 ± 0.6
Birth weight. Female pups (g)	6.3 ± 0.6	6.2 ± 0.5	6.1 ± 0.6	6.0 ± 0.4	6.1 ± 0.5
AGD. Males (units[1])	22.1 ± 0.6	22.2 ± 1.4	20.9 ± 0.4	22.5 ± 0.9	23.0 ± 0.8
AGD. Females (units[1])	12.0 ± 0.9	12.1 ± 0.8	11.5 ± 0.5	11.7 ± 0.8	12.2 ± 1.0
Body weight. PND 6. Male pups (g)	14.0 ± 2.1	13.3 ± 1.1	13.2 ± 1.4	14.2 ± 2.4	13.8 ± 0.7
Body weight. PND 6. Female pups (g)	13.3 ± 2.3	12.9 ± 1.1	13.0 ± 1.3	13.1 ± 2.1	13.3 ± 1.2
Body weight. PND 14. Male pups (g)	31.7 ± 5.8	28.0 ± 2.1	29.6 ± 3.9	30.6 ± 6.3	30.3 ± 3.4
Body weight. PND 14. Female pups (g)	30.5 ± 6.2	27.6 ± 2.4	28.8 ± 3.9	29.2 ± 5.8	29.2 ± 4.2
Body weight. PND 22. Male pups (g)	53.9 ± 10.1	51.6 ± 6.9	52.0 ± 7.9	52.5 ± 10.3	51.5 ± 4.3
Body weight. PND 22. Female pups (g)	52.8 ± 9.5	50.2 ± 6.4	51.8 ± 7.3	50.4 ± 9.3	50.52 ± 3.9

Data represent group means based on dam or litter means ± SD.
Px = Perfluorohexane sulfonate (PFHxS)

ED = EDmix (mixture of endocrine disrupting chemicals, see Table 1)
PND = Postnatal day
GD = Gestational day
AGD = Anogenital distance
^only pregnant dams giving birth to viable litters
[1]1 unit = 0.164 mm (i.e. male AGD of 22.1 units corresponds to 3.62 mm)
* $p < 0.05$ compared to control

Table 3B Pregnancy and litter data - Study 2

	Control	**EDmix**	**0.05-Px**	**0.05-Px+ED**	**5-Px**	**5-Px+ED**	**25-Px**	**25-Px+ED**	Effect in groups exposed to EDmix versus groups not exposed to EDmix
Time-mated females (no.)	20	16	20	16	20	16	20	16	
Viable litters (no.)	20	13	16	13	19	15	17	15	
Maternal bw GD7 (g)^	227.2 ± 11.7	230.2 ± 10.8	225.0 ± 12.2	230.3 ± 9.9	227.4 ± 12.6	228.9 ± 12.0	231.9 ± 14.8	228.8 ± 11.9	
Maternal bw gain GD7-GD21 (g)^	84.0 ± 13.9	92.7 ± 15.7	87.0 ± 17.7	81.3 ± 17.0	85.1 ± 19.0	83.2 ± 22.1	87.9 ± 12.0	90.0 ± 9.5	
Maternal bw gain GD7- PD1 (g)^	21.0 ± 11.6	19.0 ± 8.5	23.3 ± 10.6	19.8 ± 11.3	19.9 ± 12.6	17.9 ± 12.0	17.8 ± 8.3	15.5 ± 7.8	
Maternal bw gain PND1-PND14^	34.3 ± 9.2	43.3 ± 8.9	35.5 ± 14.5	38.2 ± 16.1	35.3 ± 16.5	39.1 ± 11.4	37.7 ± 9.0	44.1 ± 10.9	↑
Gestational length (d)	23.0 ± 0.3	22.9 ± 0.3	22.9 ± 0.3	23.0 ± 0.3	22.9 ± 0.3	23.1 ± 0.4	22.9 ± 0.3	22.9 ± 0.4	
Litter size. live pups. PD 1 (no.)	10.1 ± 3.6	11.8 ± 2.2	10.1 ± 3.8	9.5 ± 3.5	10.0 ± 3.5	10.0 ± 4.5	11.2 ± 2.1	12.2 ± 1.8	
Postimplantation loss (%)	5.9 ± 12.5	1.8 ± 3.4	5.8 ± 12.7	2.1 ± 4.1	4.3 ± 11.8	3.3 ± 7.6	7.0 ± 18.7	8.3 ± 8.4	
Perinatal loss (%)	13.3 ± 16.8	18.5 ± 24.5	23.4 ± 31.5	19.4 ± 25.8	9.6 ± 15.2	10.3 ± 18.4	14.6 ± 19.0	12.88 ± 11.84	
Birth weight. Male pups (g)	6.6 ± 0.5	6.6 ± 0.4	6.7 ± 0.5	6.7 ± 0.6	6.7 ± 0.5	6.6 ± 0.3	**6.5 ± 0.4** ↓ compared to control + EDmix	**6.1 ± 0.5 ##**	
Birth weight. Female pups (g)	6.2 ± 0.5	6.2 ± 0.3	6.4 ± 0.5	6.4 ± 0.5	6.2 ± 0.5	6.1 ± 0.4	6.1 ± 0.4	6.0 ± 0.3	
AGD. Males (units)	22.7 ± 1.6	21.8 ± 1.2	23.0 ± 1.0	22.4 ± 1.3	22.6 ± 1.3	22.2 ± 1.0	22.1 ± 1.0	21.8 ± 0.9	↓
AGD. Females (units)	11.3 ± 0.6	11.2 ± 1.1	11.6 ± 0.7	11.2 ± 0.7	11.6 ± 0.9	11.8 ± 1.1	11.3 ± 0.5	11.3 ± 0.8	
Body weight. PND 6. Male pups (g)	13.7 ± 1.9	13.9 ± 1.4	14.1 ± 2.2	14.1 ± 2.2	14.2 ± 1.5	13.3 ± 1.5	13.4 ± 1.1	13.2 ± 1.1	
Body weight. PND 6. Female pups (g)	13.4 ± 1.5	13.3 ± 1.5	13.5 ± 1.9	13.6 ± 2.1	**13.4 ± 1.6** ↓ compared to control + EDmix	**11.8 ± 1.9 #**	13.1 ± 1.2	12.9 ± 1.0	
Body weight. PND 14. Male pups (g)	30.3 ± 5.3	29.7 ± 3.4	30.9 ± 5.3	31.1 ± 5.0	30.9 ± 4.4	28.9 ± 4.5	29.0 ± 3.8	28.5 ± 3.3	
Body weight. PND 14. Female pups (g)	30.2 ± 5.1	28.9 ± 3.3	30.4 ± 5.0	30.8 ± 4.7	**29.4 ± 4.3** ↓ compared to control + EDmix	**26.6 ± 5.0**	28.5 ± 4.1	28.1 ± 3.3	
Body weight. PND 22. Male pups (g)	51.5 ± 7.6	50.6 ± 5.5	52.8 ± 7.4	53.6 ± 7.7	52.3 ± 6.8	51.6 ± 8.8	50.5 ± 5.9	50.7 ± 5.9	
Body weight. PND 22. Female pups (g)	50.9 ± 7.5	49.4 ± 5.4	51.6 ± 7.3	52.3 ± 6.6	50.6 ± 6.0	46.9 ± 8.8	49.6 ± 5.8	49.4 ± 6.2	

Data represent group means based on dam or litter means ± SD.
Px = Perfluorohexane sulfonate (PFHxS)

ED = EDmix (mixture of endocrine disrupting chemicals, see Table 1)
PND = Postnatal day
GD = Gestational day
AGD = Anogenital distance
^only pregnant dams giving birth to viable litters
[1]1 unit = 0.1626 mm (i.e. male AGD of 22.7 units corresponds to 3.69 mm)
# $p < 0.05$ compared to EDmix
$p < 0.01$ compared to EDmix
↓ or ↑ $p < 0.05$ compared to indicated exposure

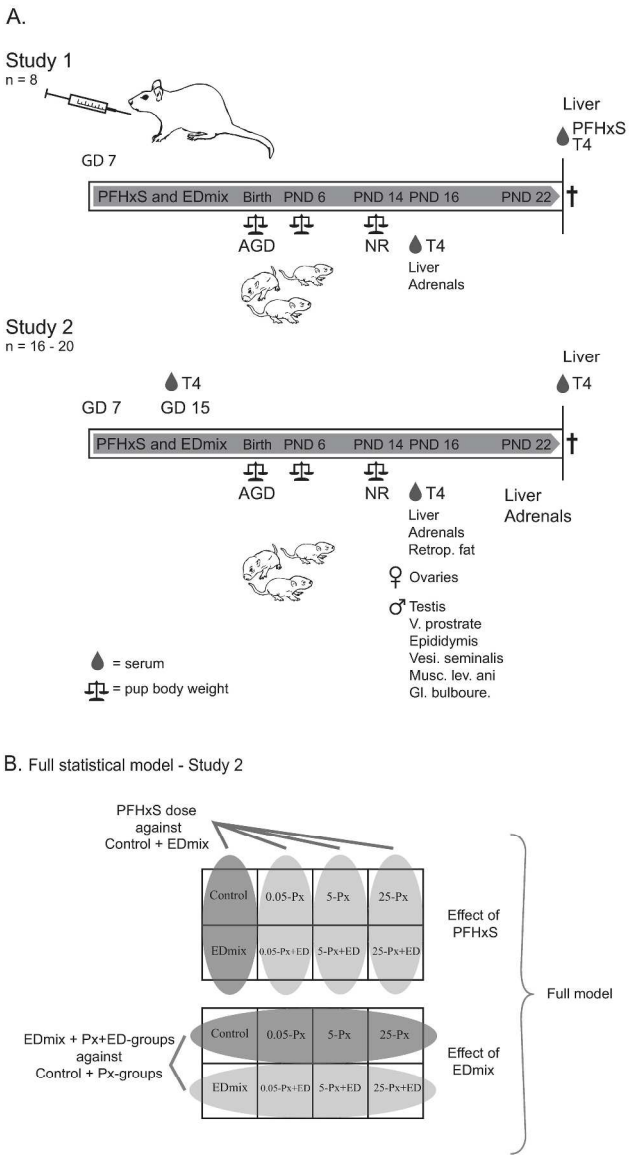

Fig. 1. Study designs. A. Study design Study 1 and 2. Two reproductive toxicity studies were performed as shown. The endpoints for dams and offspring are depicted along with time-points. Exposure of dams to vehicle, perfluorohexane sulfonate (PFHxS) and/or a mixture of endocrine disrupting chemicals (EDmix) from gestational day 7 (GD) through postnatal day 22 (PND) except day of delivery. B. The full statistical model in Study 2 was modelled on the 2 x 4 design matrix depicted, this model allowed for comparisons of PFHxS dose against no PFHxS exposure (control and EDmix groups) and for EDmix exposed groups against no EDmix groups (control and PFHxS exposed groups). AGD = anogenital distance, NR = nipple retention, T4 = thyroxine, n = time mated dams.

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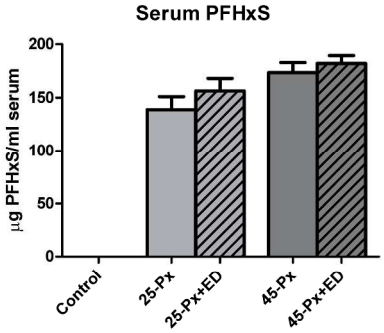

Fig. 2. Dam serum PFHxS concentrations on PND 22 (Study 1). Control mean = 0.081 µg/ml, close to detection limit due to dilutions performed in the assay. Px = Perfluorohexane sulfonate (PFHxS), ED = EDmix. Data are shown as mean + SEM. n = 5-7.

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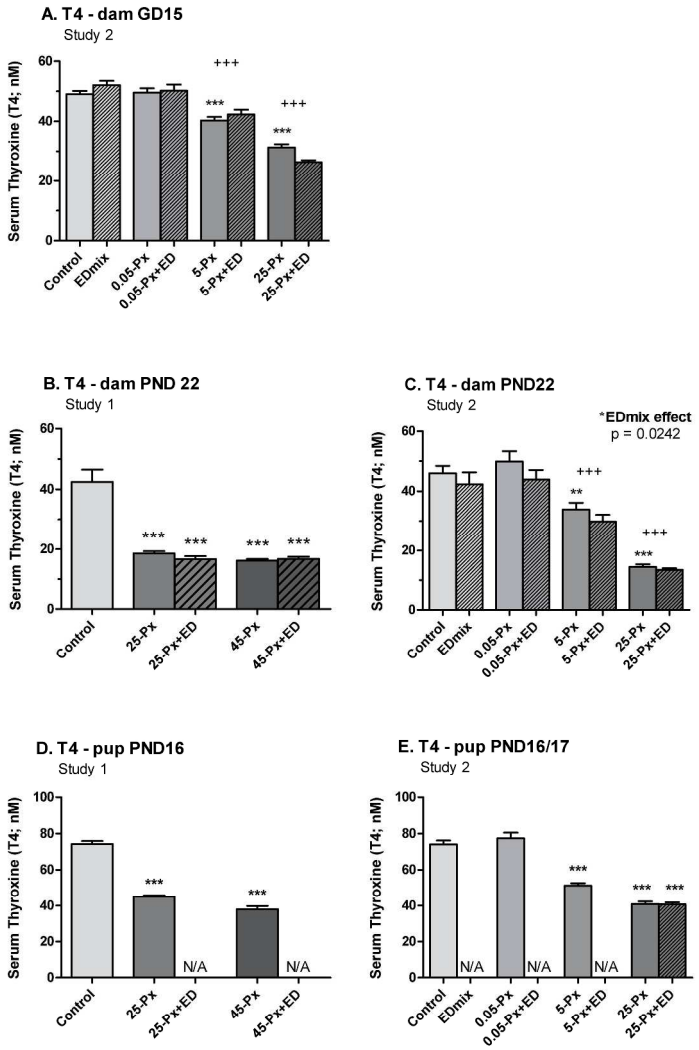

Fig. 3. Serum thyroxine (T4) after exposure to PFHxS alone and in combination with a fixed dose of endocrine disrupting chemicals (EDmix). Dams were affected by both PFHxS and EDmix, pups only by PFHxS. A. Dam serum T4 on GD 15 was reduced from 5 mg/kg bw/day PFHxS. B. Dam serum T4 on PND 22 were reduced in all 4 exposed groups, Study 1. C. Dam serum T4 at PND 22, Study 2. The background EDmix exposure overall had a decreasing effect on T4 compared to no EDmix exposure (p=0.0242). D. Pup serum T4 based on litter means of up to 1 male and 1 female pup per litter, Study 1. Doses of 25 and 45 mg/kg body weight reduced serum T4 on PND 16. E. Pup serum T4 on PND 16/17, Study 2. Doses of 5 and 25 mg PFHxS/kg body weight/day reduced serum T4 on PND 16/17. Px = Perfluorohexane sulfonate (PFHxS), ED = EDmix, N/A = not available. Data are shown as mean + SEM. A. and C: n = 13-15 (except control with n = 20) for each group. B: n = 5-7. D: n = litter means (from up to one male and one female pup per litter) of 5-7 litters. E: n = 14-18 litters represented by either a male or a female pup. ** p <0.01 compared to control, *** p <0.001 compared to control, # p <0.05 compared to EDmix, +++ p <0.0001 for full model comparison of indicated dose of PFHxS compared to no PFHxS exposure in the control and

EDmix group. Comparisons of PFHxS+EDmix groups against EDmix not shown.

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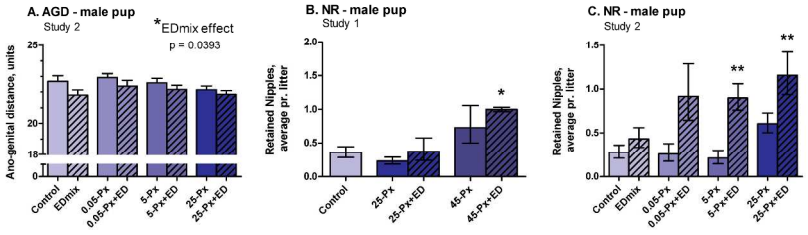

Fig. 4. Anogenital distance (AGD, A) and nipple retention (NR, B-C) in male pups following gestational exposure to PFHxS alone and in combination with a fixed dose of endocrine disrupting chemicals (EDmix). EDmix significantly decreased AGD and NR was increased at high doses of PFHxS in the presence of the EDmix. Px = Perfluorohexane sulfonate (PFHxS), ED = EDmix. A: Data are shown as litter mean + SEM. B and C: Data are shown as litter mean ± SEM A and C: n = 13-20 litters, B: n = 5-7 litters. Anogenital distance is expressed as units (1 unit = 0.1626 mm). Statistical analysis performed on data from all pups in each litter and adjusting for litter effects. * $p < 0.05$ compared to control, ** $p < 0.01$ compared to control.

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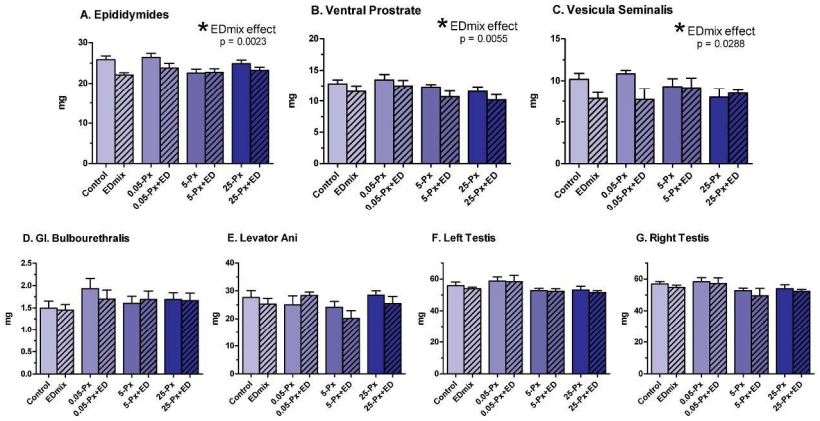

Fig. 5. Male reproductive organs in male pups on PND 16 following gestational exposure to PFHxS alone and in combination with a fixed dose of endocrine disrupting chemicals (EDmix). PFHxS exposure had no effect on organ weights. The EDmix had an overall decreasing effect on the weights of the epididymides (A), ventral prostrate (B), and the vesicular seminalis (C), but no effects on glandula bulbourethralis (D), levator ani (E) or testis weight (F and G). Px = Perfluorohexane sulfonate (PFHxS), ED = EDmix. Data are shown as mean + SEM. n = 10 – 16. * $p<0.05$ compared to no EDmix exposure.

287x412mm (300 x 300 DPI)

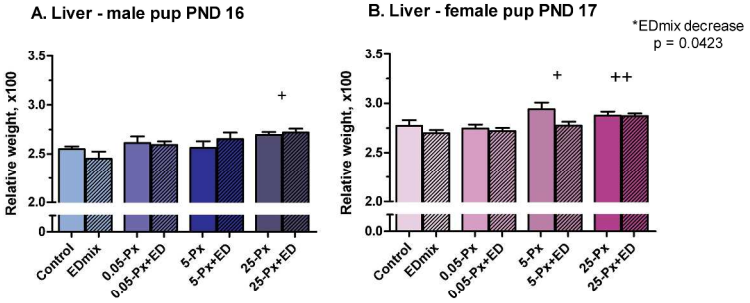

Fig. 6. Pup relative liver weights after developmental exposure to PFHxS alone and in combination with a fixed dose of endocrine disrupting chemicals (EDmix). There was a linear relationship between liver weight and body weight (male (A) PND 16: R2 = 0.85 and female (B) PND 17: R2 = 0.91). Therefore, to ease visual inspection, relative weights of the livers are depicted along with the results of the statistical analysis on relative weights, the absolute weights can be found in Table S1A & B). Relative liver weights PND 16/17 were increased by PFHxS in the presence of the background EDmix exposure (not shown) and when analyzed in the full statistical model comparing PFHxS exposure at 3 levels against no PFHxS exposure in the control and EDmix groups. Px = Perfluorohexane sulfonate (PFHxS), ED = EDmix. Data are shown as mean + SEM. n = 11-16 for each group. + <0.05 and ++ <0.01 for full model comparison of indicated dose of PFHxS compared to no PFHxS exposure in the control and EDmix group.

287x412mm (300 x 300 DPI)