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In vitro - in vivo correlations for endocrine activity of a mixture of currently used pesticides

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ABSTRACT

Two pesticide mixtures were investigated for potential endocrine activity. Mix 3 consisted of bitertanol, propiconazole, and cypermethrin, and Mix 5 included malathion and terbuthylazine in addition to the three pesticides in Mix 3.

All five single pesticides and the two mixtures were investigated for their ability to affect steroidogenesis in vitro in H295R cells. The pesticides alone and both mixtures affected steroidogenesis with both mixtures causing increase in progesterone and decrease in testosterone. For Mix 5 an increase in estradiol was seen as well, indicating increased aromatase activity.

The two mixtures were also investigated in pregnant rats dosed from gestational day 7 to 21, followed by examination of dams and fetuses. Decreased estradiol and reduced placental testosterone were seen in dams exposed to Mix 5. Also a significant increase in aromatase mRNA-levels in female adrenal glands was found for Mix5. However, either of the two mixtures showed any effects on fetal hormone levels in plasma or testis, or on anogenital distance.

Overall, potential aromatase induction was found for Mix 5 both in vitro and in vivo, but not for Mix 3, an effect likely owed to terbuthylazine in Mix 5. However, the hormonal responses in vitro were only partly reflected in vivo, probably due to some toxicokinetic issues, as the pesticide levels in the amniotic fluid also were found to be negatively affected by the number of compounds present in the mixtures. Nonetheless, the H295R assay gives hints on conceivable interference with steroidogenesis, thus generating hypotheses on in vivo effects.

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Introduction

The high prevalence of disorders related to the endocrine system, e.g. fertility problems and congenital malformations of reproductive organs, has been of growing concern for several years. Genetic, environmental and lifestyle factors are likely to be involved in these adverse effects, and one of these factors is developmental exposure to endocrine active compounds (EACs) (IPCS, 2002).

Humans are exposed to a mixture of several EACs (Blount et al., 2000; Swan et al., 2005), and during the last decade, scientific and regulatory focus has gradually shifted towards taking mixture effects into account.

In studies where experimental animals have been exposed simultaneously to several EACs, e.g. pesticides, substantial mixture effects on reproductive development have been seen, even though each of the individual compounds was present at low doses, where no effects were seen (Hass et al., 2007, 2012; Jacobsen et al., 2012; Metzdorff et al., 2007; Silva et al., 2002). In addition, there are indications that cumulative exposure to EACs such as pesticides may play a role causing adverse effects on human development. In epidemiological studies possible association between risk of cryptorchidism and maternal pesticide exposure has been reported (Andersen et al., 2008; Carbone et al., 2007; Kristensen et al., 1997; Weidner et al., 1998). Because the adverse effects of some pesticide mixtures occur at exposure levels at which the single pesticides do not cause adverse effects, it raises concerns about their potential combined impact on human health. Thus, there is a need for continued research on the effect of combined exposure in order to gain more knowledge on mixture effects.

The present study forms part of a larger project, in which an initial screening of 13 currently used pesticides was performed, applying a battery of in vitro assays, including assays for effects on the estrogen receptor (ER), the androgen receptor (AR), the aryl hydrocarbon receptor (AhR), the thyroid hormone receptor (TR), and steroidogenesis (Kjeldsen et al., 2013). The aim of the screening was to reveal potential mechanisms of action as well as to determine the potency of the

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pesticides. The selection of test compounds was based on a list of pesticides that were registered and approved for use in Denmark (except for malathion prohibited in 2008) as well as their endocrine disrupting potential, as described both in the open literature and in draft assessment reports (DARs). The overall aim of the project was to evaluate the predictability of the *in vitro* tests for potential endocrine disrupting effects *in vivo*. Based on the initial *in vitro* tests five pesticides were selected for further study. The five pesticides chosen for the current study represent different classes of pesticides, and included the fungicides bitertanol and propiconazole, the insecticides cypermethrin and malathion, and the herbicide terbuthylazine. The selection of pesticides was based on their potency and efficacy in the initial *in vitro* tests, with a special focus on data from the H295R steroidogenesis assay. The five pesticides were mixed in two different mixtures. A mixture named "Mix 3" consisting of: bitertanol, propiconazole, and cypermethrin mixed in the ratio 1:1:1 and a mixture called "Mix 5" consisting of all five pesticides mixed in a 1:1:1:1:1 ratio.

In the present study the aim was to investigate the potential endocrine disrupting effects of the selected pesticide mixtures *in vitro* using the H295R steroidogenesis assay, and *in vivo* in an *in utero* exposure rat study. In the *in vitro* experiment all five single pesticides were tested in addition to the two mixtures. For the *in vitro* study the intention was not to perform any mixture modeling or mixture predictions, but rather to compare the effect of the single pesticides to the effects of the mixtures to see if we by intuition were able to predict the qualitative response of the mixtures.

In the *in vivo* study pregnant rats were dosed with the two pesticide mixtures from gestation day (GD) 7 to 21. At GD 21 fetuses were removed by cesarean section, and various endpoints were measured to examine potential endocrine disrupting effects of the mixtures.
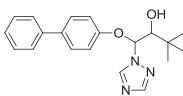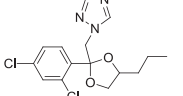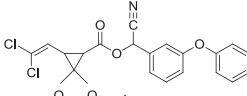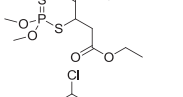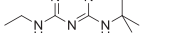
Material and methods

Chemicals

Cypermethrin, PESTANAL®, analytical standard (CAS no. 52315-07-8), malathion, PESTANAL®, analytical standard (CAS no. 121-75-5), bitertanol PESTANAL®, analytical standard (CAS no. 55179-31-2), propiconazole PESTANAL®, analytical standard (CAS no. 60207-90-1), and terbuthylazine PESTANAL®, analytical standard (CAS no. 5915-41-3) were all purchased from Sigma-Aldrich (St. Louis, USA). The test compounds were dissolved in corn oil (no. C8267-2.5L) for the *in vivo* study or in dimethyl sulfoxide (DMSO, CAS 67-68-5) for the *in vitro* studies, respectively, both from Sigma-Aldrich (St. Louis, USA). Table 1 lists the names, chemical structures and CAS numbers of the pesticides.

H295R assay

Cell culture and chemicals. The effects of the single pesticides and the two pesticide mixtures on the production of progesterone, testosterone, and estradiol were tested in the NCI-H295R human adrenocortical carcinoma cell line (ATCC no. CRL-2128, LGC Standards, Boras, Sweden) as previously described (Hecker et al., 2011). In brief cells were seeded in 24-well culture plates (Costar3524,Corning,NY, USA) with DMEM/F12 medium (Gibco, Paisley, UK) supplemented with 2.0% Nu-serum (BD Sciences Denmark) along with 1% ITS+ premix (containing6.25 μg/ml insulin, 6.25 μg/ml transferrin,6.25 ng/ml selenium,1.25 mg/ml BSA and 5.35 μg/ml linoleic acid; BD Sciences Denmark) and incubated for 24 h at37 °C in a humidified atmosphere of 5% CO_2/air. The pesticides and mixtures were added (1 ml) to the cells in triplicates at six concentrations ranging from 1.6 to 100 μM, and left to incubate for 48 h. Control wells (0 μM) contained the same amount of DMSO (0.1%) as exposed cells. After the 48 h incubation period the medium was removed and stored at −80 °C until the hormone analyses. Hormone levels were normalized to the solvent control containing 0.1% DMSO. All hormone measurements for single pesticides and mixtures were repeated in at

least three independent experiments. For evaluation of cytotoxicity, cells were added 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (cat. no. M2128, Sigma, St. Louis, USA) once the media for the hormone analysis had been removed. After an incubation period for approximately 1.5 h at 37 °C at 5% CO_2 the MTT was removed, and 0.5 ml isopropanol was added and the contents were mixed for 5 min on a plate shaker (Hecker et al., 2011). Fluorescence was next measured (excitation 560 nm, emission 590 nm) on Wallac Victor[2] 1420 multilabel counter (PerkinElmer, Massachusetts, USA).

Hormone measurements. Hormones were extracted from cell supernatants using a C18 solid phase extraction (SPE) cartridge (200 mg) (prod. no. 220-0020-B, Mikrolab Aarhus Denmark) as previously described (Vinggaard et al., 2002). The eluate was evaporated for approximately 4 1/2 h in a centrifugal vacuum concentrator (SpeedVac, Thermo Fisher Scientific, Waltham, MA, USA). Samples were resuspended in 200 ml Diluent 1 (PerkinElmer, Waltham, MA, USA) and stored at 4 °C. Samples were next placed in a water bath for 10 min at 45 °C to dissolve the steroid hormones. Estradiol, progesterone and testosterone were measured using commercially available time-resolved fluoroimmunoassay kits (Wallac DELFIA®) purchased from PerkinElmer (Skovlunde, Denmark) according to the description of the manufacturer (Estradiol: prod. no. 1244-056, Progesterone: prod. no. A066-101, Testosterone: prod. no. A050-101).

Animals and exposure

The animal study was performed under conditions approved by the Danish Animal Experiments Inspectorate and by the in-house Animal Welfare Committee. The study included 84 time-mated Wistar rats (HanTac:WH, Taconic Europe, Ejby, Denmark) supplied on gestational day (GD) 3 and upon arrival, randomly distributed in pairs and housed under standard conditions: semitransparent polycarbonate cages (15 × 27 × 43 cm) with Aspen bedding (Tapvei, Denmark) situated in an animal room with controlled environmental conditions (12 h light-dark cycles with light starting at 9 p.m., light intensity 500 lx, temperature 21 ± 2 °C, humidity 50% ± 5%, ventilation 8 air changes per h). A complete rodent diet for growing animals ALTROMIN 1314 (Soy- and alfalfa-free ALTROMIN GmbH, Lage, Germany) and acidified tap water (to prevent microbial growth) were provided *ad libitum*. The animals

Table 1
Pesticides included in the study.

Pesticide	Chemical structure	CAS number
Bitertanol		55179-31-2
Propiconazole		60207-90-1
Cypermethrin		52315-07-8
Malathion		121-75-5
Terbuthylazine		5915-41-3

were observed twice daily for signs of toxicity and body weights were recorded daily during the experimental period.

The day after arrival, *i.e.* GD 4, animals were weighed and distributed into seven groups of 12 animals, with similar weight distributions. An acclimatization period of 4 days was allowed before starting exposure. Dams were exposed by gavage to pesticide mixtures daily from GD 7 to 21. At GD 21 the last dosing was performed 1–2 h before the dams were euthanized. The study was performed in three blocks, with one week in between each block (4 dams per dose group per block). Group 1 received vehicle only. Groups 2, 3, and 4 received a 1:1:1 mixture of bitertanol, propiconazole and cypermethrin in a total dose of 3, 9 and 30 mg/kg bw/day, respectively. Groups 5, 6, and 7 received a 1:1:1:1:1 mixture of bitertanol, propiconazole, cypermethrin, malathion and terbuthylazine in a total dose of 5, 15, and 50 mg/kg bw/day, respectively.

Prior to conducting the *in vivo* study Draft Assessment Reports for the five pesticides were evaluated, including looking at the reported no observed adverse effect levels (NOAELs) and lowest observed adverse effect levels (LOAELs) particularly from 2- and 3-generation studies as well as developmental toxicity studies conducted in rats. For the selection of mixture doses it was a priority that the doses should not cause any maternal toxicity. In the design of the *in vivo* study the mixtures were composed such that the pesticides were present at equal doses (in mg/kg). The design of the mixtures was not based on equi-effective doses as we did not have a specific dose for all selected pesticides affecting the same endpoint such as *e.g.* anogenital distance, which was one of the hormone sensitive endpoints prioritized in the *in vivo* study. Furthermore, the ratios in the mixtures were not intended

to reflect human exposure — as we are lacking information on humane exposure levels for most – if not all – pesticides. The maximum dose of each compound in the mixture, *i.e.* 10 mg/kg bw/day per compound, was selected on the basis of previous toxicological data, typically from 2-generation studies, and the highest dose was equivalent to the dose at which only subtle effects were expected on maternal body weights and litter size.

Cesarean sections GD 21

At GD 21 dams were weighed, anesthetized in CO_2/O_2 and decapitated, and fetuses were removed by cesarean section as previously described (Vinggaard et al., 2005).

Uteri were taken out, and the number of live fetuses, location in uterus, resorptions, and implantations were registered. Amniotic fluid from each dam was collected in glass tubes and stored at -80 °C until it was used for analysis of pesticides and metabolites as described by Bossi et al. (2013). Body weight, sex, and any anomalies were recorded. Anogenital distance (AGD), measured as the distance between the genital papilla and the anus, was measured in all fetuses using a dissecting scope with an ocular reticle. AGD was analyzed both with and without the cubic root of the body weight as a covariate. This was done in order to take into account that the length of the AGD may be influenced by the size of the fetus, and that while body weight is a three dimensional measurement, AGD is one-dimensional. The measurements were performed blinded with respect to treatment group by a skilled technician.
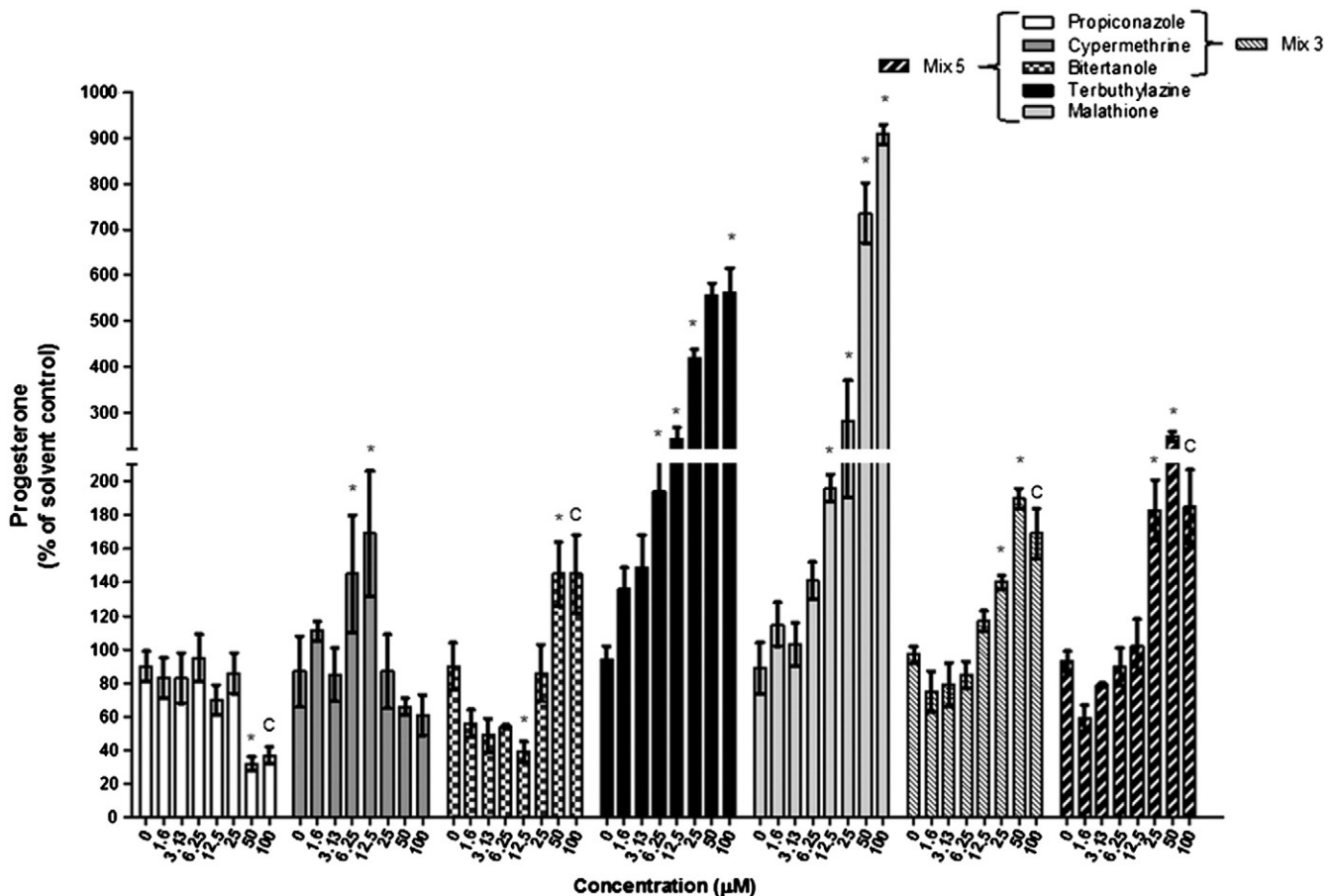

Fig. 1. Effect on progesterone production *in vitro*. *In vitro* effects of the five single pesticides as well as Mix 3 and Mix 5 on progesterone production in H295R cells. Data represent the mean ± SD for one representative experiment out of three independent experiments. *Statistically significantly different from control (P < 0.05). C = cytotoxicity.

Hormone levels and histopathology

Trunk blood from dams and fetuses was collected immediately after decapitation into heparin-coated vials for hormone analysis; from the fetuses one pool per litter was made for all male and female fetuses, respectively. Steroid hormones were extracted on ISOLUTE SPE columns from the serum as previously described (Birkhoj et al., 2004; Vinggaard et al., 2002). The thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) were analyzed as previously described (Taxvig et al., 2008), and all hormones were measured by use of Delfia time-resolved fluorescence kits (PerkinElmer), using a Wallac Victor 1420 multi-label counter (PerkinElmer Life Sciences).

From the control group and each of the high dose mixture-groups (Mix 3 30 mg/kg and Mix 5 50 mg/kg) placentas were taken from 3–4 dams. The level of testosterone and estradiol in the placentas was measured by pooling two placentas from one dam, placing the tissue in a glass vial containing 1000 µl water. The tissue was homogenized, 4 ml heptane was added to the vial, and then blended using a metal spatula. The homogenate was placed in a "bath" consisting of dry ice and acetone until the water fraction was frozen. The supernatant was transferred to a new glass vial. The water fraction was extracted once more with 4 ml heptane, cooled, and the supernatant pooled with the first heptane fraction. Heptane was evaporated by placing the vials in a water bath of 45 °C under nitrogen using TurboVap®LV (Biotage, Sweden). Before analyzing, the samples were re-suspended in 100 µl Diluent 1 (PerkinElmer), and hormone concentrations were measured with a Delfia time-resolved fluorescence kit (PerkinElmer) and a Wallac Victor 1420 multi-label counter (PerkinElmer Life Sciences) as described above.

Fetal testes were excised and sampled for 1) measurement of testicular testosterone content, 2) incubation for 3 h to assess testicular testosterone production *ex vivo*, and 3) fixation for histopathological examination. One testis from each of 2 male fetuses per litter was incubated in media for 3 h at 37 °C, and frozen at −80 °C until measurement of testosterone in the incubation media, as described previously (Borch et al., 2006). One testis from each of two male fetuses per litter was snap-frozen in empty tubes in liquid nitrogen before measurement of testicular testosterone content. One testis from each of two males per litter was placed in Bouin's fixative and processed for histology as described in Borch et al. (2006). Hematoxylin and eosin stained fetal testes were evaluated by an observer blinded to treatment groups and scored for the presence of the following parameters: presence of multinuclear gonocytes, clustering of gonocytes, increased numbers of gonocytes, altered morphology of Leydig cells, clustering of Leydig cells, and disorganization of structure of seminiferous chords or interstitial area.

mRNA levels determined by real-time RT-PCR

The adrenal gland from one male and one female per litter was weighed and stored in RNAlater (Qiagen; Ballerup, Denmark) for mRNA level analyses. RNA purification and cDNA production were done according to the procedure from the manufacturer and as described in Laier et al. (2006).

mRNA levels were quantified on a Taqman 7900 HT qPCR apparatus (Applied Biosystems) by standard TaqMan technology using TaqMan® Fast Universal PCR Master Mix (Applied Biosystems). All mRNA levels were quantified by the delta Ct method, and mRNA expression levels of each target gene were normalized to the expression level of the housekeeping gene 18S rRNA. Normalization was done by subtracting the Ct value of 18S rRNA from the Ct value of the target gene, giving a value named the delta Ct value (dCt). The 2^{-dCt} values were then
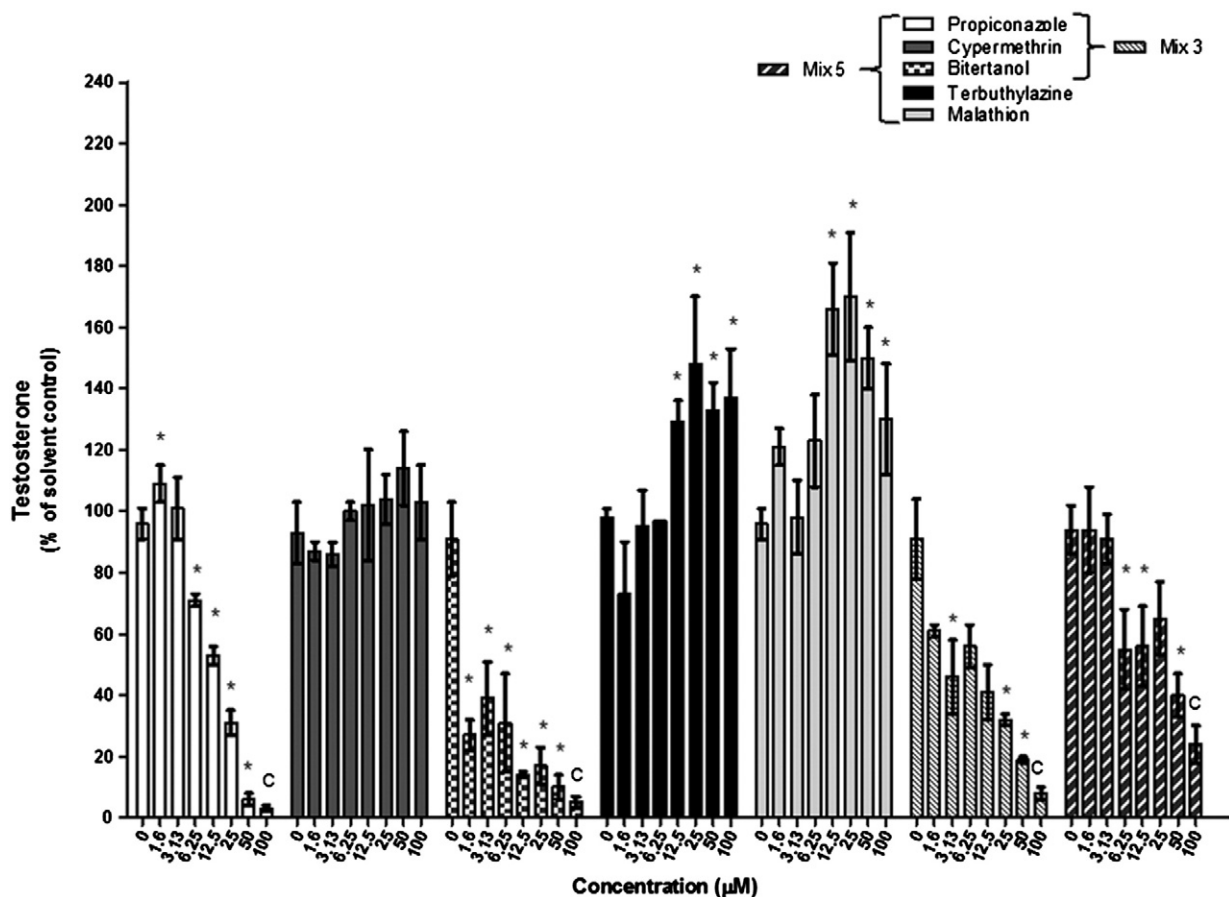

Fig. 2. Effect on testosterone production *in vitro*. *In vitro* effects of the five single pesticides as well as Mix 3 and Mix 5 on testosterone production in H295R cells. Data represent the mean ± SD for one representative experiment out of three independent experiments. *Statistically significantly different from control (P < 0.05). C = cytotoxicity.

used for graphs and to determine statistical significant differences between groups.

The following genes were quantified in male and female adrenal glands: 18S rRNA, steroidogenic acute regulatory protein (StAR), cytochrome P450-side-chain cleavage (P450scc), Cyp17a1 (P450c17), peripheral benzodiazepine receptor (PBR/Bzrp), and the aromatase (Cyp19) gene. The primer and probe sets were generated using the Primer Express Software v2.0, from Applied Biosystems; nucleotide sequences are described in Taxvig et al. (2008).

Statistics

The statistical analyses of data with normal distribution and homogeneity of variance were analyzed using analysis of variance (ANOVA), followed by Dunnett's post hoc test. When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor in ANOVA. Birth weights were analyzed with the number of pups per litter as a covariate. In cases where normal distribution and homogeneity of variance could not be obtained by data transformation, a non-parametric Kruskal–Wallis test was used (post-implantation loss). The statistical analyses were performed using SigmaPlot version 11 (Systat Software, Inc., Chicago, IL, USA) or SAS statistical software (SAS Institute Inc., Cary, NC, USA).

Results

Effects on steroidogenesis in vitro

The influence of the five single pesticides and the two pesticide mixtures on steroidogenesis was analyzed in the H295R assay.

For four out of the five pesticides an increase in progesterone production was seen, with malathion and terbuthylazine as the most

Table 2
Summery of in vitro effects.

	Progesterone	Testosterone	Estradiol	Max. E2/T
In vitro				
Propiconazole	↓ (50 μM)	↓ (50 μM)	↓ (2 μM)	0.6 (2 μM)
Cypermethrin	↑ (6–13 μM)	↔	↑ (3 μM)	1.7 (13 μM)
Bitertanol	↓(33 μM) ↑(50 μM)	↓ (50 μM)	↓ (13 μM)	4.7 (13 μM)
Terbuthylazine	↑ (6 μM)	↑ (13 μM)	↑ (3 μM)	2.5 (50 μM)
Malathion	↑ (13 μM)	↑ (13 μM)	↑ (13 μM)	1.4 (25 μM)
Mix 3	↑ (25 μM)	↓ (3 μM)	↓ (2 μM)	1.5 (3 μM)
Mix 5	↑ (25 μM)	↓ (6 μM)	↑ (3 μM)	3.5 (6 μM)

↑: increase; ↓: decrease; ↔: no effect.
The concentrations listed in brackets under the hormones are LOECs. The concentrations listed after the E2/T ratio is the concentration giving the largest ratio.

potent, with the lowest observed effect concentrations (LOECs) at 12.5 and 6.3 μM, respectively (Fig. 1). Propiconazole showed a decreased progesterone production, but only at the highest, non-cytotoxic concentration of 50 μM (Fig. 1). For both Mix 3 and Mix 5 an increase in progesterone production was seen, with LOECs of 25 μM (Fig. 1).

The effect on testosterone production was different for the five single pesticides. Malathion and terbuthylazine caused an increase in testosterone production, whereas bitertanol and propiconazole showed a decrease, with bitertanol being the most potent, with a LOEC of 1.6 μM (Fig. 2). Cypermethrin did not show any effect on testosterone production (Fig. 2). However, both Mix 3 and Mix 5 led to a significant decrease in the testosterone production in the H295R cells with LOECs of 3.1 and 6.3 μM, respectively (Fig. 2). In accordance with the effect of the single pesticides, Mix 3 caused the most pronounced testosterone-lowering effect.

Concerning effects on estradiol production an increase was seen for malathion, cypermethrin, and terbuthylazine, where terbuthylazine
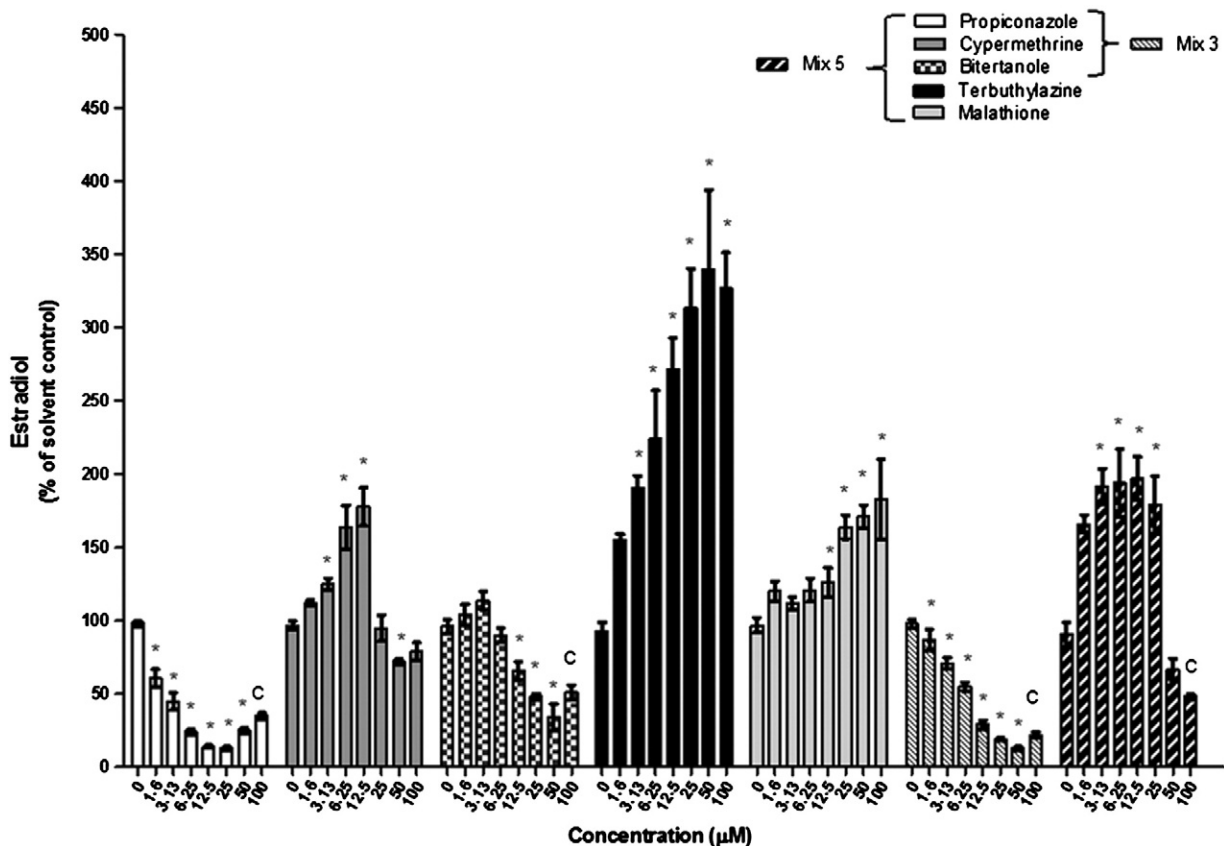

Fig. 3. Effect on estradiol production in vitro.In vitro effects of the five single pesticides as well as Mix 3 and Mix 5 on estradiol production in H295R cells. Data represent the mean ± SD for one representative experiment out of three independent experiments. *Statistically significantly different from control (P < 0.05). C = cytotoxicity.

Table 3
Pregnancy data, fetal body weight and anogenital distance at GD21.

	Control	Mix3 3 mg/kg	Mix3 9 mg/kg	Mix3 30 mg/kg	Mix5 5 mg/kg	Mix5 15 mg/kg	Mix5 50 mg/kg
No. of dams	11	12	11	12	12	10	12
Maternal bw (g)	315.0 + 18.7	314.8 + 12.7	311.7 + 12.6	303.0 + 16.1	310.8 + 16.0	321.7 + 18.6	316 + 16.4
Adjusted bw (g)	243.7 + 13.2	246.5 + 14.1	246.4 + 12.8	245.6 + 11.7	247.5 + 10.2	249.9 + 18.2	251.3 + 9.6
No. implantations	12.8 + 1.2	13.3 + 1.2	12.3 + 1.3	11.6 + 2.1	12.5 + 1.9	13.0 + 0.9	13.1 + 1.4
No. fetuses	12.5 + 1.3	12.3 + 2.0	11.9 + 1.7	10.5 + 3	11.1 + 2.6	12.6 + 1.2	12.2 + 2.2
% post implantation loss	2.1 + 3.6	8.6 + 10.6	2.5 + 6.2	11.0 + 14.8	10.6 + 20.3	3.1 + 4.1	6.8 + 14.1
% males	45.1 + 14.2	49.9 + 10.2	50.0 + 6.9	45.4 + 22.6	53.9 + 10.7	52.0 + 11.2	43.7 + 13.1
Fetal weight male (g)	3.74 + 0.38	3.63 + 0.52	3.52 + 0.34	3.52 + 0.30	3.80 + 0.25	3.91 + 0.34	3.62 + 0.24
Fetal weight female (g)	3.56 + 0.36	3.49 + 0.42	3.39 + 0.33	3.44 + 0.34	3.59 + 0.31	3.67 + 0.27	3.40 + 0.19
Male AGD (units)	21.40 + 0.79	20.76 + 1.39	21.17 + 0.78	21.56 + 0.63	21.35 + 0.96	21.47 + 0.82	21.02 + 1.25
Female AGD (units)	11.58 + 0.72	11.58 + 0.85	11.64 + 0.80	11.46 + 0.42	11.68 + 0.71	11.56 + 0.56	11.16 + 0.55

Data represent means ± SD of litter means.

showed the most pronounced effect (LOEC 3.1 μM) (Fig. 3). For bitertanol a decrease was seen at the highest concentrations (LOEC of 12.5 μM) (Fig. 3), and for propiconazole a significant decrease in estradiol levels was seen with a LOEC of 2 μM (Fig. 3). For Mix 3 and Mix 5 the effect on estradiol production was opposite; Mix 3 showed a clear decrease with a LOEC of 2.6 μM, whereas Mix 5 showed a significant increase, with a LOEC of 3.1 μM (Fig. 3). Table 2 gives a summary of the in vitro effects.

In vivo study

No effects were seen on maternal body weights or on the adjusted maternal body weight, which is the body weight of the dam minus the weight of the uterus with fetuses, placentas and fluids. The number of implantations and live fetuses as well as percent post-implantation loss and percent males per litter were unaffected by the prenatal pesticide exposure (Table 3). No morphological or histological changes were
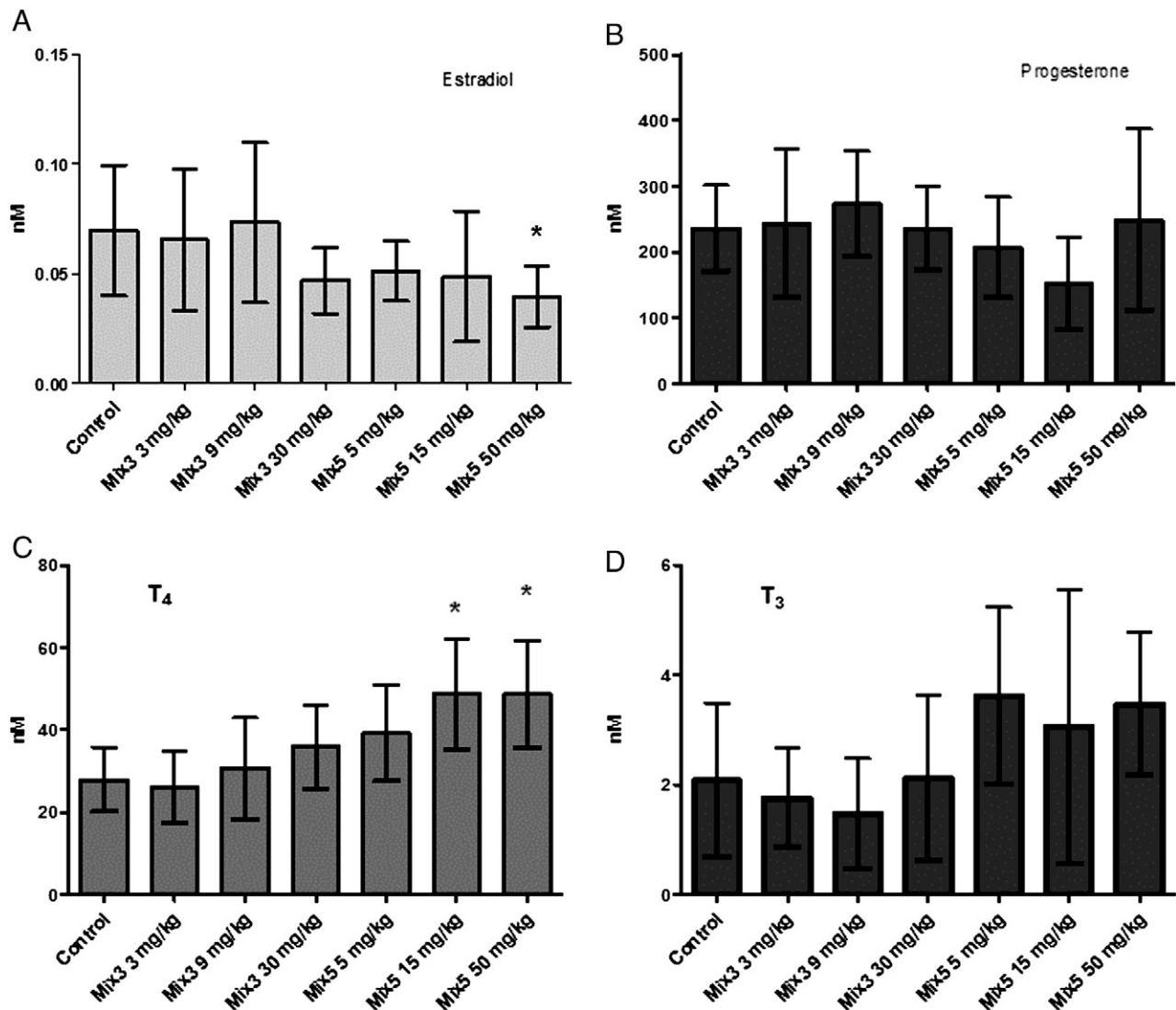

Fig. 4. Plasma hormone levels in dams GD21. Plasma concentrations of A) estradiol, B), progesterone, C) T_4, and D) T_3 in dams at GD21 following exposure to mixtures of pesticides from GDs 7 to 21. Data represent the mean ± SD. n = 9–12 dams pr. dose group). *Indicate a statistically significant difference compared to controls (P < 0.05).

observed in the *in vivo* study, including no effects on anogenital distance (AGD) in male and female fetuses (Table 3). Weights of male and female fetuses were around 4–5% lower in the groups exposed to the highest doses of the two pesticide mixtures, *i.e.* in Mix 3 30 mg/kg and Mix 5 50 mg/kg, but no statistically significant effects were seen (Table 3).

Effect on hormone levels in dams. In plasma of dams exposed to the highest dose of Mix 5 (50 mg/kg) a slight but significant decrease was found in the estradiol level (Fig. 4A) at GD 21. A significant increase in T_4 was seen at 15 and 50 mg/kg Mix 5 (Fig. 4C), and for T_3 a tendency towards an increase with Mix 5 was seen as well, but the effect was not statistically significant (Fig. 4D). No significant effect was found on progesterone plasma concentrations in the dams at GD 21 (Fig. 4B).

Testosterone levels were measured in placentas from controls, from the high dose Mix 3 (30 mg/kg), and high dose Mix 5 (50 mg/kg). A statistically significant decrease in the level of testosterone was found in placentas from the Mix 5 in the 50/mg/kg dose group (Fig. 5). The level of estradiol in placental tissue could not be measured by the applied method (because the level was below the limit of detection for the analysis).

Effect on testis hormones and testicular histopathology GD 21. Testosterone levels were measured in testis from the male fetuses taken by cesarean section on GD 21, but no effects were found for either Mix 3 or Mix 5 dose group (data not shown). Likewise, neither mixture had any effects on the *ex vivo* testicular testosterone production (data not shown).

No dose-related changes in testicular histopathology were observed. A number of animals were noted as having disorganization of the interstitial area, but this finding was present in all dose groups and there was no sign of dose-relationship (data not shown).

Effects on gene expression levels. Gene expression analysis was performed on fetal adrenal glands. In female fetuses exposed to 50 mg/kg Mix 5 a statistically significant increase in the mRNA level of the aromatase gene in adrenal glands was found (Fig. 6). In male fetuses no significant effect on adrenal aromatase mRNA level was found (data not shown). No other steroidogenic genes were affected in either male or female fetuses (data not shown).

Pesticide levels in amniotic fluid. The measurements of the pesticides and their metabolites in the amniotic fluid showed increasing concentrations with increasing exposure levels (Fig. 7). This is evident in most cases, but for bitertanol in Mix 3 there is a 7-fold difference in pesticide level when the dose is increased 3-fold. However, this non-
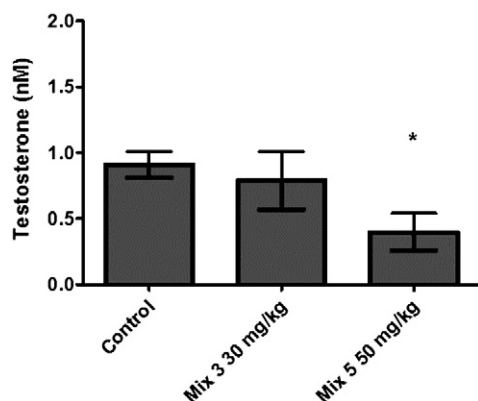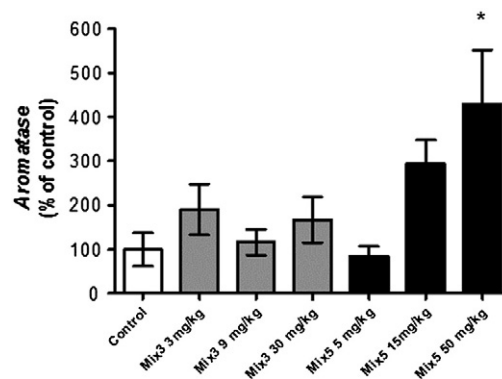

Fig. 6. mRNA expression levels of the aromatase gene. Expression of aromatase mRNA in adrenal glands from female rat fetuses determined by quantitative PCR at GD 21. The fetuses were exposed to the pesticide mixtures from GDs 7 to 21. The data represent the values of the specific mRNA levels relative to the housekeeping gene 18S rRNA. Data are mean $\pm$ SEM of 2^{-dCt} values with all data in percent of the mean of the control group (n = 10–12). *Indicates statistically significant difference compared to controls (p < 0.05).

linearity disappears when bitertanol is dosed together with the four pesticides in Mix 5.

The measurements showed that propiconazole was the parent pesticide found in the lowest concentrations, whereas bitertanol was the pesticide measured at the highest concentration. Relatively high concentrations of the cypermethrin metabolite 3-phenoxybenzoic acid (3-PBA) and the terbuthylazine metabolite desethylterbuthylazine were also recovered in the amniotic fluid (Fig. 7).

Discussion

The overall aim of the current study was to investigate the potential endocrine activity of two selected pesticide mixtures consisting of fungicides, insecticides, as well as an herbicide. Individually, these pesticides were able to alter steroidogenesis in adrenal cells *in vitro*, with four out of five pesticides increasing progesterone levels. Opposing effects of the various pesticides were seen on estradiol and testosterone levels.

Gametogenesis and the early development of the fetus is a particularly sensitive period for adverse effects of exposure to EACs (Hardell et al., 2006; Sharpe, 2006; Skakkebaek, 2002). In the current study the two selected pesticide mixtures, Mix 3 (bitertanol, propiconazole, and cypermethrin) and Mix 5 (bitertanol, propiconazole, cypermethrin, malathion, and terbuthylazine), were investigated *in vivo* by dosing pregnant rats from GD 7 to GD 21, followed by examination of endocrine effects in dams, but in particular in the fetuses, which are considered very sensitive. Several currently used pesticides have recently been reported being anti-androgens *in vitro* (Orton et al., 2011), and anti-androgenic effects both *in vitro* and *via* maternal exposure *in vivo* have been reported for several pesticides including prochloraz, procymidone, linuron, tebuconazole, and vinclozolin both singly and in mixtures (Gray et al., 1999; Hass et al., 2012; Lambright et al., 2000; Ostby et al., 1999; Taxvig et al., 2007; Uzumcu et al., 2004; Vinggaard et al., 2005). In the current study no anti-androgenic effects or changes in the measured steroid hormone levels were found in the fetuses, and no effects on AGD, testosterone levels, or histopathology were observed. This means that it is likely that if any androgen receptor blocking or inhibition of testosterone synthesis took place in the gonads of the fetuses at the applied dose levels, this was not detectable as a biological effect on downstream endpoints. In contrast, a small but significant decrease in the estradiol plasma level was seen in the dams exposed to the highest dose of Mix 5. Additionally, a significant increase in the T_4 plasma concentration was found in dams exposed to the two highest doses of Mix 5 (15 and 50 mg/kg). In contrast, in pregnant rats other pesticides such as mancozeb have been found to reduce T_4 levels in dams,

Fig. 5. Testosterone levels in placenta. The level of testosterone was measured in placentas following maternal exposure to mixtures of pesticides from GD 7 to 21. At GD 21 cesarean sections were performed and placentas were collected from dams in the Mix3 30 mg/kg, and the Mix 5 50 mg/kg dose groups, respectively. Data represent the mean $\pm$ SD. n = 3–4 dams pr. dose group. *Indicate a statistically significant difference compared to controls (P < 0.05).
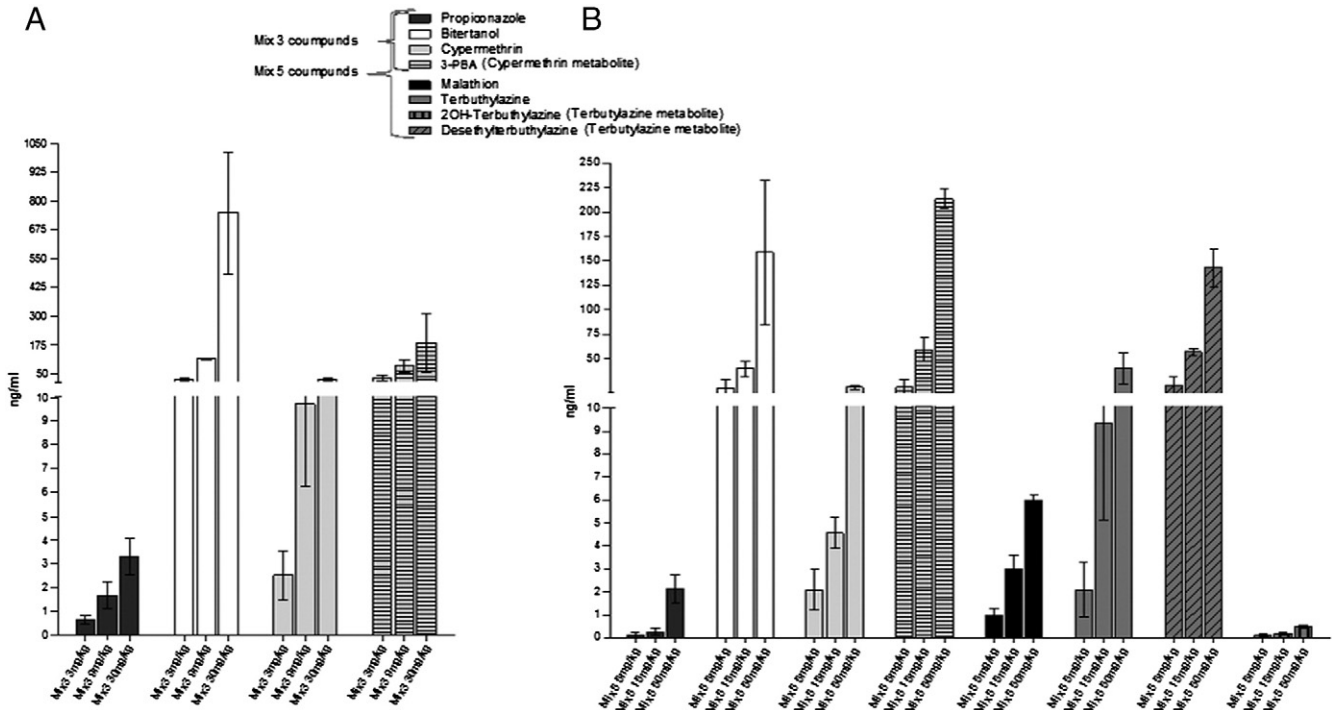
Fig. 7. Concentrations of pesticides and metabolites in amniotic fluid. The level of pesticides and metabolites were measured in amniotic fluid from rats exposed to pesticide A) Mix 3 or B) Mix 5 from GDs 7 to 21. Data represents mean ± SD (n = 5). The data presented in this figure are also presented in a different way and in a different context in the method paper by Bossi et al. (2013).

although giving no behavioral effects in the offspring (Axelstad et al., 2011), and prochloraz and p,p′-DDE exposure has also been shown to reduce T_4 levels in adult male rats (O'Connor et al., 1999; Vinggaard et al., 2002). As dams transfer thyroid hormones (THs) to their fetuses, it may be expected that a modest increase in T_4 would not result in a severe adverse effect (Morreale de Escobar et al., 1987). This is also supported by the fact that, in humans as well as in rats, reduced levels of THs during early prenatal life have been associated with adverse effects e.g. impaired neurological development (Crofton, 2004; Haddow et al., 1999; Hendrich et al., 1984; Li et al., 2010; Porterfield and Hendrich, 1981).

In addition to the reduced estradiol levels in the dams from the Mix 5 50 mg/kg dose group, a significant decrease in the level of testosterone

was also found in the placentas from the Mix 5 50 mg/kg dose group. As the placenta is the primary site of estrogen synthesis during pregnancy (Kaludjerovic and Ward, 2012), it is likely that steroidogenesis in placenta is inhibited, as indicated by the lowered testosterone levels. As the precursor for placental steroidogenesis is dehydroepiandrosterone (DHEA) produced primarily by the fetal adrenal cortex (Kaludjerovic and Ward, 2012), we hypothesize that a general inhibition or reduction of DHEA synthesis takes place in the fetus (Fig. 8). In addition, an increase in the mRNA level of the aromatase gene in fetal adrenal glands from the highest Mix 5 dose group was seen; however this effect was only observed in the female offspring indicating important sex differences in either ADME (absorption, distribution, metabolism, and excretion) related issues, Cyp19 gene regulation, or differences related to steroid
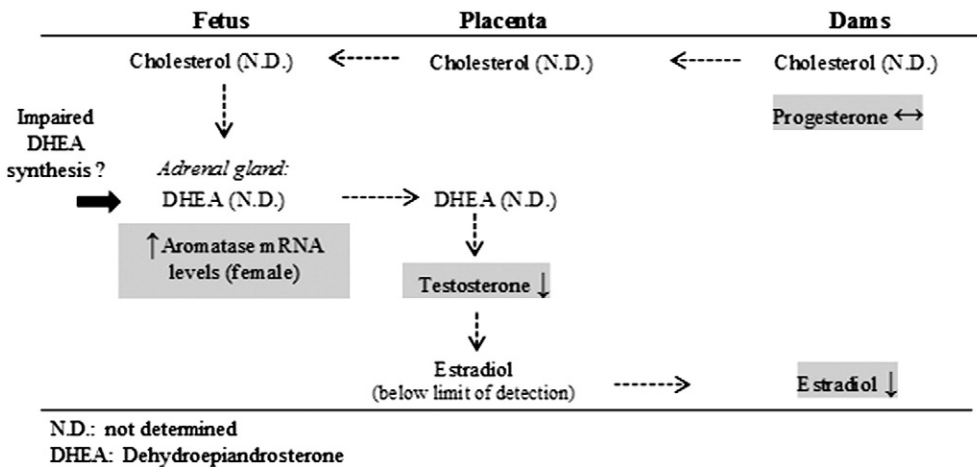

Fig. 8. Summary of Mix 5-induced effects in vivo. The figure presents a hypothesis for a link between the effects observed for Mix 5: in vivo estradiol levels were reduced in the dams dosed with Mix 5 50 mg/kg. The placenta is the primary site of estradiol synthesis during pregnancy. In placenta we found a significant decrease in testosterone indicating that steroidogenesis in the placenta is inhibited. As the precursor for placental steroidogenesis is dehydroepiandrosterone (DHEA), mainly produced by the fetal adrenal cortex (Kaludjerovic and Ward, 2012), we hypothesize that a general inhibition of DHEA synthesis has taken place in the fetus. In addition, an increase in aromatase mRNA levels were found in female fetal adrenal glands with Mix 5 50 mg/kg. Whether the aromatase induction in the fetus is a secondary effect to the proposed inhibition of DHEA synthesis, or it is a separate event, we do not know.

hormone production. It remains unknown whether the aromatase induction in the female fetus is a secondary effect to the suggested inhibition in DHEA synthesis, or it is a separate independent event (Fig. 8).

The *in vitro* data from the H295R steroidogenesis assay showed a decrease in the testosterone production for both mixtures, and for Mix 5 an increase in estradiol was also observed. This effect of Mix 5 can be explained by an increased aromatase activity, which would be in accordance with the observed increase in the aromatase mRNA level seen in the female adrenal glands. Still, the induction of aromatase mRNA only gives an indication that the aromatase enzyme activity might be increased.

EACs can act *via* multiple modes of action including direct interaction with steroid hormone receptors, or altered steroidogenesis. Cytochrome P450 (Cyp) enzymes play a critical role in steroidogenesis, and it is known from previous studies that many pesticides have the ability to alter the activity of Cyp enzymes. Particularly the aromatase (Cyp19) enzyme has been found as a target for many pesticides, some pesticides inducing the activity and others inhibiting it (Sanderson et al., 2002; Vinggaard et al., 2000; Zarn et al., 2003). Propiconazole and other azole fungicides have been shown to inhibit aromatase activity (Kjeldsen et al., 2013), whereas cypermethrin and triazine herbicides have been reported to induce the activity *in vitro* (Laville et al., 2006; Sanderson et al., 2001; Trosken et al., 2006; Zarn et al., 2003). As the observed effects *in vivo* were only seen for Mix 5, it suggests that one or both pesticides specific for Mix 5, terbuthylazine and/or malathion are driving the effect. The *in vitro* data in H295R cells indicate that terbuthylazine and malathion by themselves have an overall stimulatory effect on the steroid hormone synthesis, increasing progesterone, testosterone and estradiol. Terbuthylazine is however causing relatively higher increases in estradiol production in H295R cells (maximum estradiol/testosterone ratio of 2.5 and 1.4 for terbuthylazine and malathion, respectively) suggesting aromatase induction. Thus, it is likely that terbuthylazine is the compound driving the effect of Mix 5 on aromatase activity *in vivo*. However, data from this *in vivo* study do not provide the full evidence that aromatase induction is the dominating mechanism of action for Mix 5 *in vivo*.

In the current *in vivo* study a novel approach was applied to analyze for pesticides and their metabolites in amniotic fluid (Bossi et al., 2013), and these levels are close to representing the actual dose at the target *i.e.* in the fetus. It was possible to detect all five pesticides in the amniotic fluid, demonstrating that the fetuses were exposed to these chemicals (Bossi et al., 2013). However, it is possible that the internal dose in the fetuses was too low to cause any effect on AGD or fetal hormone levels.

Overall, it is an interesting observation that the levels of the three pesticides present in both Mix 3 and Mix 5 were lower, when they were combined with terbuthylazine and malathion in Mix5 (Fig. 7 and Bossi et al., 2013). This indicates ADME interactions resulting in lower exposure when more chemicals are given at relatively high doses (10 mg/kg). Whether this is due to an affected absorption, distribution, metabolism, and/or excretion of the pesticides remains unknown. However, the lower levels of the same pesticides in Mix 5 compared to Mix 3 indicate that this effect is not primarily due to saturated metabolism. The bitertanol levels in Mix 3 increase in a non-linear fashion and this might be explained by saturated metabolism. However, this non-linearity is not observed for bitertanol in Mix 5, indicating that the presence of terbuthylazine and/or malathion reduce bitertanol levels or lead to more complex ADME interactions.

Altogether, it is likely that discrepancies between *in vitro* and *in vivo* results could partly be due to ADME interactions and metabolism of parent compounds that occur *in vivo*, but not *in vitro*. Nevertheless, for Mix 5 alone there seems to be reasonable agreement between *in vivo* and *in vitro* observations, as aromatase induction is evident both *in vitro* and *in vivo*. Terbuthylazine alone has an overall stimulatory effect on testosterone production, but causes a more potent and efficient increase in estradiol levels, which is in agreement with the *in vitro* data on Mix 5 showing a clear indication of increased aromatase activity.

In conclusion, all five single pesticides, as well as both mixtures, had the ability to affect steroidogenesis *in vitro*, and at the highest dose Mix 5 also exerted endocrine activity in dams as well as female fetuses, in terms of disrupted hormone levels and affected the aromatase mRNA level (Fig. 8).

Overall, the human H295R cell assay can give important hints on potential chemically-induced effects on steroidogenesis that can be further investigated *in vivo*. Supplementing the examination on steroidogenesis with a battery of *in vitro* assays covering various mechanisms of action will provide a good basis for generating hypotheses on relevant mechanisms and *in vivo* effects.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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