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# Combined exposure to endocrine disrupting pesticides impairs parturition, causes pup mortality and affects sexual differentiation in rats

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## Summary

Risk assessment is currently based on the no observed adverse effect levels (NOAELs) for single compounds. Humans are exposed to a mixture of chemicals and recent studies in our laboratory have shown that combined exposure to endocrine disrupters can cause adverse effects on male sexual development, even though the doses of the single compounds are below their individual NOAELs for anti-androgenic effects. Consequently, we have initiated a large project where the purpose is to study mixture effects of endocrine disrupting pesticides at low doses. In the initial range-finding mixture studies, rats were gavaged during gestation and lactation with five doses of a mixture of the fungicides procymidone, mancozeb, epoxyconazole, tebuconazole and prochloraz. The mixture ratio was chosen according to the doses of each individual pesticide that produced no observable effects on pregnancy length and pup survival in our laboratory and the dose levels used ranged from 25 to 100% of this mixture. All dose levels caused increased gestation length and dose levels above 25% caused impaired parturition leading to markedly decreased number of live born offspring and high pup perinatal mortality. The sexual differentiation of the pups was affected at 25% and higher as anogenital distance was affected in both male and female offspring at birth and the male offspring exhibited malformations of the genital tubercle, increased nipple retention, and decreased prostate and epididymis weights at pup day 13. The results show that doses of endocrine disrupting pesticides, which appear to induce no effects on gestation length, parturition and pup mortality when judged on their own, induced marked adverse effects on these endpoints in concert with other pesticides. In addition, the sexual differentiation of the offspring was affected. This as well as the predictability of the combination effects based on dose-additivity modelling will be studied further in a large dose-response study.

## Introduction

Animal laboratory experiments have shown that in utero exposure to endocrine disrupting chemicals (EDCs) including some pesticides can cause adverse effects on male reproductive development (Foster, 2006; Gray *et al.*, 2006; Hass *et al.*, 2007; Metzdorff *et al.*, 2007; Christiansen *et al.*, 2008). Individual pesticides alone have so far not been shown to contribute to adverse human effects at relevant exposure levels. However, some studies indicate increased prevalence of cryptorchidism and decreased penile length in sons of women working as gardeners or living on farms where pesticides have been used (Kristensen *et al.*, 1997; Weidner *et al.*, 1998; Carbone *et al.*, 2007; Andersen *et al.*, 2008).

Many EDCs have been found as mixtures in humans (Blount *et al.*, 2000; Swan *et al.*, 2005), including children (Brock *et al.*, 2002; Swan *et al.*, 2005; Main *et al.*, 2006). Damgaard *et al.* (2006) observed an association between congenital cryptorchidism and the levels of certain organochlorine pesticides in mothers' milk (Damgaard *et al.*, 2006). Earlier, Pierik *et al.* (2004) identified paternal exposures to pesticides and smoking as factors associated with these congenital malformations. These initial

observations in epidemiological studies points in the same direction as laboratory experiments with oestrogenic or anti-androgenic chemicals in which substantial mixture effects occurred even though each individual chemical was present at low, ineffective doses (Rajapakse *et al.*, 2002; Silva *et al.*, 2002; Hass *et al.*, 2007; Metzdorff *et al.*, 2007; Christiansen *et al.*, 2008, 2009).

Some pesticides such as vinclozolin and procymidone antagonize competitively the androgen receptor (AR) binding of androgens and affect mainly the reproductive development in male offspring (Kelce *et al.*, 1997; Ostby *et al.*, 1999). Other pesticides such as mancozeb and propineb act mainly via disruption of the thyroid hormones and are mainly suspected to disrupt brain development (Hurley, 1998; Hass & Axelstad, Personal Communication).

Our detailed research on prochloraz, combined with studies on other azole fungicides such as tebuconazole and epoxyconazole, indicates that these pesticides have the ability to react through several endocrine disrupting mechanisms, and to induce various endocrine disrupting effects (Vinggaard *et al.*, 2005a,b; Taxvig *et al.*, 2007). We have shown that prochloraz induced anti-androgenic effects in rats in vivo in a Hershberger test as well as in a developmental toxicity study (Vinggaard *et al.*, 2002, 2005a). In addition, our studies show that prochloraz increases gestation length and indicate that prochloraz may also affect thyroid hormone levels and cause effects on the sexually dimorphic development of the brain (Vinggaard *et al.*, 2002, 2005a). Both tebuconazole and epoxyconazole increase gestation length and pup mortality and furthermore, these pesticides virilise female pups, and affect steroid hormone levels in foetuses and/or dams (Taxvig *et al.*, 2007).

In this article, we present data from two range-finding studies on the effects of a mixture of five endocrine disrupting pesticides. We selected procymidone, prochloraz, tebuconazole, epoxyconazole and mancozeb for our experiments. The choice of these pesticides was motivated by their common use as pesticides and their multiple mechanisms. The main aim of our range-finding studies was to assess whether there would be joint effects on pregnancy length and pup survival when every mixture component was present at doses that individually did not, in our earlier studies, produce observable effects on these endpoints. In addition, the aim was to obtain preliminary data on effects on the sexual development of the offspring to plan a large dose-response study.

## Materials and methods

### Animals and exposure

Two range finding studies, hereafter referred to as study 1 and study 2, were performed 2 months apart. In both studies, time-mated nulliparous, young adult Wistar rats (HanTac : WH, Taconic Europe, Ejby, Denmark) were supplied at gestation day 3 (GD3) of pregnancy.

The animals were housed in pairs until GD18 and alone thereafter under standard conditions in semi-transparent polycarbonate cages (15 × 27 × 43cm) with Aspen bedding (Tapvei, Denmark) situated in an animal room with controlled environmental conditions (12 h light-dark cycles with light starting at 21.00 PM, light intensity 500 lux, temperature 21 ± 2 °C, humidity 50 ± 5%, ventilation 8 air changes per hour). 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.

On the day after arrival (GD4), the dams were pseudorandomly distributed into groups of eight animals with similar body weight (bw) distributions. Mixtures were administered by gavage from GD7 to the day before expected birth (GD21) and from pup day (PD) 1 until PD13. However, as most of the exposed dams in study 1 were unable to give birth, only dams from study 2 were dosed from PD1 to PD13.

The substances used were corn oil (vehicle) (Sigma-Aldrich, Brøndby, Denmark), and procymidone, epoxyconazole, tebuconazole, mancozeb and prochloraz. All chemicals were purchased in a technical quality from VWR- Bie & Berntsen (Herlev, Denmark).

The composition of the pesticide mixture (Pmix) was chosen according to the doses of each individual pesticide that caused no major effects on pregnancy length and pup survival in our earlier studies (Vinggaard *et al.*, 2005a; Taxvig *et al.*, 2007). The animals were dosed with vehicle (control) or 25, 50, 75, 100 or 125% of Pmix (see Table 1). The doses used in study 1 were 75, 100 and 125%, whereas the doses studied in study 2 were 25 and 50% of Pmix. However, the dams dosed with 125% of Pmix exhibited signs of acute neurotoxicity after 2 days of dosing. Consequently, the dose was decreased to 100% of Pmix and the dams were included in the 100% group. The dams were inspected twice a day for general toxicity

**Table 1** Doses of the five pesticides in mg/kg/day in the Pmix doses

| Pesticide | Pmix-25% | Pmix-50% | Pmix-75% | Pmix-100% | Pmix-125% |
|---|---|---|---|---|---|
| Epoxiconazole | 3.75 | 7.50 | 11.25 | 15.00 | 18.75 |
| Mancozeb | 6.25 | 12.50 | 18.75 | 25.00 | 31.25 |
| Prochloraz | 8.75 | 17.50 | 26.25 | 35.00 | 43.75 |
| Tebuconazole | 12.5 | 25.00 | 37.50 | 50.00 | 62.50 |
| Procymidone | 12.5 | 25.00 | 37.50 | 50.00 | 62.50 |
| Pestimix, total | 43.75 | 87.5 | 131.25 | 175.00 | 218.75 |

The mixture ratio was based on doses causing no effects on gestation lengths for the individual pesticides, i.e. Pmix-100%.

 

including changes in clinical appearance (e.g. sedation and tremor). Body weights were recorded on GD4 and daily during the dosing period to monitor a decrease or increase in weight gain and the number of pregnant dams is presented in Table 2.

The animal studies were performed under conditions approved by the Danish Agency for Protection of Experimental Animals and by the in-house Animal Welfare Committee.

### Delivery and post-natal development

In study 1, all control animals gave birth as expected on GD22–23, whereas most of the exposed dams exhibited severe problems with parturition. It was therefore decided to end the study on GD25 and perform caesarean section on animals that had not yet given birth. The dams that had already given birth and their pups were sacrificed the same day. The dams were weighed and decapitated after $CO_2/O_2$ anaesthesia, uteri were taken out, and the number of live or dead fetuses, resorptions and implantations were registered.

The weights of dams and individual pups were recorded after delivery both in the animals in study 1 which were able to give birth and in all the pregnant animals in study 2. The pups were counted, sexed and checked for anomalies. Pups found dead were macroscopically investigated for changes when possible. The expected day of delivery, GD23, was designated pup day (PD) 1 for the pups. Thereby, the age of the pups was not related to the time of conception, but was rather similar to post-natal age as the animals gave birth on GD22–24. Body weight of offspring in study 2 was recorded on PD6 and 13.

*Anogenital distance and nipple retention*

Anogenital distance (AGD) was measured in the offspring at birth (PD1) using a stereomicroscope. On PD13, all male and female pups were examined for the presence of areolas/nipples (NR), described as a dark focal area (with or without a nipple bud) located where nipples are normally present in female offspring. Female rats normally have 12–13 nipples.

*Section PD13, organ weights and assessment of malformations in male external genitalia*

The animals were weighed and decapitated after $CO_2/O_2$ anaesthesia. Testis, epididymis, ventral prostate, seminal vesicles, and liver were excised and weighed from one male per litter. From one female per litter, the uterus and ovary were excised and weighed. The external genitalia of all male offspring were inspected for genital dysgenesis and scored on a scale from 0 to 3, with the observer being blinded with respect to dose group. The scores were:

Score 0 (no effect): Normal genital tubercle, with the urethral opening found at the tip of the genital tubercle and the preputial skin intact.

Score 1 (mild dysgenesis of the external genitals): A small cavity on the inferior side of the genital tubercle or a minor cleft in the preputial opening was observed, estimated 0.5–1.4 on an arbitrary scale. The size of the genital tubercle was decreased.

**Table 2** Pregnancy and weight data

| | Control[a + b] | Pmix-25%[b] | Pmix-50%[b] | Pmix-75%[a] | Pmix-100%[a] |
|---|---|---|---|---|---|
| No. pregnant dams | 8 | 4 | 8 | 7 | 14 |
| Maternal bw gain GD7–21 (g) | 83.4 ± 3.8 | 82.6 ± 3.4 | 74.4 ± 4.7 | 75.0 ± 10.0 | 53.7 ± 6.9** |
| Maternal bw gain GD7–PD1 (g) | 9.3 ± 1.8 | 4.8 ± 3.5 | 4.9 ± 3.5 | – | – |
| Gestational length (d) | 23 ± 0.0 | 23.5 ± 0.1* | 24.1 ± 0.2** | 24.8 ± 0.1** | 24.6 ± 0.2** |
| Pup perinatal mortality (%) | 7.7 ± 3.6 | 15.7 ± 12.9 | 72.8 ± 10.7** | 93.9 ± 2.8** | 92.8 ± 4.4*** |
| Birth weight, male pups (g) | 6.3 ± 0.2 | 6.2 ± 0.3 | 6.0 ± 0.1 | – | – |
| Birth weight, female pups (g) | 6.1 ± 0.1 | 6.0 ± 0.2 | 5.5 ± 0.03** | – | – |
| Prostate weight (mg) | 5.8 ± 0.4 | 3.6 ± 0.4* | 2.1 ± 0.3* | – | – |
| Left testis weight (mg) | 36.9 ± 1.6 | 33.3 ± 2.5 | 33.7 ± 1.9 | – | – |
| Right testis weight (mg) | 36.0 ± 2.0 | 33.2 ± 3.0 | 33.0 ± 1.6 | – | – |
| Epididymis weight (mg) | 23.6 ± 0.5 | 17.4 ± 1.6* | 14.9 ± 1.2* | – | – |
| Uterus weight (mg) | 17.1 ± 2.4 | 14.0 ± 1.3 | 12.1 ± 2.0 | – | – |
| Liver weight – male pups (g) | 0.64 ± 0.03 | 0.69 ± 0.09 | 0.85 ± 0.1* | – | – |
| Liver weight – female pups (g) | 0.61 ± 0.02 | 0.7 ± 0.06 | 0.72 ± 0.01** | – | – |
| Liver weight dams (g) | 10.4 ± 0.3 | 12 ± 0.4 | 11.2 ± 0.2 | – | – |

Data represent group means, based on litter mean ± SEM.

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.0001$.

Birth weight was analysed using the number of offspring as a covariate; organ weights were analysed using body weight as a covariate.

–, no data because of caesarean section in the groups Pmix-75% and Pmix-100%.

[a]Study number 1; [b]Study number 2; [a + b]The control group is representing both study 1 and 2.

Score 2 (moderate dysgenesis of the external genitals): The preputial cleft was larger, estimated 1.5–2.4 on an arbitrary scale. The urethral opening was situated half-way down towards the base of the genital tubercle (hypospadias).

Score 3 (severe dysgenesis of the external genitals): The preputial cleft was large, estimated 2.5–3.5 on an arbitrary scale. The urethral opening was situated further than half-way down the inferior side of the genital tubercle to the base of the genital tubercle (hypospadias). At the base of the genital tubercle, a groove extending laterally was observed (similar to control females at PD13).

### Hormone analysis

Progesterone were analysed in serum from 1–5 male and 1–3 female pups in 4–5 litters pr. dose group at PD13. Testosterone and estradiol were analysed in serum from 1 to 3 male or 1 to 3 female pups in 3–5 litters respectively. Serum from the pups in each litters were pooled by sex. Testosterone, estradiol and progesterone were extracted from the serum as previously described (Vinggaard *et al.*, 2005b) and the hormones were measured by time-resolved fluorescence using commercially available fluoroimmunoassay kits (PerkinElmer Life Sciences, Turku, Finland).

### Statistics

For all analyses, the alpha level was set at 0.05 and the litter was the statistical unit. Data were examined for normal distribution and homogeneity of variance, and if relevant, transformed. In cases where normal distribution and homogeneity of variance could not be obtained by data transformation, a non-parametric Kruskall–Wallis test was used, followed by Wilcoxon's test for pair wise comparisons. Data with normal distribution and homogeneity of variance were analysed using analysis of variance (ANOVA). 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 analysed using the number of offspring per litter as covariate and organ weights were analysed using body weight as a covariate.

Anogenital distance data were analysed by the calculated AGD-index, namely, AGD divided by the cube root of body weight. The cube root was used because this converts a three-dimensional end point (weight) into a one-dimensional such as the AGD (Gallavan *et al.*, 1999; Gray *et al.*, 1999).

Analysing the level of demasculinization of male pups, the scores were categorized into a binary variable with scores 0 and 1 (no hypospadias) and scores 2 and 3 (mild and severe hypospadias). Statistical analyses of the effects on level of demasculinization were performed using Fisher's exact test.

The number of nipple/areolas was assumed to follow a binomial-distribution with a response range between 0 and $\theta_{max}$, with $\theta_{max}$ being equal to the biologically possible maximal number of nipples in rats, either 12 or 13. The choice of $\theta_{max}$ was decided on considering the global fit (information criterion of Schwarz). To account for litter effects on NR, correlation structures between number of nipple/areolas and litter were modelled by the Generalized Estimating Equations method as in Hass *et al.* (2007). All statistical analyses were performed using the SAS procedure PROC GENMOD (SAS Institute Inc, Cary, NC, USA).

## Results

### Pregnancy data and post-natal survival

There were no significant effects on maternal body weight gain from GD7–21 and GD7–PD1 in dams exposed to Pmix-75% or lower (Table 2). However, maternal body weight gain from GD7–21 in dams exposed to the highest dose of the mixture (Pmix-100%) was significantly decreased (Table 2).

Gestation length was significantly increased in all dosed groups (Table 2) and 5 of 7 dams in Pmix-75% and 9 of 14 dams in Pmix-100% were unable to give birth and had to be sacrificed on GD25 (Fig. 1a).

The number of liveborn pups significantly decreased and the perinatal pup loss was significantly increased at Pmix-50% and higher when compared with controls (Table 2, Fig. 1b). No effects on birth weight were observed in male pups compared with controls, whereas the female pups exposed to mix-50% had a significantly decreased birth weight (Table 2).

No data are shown on birth weight in pups exposed to mix-75% and mix-100%, as there were too few live pups to asses this end point properly.

### AGD and NR

It was only possible to record AGD in a few litters in study 1 as most of the dams were unable to give birth (data not shown). In study 2, the mixture produced dose-dependent changes in AGD index with a significant increase seen in females and a decrease in males (Fig. 2a). Nipple retention was significantly and dose-dependently increased in male pups in both groups exposed to the mixture i.e. Pmix-25% and Pmix-50% (Fig. 2b).

### Autopsy, organ weight and dysgenesis PD13

No effects were observed on weight of the testes or the uterus in male and female offspring respectively. Weights

 

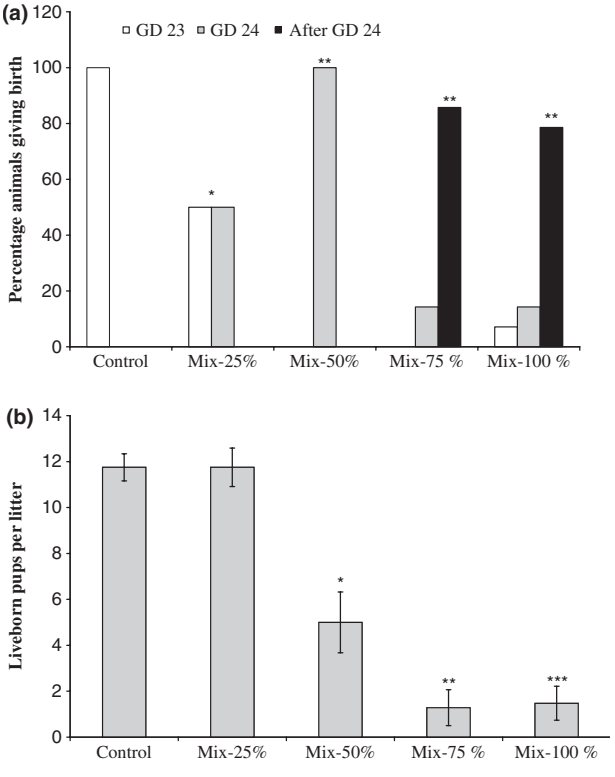


**Figure 1** Effects of combined exposure to procymidone, prochloraz, tebuconazole, epoxyconazole and mancozeb on parturition (a) and live litter size in rats (b). The parturition results are shown as percentage of animals giving birth on gestation days 23, 24 and after day 24. A few of the animals in the latter group gave birth on gestation day 25, but most of them were unable to give birth. Results shown for number of liveborn pups per litter are group mean ± SD. *p*-values are *<0.05, **<0.01 and ***<0.001. *N* = 8(94), 4(47), 7(40), 7(9) and 14(22) litters (liveborn pups) in Control, Mix-25%, Mix-50%, Mix-75% and Mix-100% respectively.

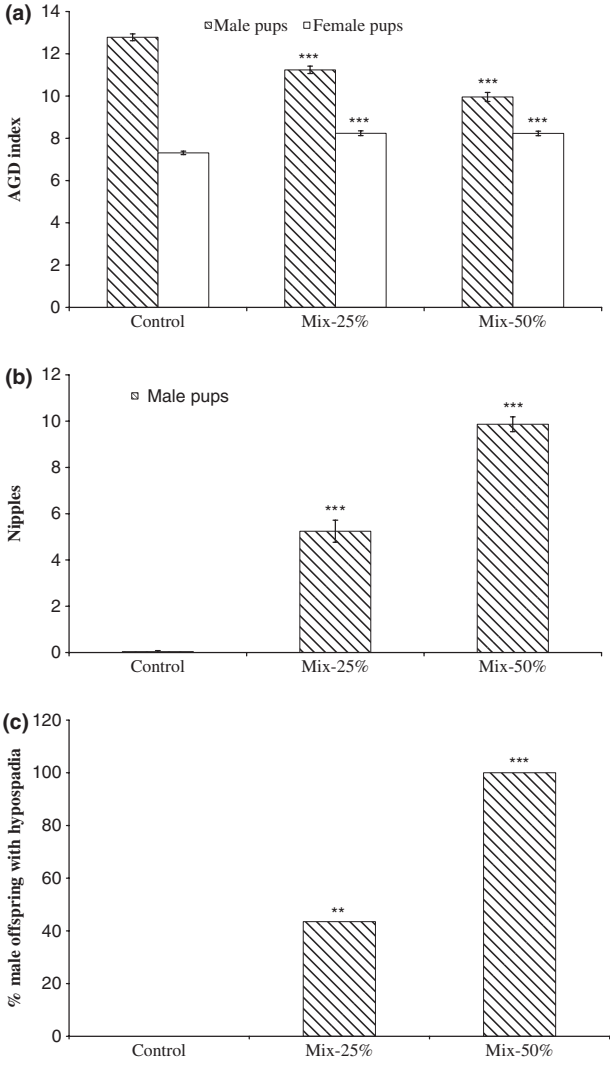


**Figure 2** Effects of combined exposure to procymidone, prochloraz, tebuconazole, epoxyconazole and mancozeb on anogenital distance (AGD) index at birth (a), nipple retention on pup day 13 (b) and malformation score on pup day 13 (c). Results shown are group mean ± SD. The number of nipples in female controls is generally 12. See text for details on the endpoints. *p*-values are *<0.05, **<0.01 and ***<0.001. *N* = 8(45 : 49), 4(26 : 21), and 7(24 : 15) litters (male : female pups) in Control, Mix-25% and Mix-50% respectively.

of prostate and epididymis in male pups were decreased in Pmix-25% and Pmix-50% exposed animals (Table 2). The liver weights of both male and female pups were elevated in the Pmix-50%-treated animals, but no effects were observed on liver weights of the dams (Table 2).

The incidence of hypospadias was increased with increasing dose (Fig. 2c). In the Pmix-25%, the males had either no, mild or moderate dysgenesis (score 0–2), whereas all of the males in the Pmix-50% group showed severe dysgenesis of the genitalia (score 3). No animals in the control group showed any malformations.

### Hormone levels

No statistically significant effects of exposure of Pmix (25, 50%) on progesterone, testosterone or estradiol serum levels were revealed in dams, male or female pups (Table 3). However, large standard deviations and the small number of samples may conceal any real effects.

## Discussion

The aim of these range-finding studies was to assess whether there would be joint effects on pregnancy length and pup survival when the five pesticides were present at doses that individually did not produce observable effects on these endpoints in our earlier studies. Moreover, an aim was also to find the dose that should be the highest

**Table 3** Hormone levels in study 2

| | Control | Pmix-25% | Pmix-50% |
|---|---|---|---|
| Progesterone levels, males (nM) | 0.7 ± 0.4 | 1.5 ± 0.4 | 1.3 ± 0.7 |
| Progesterone levels, females (nM) | 1.2 ± 0.5 | 0.9 ± 0.8 | 0.7 ± 0.2 |
| Progesterone levels, dams (nM) | 129.5 ± 44.5 | 95.0 ± 25.4 | 82.5 ± 65.6 |
| Testosterone levels, males (nM) | 0.3 ± 0.1 | 0.3 ± 0.2 | 0.7 ± 0.4 |
| Estradiol levels, females (nM) | 0.03 ± 0.01 | 0.04 ± 0.02 | 0.03 ± 0.01 |

Data represent group mean, based on pooled serum ± SD, $N$ = 3–5 litters in each group.

dose in a later large dose response study. Detailed molecular and endocrine analyses were not performed in the current study because of the limited group sizes, but will be targeted in a larger study.

Overall, the findings showed clearly that the combined exposure induced severe effects manifested as dystochia (impaired parturition) and high perinatal pup mortality.

Effect on gestation length was evident in all exposed groups, including the lowest mixture dose, where each pesticide was present at 25% of the doses that individually previously had not caused effect on gestation length. The effect is probably because of the presence of the three azole fungicides in the mixture, which have previously been shown to elicit such effects (Noriega *et al.*, 2005; Vinggaard *et al.*, 2006; Taxvig *et al.*, 2007). The prolonging of the gestation period may possibly be as a result of an increase in progesterone in the dams as suggested for prochloraz, epoxyconazole and tebuconazole (Vinggaard *et al.*, 2005a; Taxvig *et al.*, 2007).

The dystochia and pup mortality seen in the present studies have previously been observed as common effects of several of the azole fungicides (Wolf *et al.*, 1999; Moser *et al.*, 2001; Noriega *et al.*, 2005; Taxvig *et al.*, 2007). Neither mancozeb nor procymidone has earlier been shown to cause effects on pregnancy length or perinatal survival at the doses studied (Metzdorff *et al.*, 2007; Axelstad, Christiansen & Hass, unpublished data from our laboratory). In our large follow-up study, we will aim to avoid dystochia by including the Pmix-25% dose group as the highest exposure group. Another possible way to avoid dystochia without reducing the exposure level would be to stop the dosing of the animals for some days before expected birth. However, as increased pregnancy length is also an important endpoint for endocrine disrupters, we have not chosen this approach. We thereby have also selected the most human relevant dosing period, as pesticides are environmental contaminants that humans can be exposed to throughout pregnancy.

The observed anti-androgenic effects on the sexual differentiation of the male offspring seen as decreased AGD, nipple retention, decreased prostate and epididymis weight and hypospadias are likely because of the combined exposure to the three azole fungicides and procymidone as similar effects to some extent have been seen for the individual pesticides in our earlier studies and by others (Ostby *et al.*, 1999; Wolf *et al.*, 1999; Noriega *et al.*, 2005; Vinggaard *et al.*, 2005a; Laier *et al.*, 2006; Hass *et al.*, 2007; Taxvig *et al.*, 2007; Christiansen *et al.*, 2009).

Prochloraz caused increased AGD at 50 mg/kg and nipple retention at 30mg/kg in male offspring (Vinggaard *et al.*, 2005a; Laier *et al.*, 2006). Genital malformations were observed at 150 mg/kg prochloraz, but no effects on epididymis or prostate were found at the same dose (Laier *et al.*, 2006; Christiansen *et al.*, 2009). Prochloraz is also able to induce increased testicular progesterone concentrations in male rat foetuses (Vinggaard *et al.*, 2005a; Laier *et al.*, 2006; Blystone *et al.*, 2007). Recent studies suggest a previously unidentified role for the progesterone receptor, possibly interacting with the androgen receptor, in disturbed genital tubercle development (Willingham *et al.*, 2006).

Tebuconazole caused nipple retention at 50 and 100 mg/kg/day, whereas epoxyconazole did not induce observable nipple retention at 15 and 50 mg/kg/day (Taxvig *et al.*, 2007). Decreased weight of prostate, but not epididymis, has been observed at 50 mg/kg epoxyconazole, whereas tebuconazole did not affect prostate or epididymis weights up to 100 mg/kg in studies performed in our laboratory (Taxvig *et al.*, 2007).

Procymidone has in our laboratory induced decreased AGD and nipple retention at 25 mg/kg/day, but not at 10 mg/kg/day, while a decreased prostate weight was observed at 10 mg/kg (Hass *et al.*, 2007; Metzdorff *et al.*, 2007). No hypospadias was observed at 25 mg/kg in adult male rats or at 14.1 mg/kg in immature male rats (Metzdorff *et al.*, 2007; Christiansen *et al.*, 2008).

In a similarly designed study in our laboratory, Mancozeb has not shown effects on NR and AGD at doses below 100 mg/kg (Axelstad, Christiansen & Hass, unpublished data from our laboratory).

Thus, the individual doses (Table 1) of each of the three azole fungicides and mancozeb in the pesticide mixture Pmix-25% were clearly lower than those causing no effects on male sexual differentiation, whereas the dose of procymidone was close to this dose level (12.5 mg/kg).

The increased AGD observed in the female offspring is likely to be caused by the combined exposure to the three azole fungicides as our earlier studies have documented similar effects of prochloraz, epoxyconazole and tebuconazole (Laier *et al.*, 2006; Taxvig *et al.*, 2007). This effect may be caused by increased progesterone levels in

 

the dams (Willingham *et al.*, 2006). The progesterone as well as testosterone and estradiol levels in the current study were not significantly changed in the dams or in the pups, but this may be as a result of the low number of samples taken in this range finding study with a limited number of litters per group.

Prostate and liver weights were reduced in the present study and it would be relevant to measure changes in gene expression in these organs. In the prostate, androgen-regulated genes such as ornithine decarboxylase and Prostate binding protein subunit C3 (PPB C3) are known to be altered by anti-androgenic compounds (Nellemann *et al.*, 2005). Hepatic expression of growth hormone as well as drug metabolizing enzymes is sexually dimorphic and may be altered by xenobiotics (Waxman & Holloway, 2009). As mentioned above, detailed molecular and endocrine endpoints will be addressed in a later study with more litters per group.

In conclusion, the findings from these range-finding studies showed that combined exposure to the five pesticides induced marked adverse effects on parturition and pup survival at doses where the individual pesticides appear to induce no such effects. The significance of these findings for human risk assessment must be emphasized because they clearly indicate that risk assessment based on single endocrine disrupters alone underestimates the risk for adverse effects when exposure is to several pesticides with common effect outcomes regardless of mechanism.

The sexual differentiation of the offspring was also significantly affected. However, based on a range-finding study with a limited number of litters, only cautious conclusions can be drawn. Consequently, sexual differentiation of the offspring, as well as the predictability of the combination effects based on dose-additivity modelling, is currently studied more thoroughly in a large mixture dose-response study in our laboratory.

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## Panel discussion

### Ana Soto (Boston, USA)

In your 5 chemical mixture experiment with antiandrogens on fetal rats you observed decreased anogenital distance (AGD) in males and increased AGD in females. What is the mechanism causing this effect in females?

### Ulla Hass (Søborg, Denmark)

Not many such studies have been performed in females and the mechanisms are unknown. It is hypothesised that these chemicals in addition to their antiandrogenic effect also have very weak androgenic activity. They bind to the androgenic receptor (AR) but have a very poor stimulatory effect. In males, the binding to AR competes with the much more strongly acting testosterone and therefore the receptor is blocked causing reduced AGD. In the females where there is no testosterone, the weak androgenic activity causes increased AGD. This is one possibility but more research is required and the role of progesterone must also be considered.

### Fred vom Saal (Columbia, USA)

Literature from the 1970s described elongated AGD in female rodents developing in utero between two males. The testosterone in the females come from the rodent placenta which, unlike the human placenta, has no aromatase activity and has high levels of C17-20 lyase and 17β hydroxylase: the major sex steroids from the rodent placenta are androstenedione and testosterone, and not progesterone. Pregnant rodent females have serum levels of >1ng/ml testosterone, and 10ng/ml androstenedione. The AGD can be reduced in these masculinised rodent fetuses by antiandrogens such as flutamide or cyproterone acetate. Papers on this finding have been published by Clemens and myself.

### Ulla Hass

Thank you for that valuable information which we shall now be assessing.

 

### Vasantha Padmanabhan (Ann Arbor, USA)

John Marshall has studied the interaction of progesterone and androgen in the human polycystic ovary syndrome. Progesterone is a major negative feedback regulator of LH. High androgen levels such as seen in PCOS can reduce sensitivity to progesterone. A similar scenario may occur in your studies. You are looking at several antiandrogens in unison. Have you plans to assess the response to antiandrogenic with androgenic, or antiandrogenic with oestrogenic EDCs in combination as occurs in real life situations?

### Ulla Hass

We are about to test the Contamed mix, which is a mixture of antiandrogen and oestrogen; but not antiandrogen with androgen because I am unaware of environmental pollutants which are androgenic and therefore this might not be of relevance.

### Niels E Skakkebæk (Copenhagen, Denmark)

Your hypothesis concerning increased AGD in females is supported by older studies performed in the 1970s using cyproterone acetate (CA) which showed weak virilisation effects on female fetuses when the pregnant dams were exposed to CA.