Androgens are key regulators of male sexual differentiation during the \textit{in utero} and early postnatal development. Exposure to chemicals that counteract androgen action at some stage in this period can permanently feminize male fetuses and lead to malformations of the reproductive tract. Examples of chemicals known to disrupt sexual differentiation in this way include pesticides and their metabolites, such as vinclozolin, procmidone, 1,1-dichloro-2,2-bis(4-chlorophenyle) ethylene (\textit{p,p' DDE}), and linuron, and certain phthalate esters such as di-ethylhexyl phthalate and di-butyl phthalate (Gray et al. 2001, 2000, 2001; Nellemann et al. 2003), but little is known about the developmental effects of vinclozolin metabolites competing and other receptor-mediated events occur in an additive fashion (Birkhoj et al. 2004; Gray et al. 2001; Nelleman et al. 2003), but little is known about the developmental effects of \textit{in utero} and early postnatal exposure to multiple anti-androgenic chemicals.

In this article, we present data from detailed investigations of the ability of combinations of androgen receptor (AR) antagonists to induce disruption of male sexual differentiation after long-term exposures \textit{in utero} and postnatally. We selected a mixture of vinclozolin, procmidone, and flutamide for our experiments. Vinclozolin metabolites compete with androgens for AR binding (Kelce et al. 1994), suppress androgen-dependent gene transcription (Kelce et al. 1997), and affect reproductive development. Procmidone and flutamide also antagonize competitively the AR binding of androgens, with consequent inhibition of AR-mediated gene expression (Ostby et al. 1999; Simard et al. 1986). Common developmental effects of all three chemicals after \textit{in utero} exposure of male rats include reduced anogenital distance (AGD), nipple retention (NR), hypospadias, diminished prostate weight, reduced testis and epididymal weights, and altered behavior in male offspring (Foster and McIntyre 2002; Gray et al. 1994; Hellwig et al. 2000; Hib and Poncio 1995; Hotchkiss et al. 2002; McIntyre et al. 2001; Miyata et al. 2002; Ostby et al. 1999; Shimamura et al. 2002). There is no particular environmental relevance to this mixture. The choice of compounds was motivated by our interest to explore the predictability of combination effects caused by similarly acting anti-androgens rather than to emulate "real world" mixtures.

Conclusive answers to the question of combination effect predictability require quantitative comparisons between predicted and experimentally observed mixture effects. Experimentally, we have approached this task in a step-wise fashion: \textit{a)} Dose–response curves for all single-mixture components were recorded. \textit{b)} These data were used for the calculation of additivity expectations for a mixture of specific composition using "fixed mixture ratio design" (Altenburger et al. 2000; Hewlett and Plackett 1959). \textit{c)} The mixture experiments were conducted. \textit{d)} The observed combination effects were compared with the predicted responses.

The choice of an appropriate model for the calculation of additivity expectations is essential for assessments of mixture effects because it is in relation to these additivity expectations that combination effects are judged in terms of synergisms or antagonisms. Several concepts for the computation of expected additive effects of anti-androgens have been used. The simple method of summarizing the individual effects of chemicals in the combination, termed "effect summation", has been drawn on previously (Gray et al. 2000; 2001; 2006; 2001; 2000, 2001; 2000, 2001; 2000; 2001; 2000, 2001; 2000, 2001; 2000, 2001; 2000, 2001; 2000, 2001).
use of existing single-chemical databases for the prediction of mixture effects. A second aim was to determine whether there would be joint effects when every mixture component was present at doses that individually do not produce observable responses.

**Materials and Methods**

**Chemicals.** The chemicals used were vinclozolin (CAS No. 50471-44-8, purity 99%, ChemService catalogue no. PS-1049; Bie & Berntsen, Herlev, Denmark), procymidine (CAS No. 32809-16-8, purity 99%, ChemService catalogue no. PS-2126; Bie & Berntsen), flutamide (CAS No. 13311-84-7, purity 99%, catalogue no. F9397; Sigma Aldrich, Bronby, Denmark), and corn oil used as vehicle (Bie & Berntsen).

**Studies and dose levels.** Before the mixture experiment, dose–response studies for each chemical were conducted. The dose ranges were chosen with the aim to cover the entire range of effects from no effect up to maximum effects, as determined by measurement of AGD and NR. At the same time, it was attempted to select doses that would not cause marked effects on body weights in the dams, and especially in the offspring, as this would complicate evaluation of the effects on AGD and NR. The dose levels selected for the dose–response studies were based on the reductions of AGD and increase of NR reported for vinclozolin (Gray et al. 1994, 1999; Hellwig et al. 2000; Hotchkiss et al. 2002; Shimamura et al. 2002), flutamide (Foster and McIntyre 2002; Hib and Ponzi 1995; Hotchkiss AK et al. 2002; McIntyre et al. 2001; Miyata et al. 2002), and procyomidine (Osby et al. 1999). As data on procyomidine were relatively limited, a range-finding study was performed before the dose–response study. To gain information about variability of effects between studies, we ran selected doses of vinclozolin, flutamide, and procyomidine in parallel with the mixture experiment. An overview of the studies including dose levels and number of animals is shown in Table 1. A similar study design was used for all studies (see below).

| Study | Groups and doses | No. of animals per group |
|-------|------------------|-------------------------|
| 1. Vinclozolin and flutamide, dose–response | Control: vehicle-dosed | 16 |
| | 6 doses of vinclozolin: 5, 10, 20, 40, 80, or 160 mg/kg/day | 8 |
| | 6 doses of flutamide: 0.5, 1.0, 2.0, 4.0, 8.0, or 16 mg/kg/day | 8 |
| | 2 doses of procyomidine: 25 or 200 mg/kg/day | 4 |
| 2. Procymidine, range-finding | Control: vehicle-dosed | 16 |
| | 6 doses of procymidine: 5, 10, 25, 50, 100, or 150 mg/kg/day | 8 |
| 3. Procymidine, dose–response | Control: vehicle-dosed | 16 |
| | 6 doses of procymidine: 5, 10, 25, 50, 100, or 150 mg/kg/day | 8 |
| 4. Mixture study of vinclozolin, flutamide, and procyomidine | Control: vehicle-dosed | 16 |
| | 5 doses of mixture: 7.87, 19.67, 39.33, 70.80, or 106.19 mg/kg/day | 16 |
| | 2 doses of vinclozolin: 24.5 or 95.9 mg/kg/day | 16 |
| | 2 doses of flutamide: 0.77 or 3.86 mg/kg/day | 8 |
| | 2 doses of procyomidine: 14.1 or 61.8 mg/kg/day | 8 |
PND1 until PND16. The dosing volume of 2 mL/kg bw was calculated on the basis of the body weight of the animal on the day of dosing. The dose levels and group sizes are shown in Table 1. Animals were inspected for general toxicity twice daily. The studies were performed using four blocks (with 1 week in between), and all dose groups were equally represented in the blocks.

**Anogenital distance and nipple retention.** In all studies, AGD and NR were recorded by the same technician who was blinded with respect to exposure groups. After birth all live pups in the litter were weighed, sexed, and AGD was measured using a stereomicroscope. The sex of several of the pups in the highest dose groups could not be determined based on the AGD, as the AGDs were similar to female values in all pups in some litters. In these cases, the sex of the pups was determined later by internal inspection of reproductive organs with the presence of testes defining a male. The highest AGD values obtained in the litter were used as the values for male pups. This approach was chosen because these values were most likely to represent the males, as males normally have longer AGDs than females. For dose–response analysis, AGD data were analyzed 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 two-dimensional ratio of the dose of each compound to total mixture dose.

**Table 2.** Statistical dose–response descriptors for single and mixture exposures.

| Substance | Fraction in mixture | RM | $\theta_1$ | $\theta_2$ | $\theta_3$ | $\theta_{\text{max}}$ | Effect doses (mg/kg/day) | NOAEL a |
|-----------|---------------------|----|-----------|-----------|-----------|-----------|----------------|---------|
| **Nipple retention** | | | | | | | | |
| Vinlozolin | 0.622301 | BP-probit | –1.76 | 0.025 | 1.024 | 12 | 67.18 (55.78–77.08) | 5.72 (0.67–25.58) | < 5.0 |
| Flutamide | 0.019558 | Probit | –0.61 | 2.01 | — | 12 | 2.02 (1.71–2.40) | 0.41 (0.28–0.60) | < 0.5 |
| Procymidon | 0.358141 | BP-logit | –5.09 | 1.21 | 0.1 | 12 | 33.91 (24.81–45.74) | 7.51 (1.0–10.76) | 10.0 |
| Mixture | | bw-Weibull | –19.66 | 16.83 | –0.8 | 13 | 20.76 (17.76–23.52) | 8.21 (6.67–10.42) | < 7.87 |
| **AGD index** | | | | | | | | |
| Vinlozolin | 0.622301 | Logit | –6.80 | 3.59 | — | 1 | 78.65 (67.43–83.32) | 9.21 (1.15–29.08) | 5.0 |
| Flutamide | 0.019558 | Weibull | –1.38 | 1.85 | — | 1 | 5.34 (2.84–4.37) | 0.34 (0.19–0.57) | < 0.5 |
| Procymidon | 0.358141 | bw-Weibull | –6.31 | 2.087 | –0.20 | 1 | 69.38 (56.69–86.38) | 1.84 (1.42–22.47) | 10.0 |
| Mixture | | Glogit II | –9.24 | 7.21 | 0.29 | 1 | 39.77 (32.51–49.48) | 4.68 (6.79–20.73) | 19.67 |

*aNOAEL – no observed adverse effect level, marked as “<” when the lowest tested dose already produced a significant effect. bRM – regression models as defined by Scholze et al. (2001); for more details see “Material and Methods”; $\theta_1$, $\theta_2$, $\theta_3$, $\theta_{\text{max}}$ – statistical estimates of model parameters, given for doses expressed as mg/kg/day (rounded values); $\theta_{\text{max}}$ – upper model asymptote. c$ED_{50}$, $ED_{90}$ – effect doses for 50% and 90% normalized AGD index, calculated from the respective dose–response function. d$ED_{50}$, $ED_{90}$ – effect doses for 50% and 90% normalized AGD index, calculated from the respective dose–response function; 95% CI – 95% confidence intervals for mean effect doses given in mg/kg/day.

The analyses for single compounds were carried out using effect data pooled from the initial dose–response studies (Table 1, studies 1–3) and the repeat experiments run concurrently with the mixture study (Table 1, study 4). “Study run” was implemented as an additional model factor in data analysis. The effect doses ($ED_x$) shown in Table 2 were selected for low and median response levels and were calculated from the functional inverse of the best-fitting model. Statistical uncertainties for the estimated effect doses were expressed as 95% confidence intervals and approximately determined by applying the bootstrap method (Efron and Tibshirani 1993).

NOAELs were estimated using multiple contrast tests (Hothorn 2004). These tests were chosen as they are already implemented in the SAS procedures PROC NLMIXED and PROC GENMOD. Corresponding optimal contrasts were determined according to the best-fit regression model (Bretz et al. 2005).

**Calculation of mixture–effect predictions using dose addition.** To assess whether the joint effect of the three chemicals was dose additive, we predicted mixture effects based on information about the dose–effect relationships of all individual mixture components. These data were derived from the best-fit regression analyses of AGD data were carried out using the SAS procedure PROC NLMIXED (SAS Institute Inc., Cary, NC, USA).

The number of nipples/areolas was assumed to follow a binomial distribution with a response range between 0 and $\theta_{\text{max}}$, with $\theta_{\text{max}}$ being equal to the biologically possible maximal number of nipples in rats, either 12 or 13 (Table 2). The choice of $\theta_{\text{max}}$ was decided on considering the global fit (information criterion of Schwarz). To account for litter effects on NR, correlation structures between number of nipples/areolas and litter were modeled by the generalized estimating equations method (Vonesh and Chinchilli 1996). All statistical analysis was performed using the SAS procedure PROC GENMOD (SAS Institute, Inc., Cary, NC, USA).

The analyses for single compounds were carried out using effect data pooled from the initial dose–response studies (Table 1, studies 1–3) and the repeat experiments run concurrently with the mixture study (Table 1, study 4). “Study run” was implemented as an additional model factor in data analysis. The effect doses ($ED_x$) shown in Table 2 were selected for low and median response levels and were calculated from the functional inverse of the best-fitting model. Statistical uncertainties for the estimated effect doses were expressed as 95% confidence intervals and approximately determined by applying the bootstrap method (Efron and Tibshirani 1993).

**Note:**

- Table 2 shows the statistical dose–response descriptors for single and mixture exposures.
- The analyses for single compounds were carried out using effect data pooled from the initial dose–response studies (Table 1, studies 1–3) and the repeat experiments run concurrently with the mixture study (Table 1, study 4).
- “Study run” was implemented as an additional model factor in data analysis.
- The effect doses ($ED_x$) shown in Table 2 were selected for low and median response levels and were calculated from the functional inverse of the best-fitting model.
- Statistical uncertainties for the estimated effect doses were expressed as 95% confidence intervals and approximately determined by applying the bootstrap method (Efron and Tibshirani 1993).

**Table 2.** Statistical dose–response descriptors for single and mixture exposures.

| Substance | Fraction in mixture | RM | $\theta_1$ | $\theta_2$ | $\theta_3$ | $\theta_{\text{max}}$ | Effect doses (mg/kg/day) | NOAEL a |
|-----------|---------------------|----|-----------|-----------|-----------|-----------|----------------|---------|
| **Nipple retention** | | | | | | | | |
| Vinlozolin | 0.622301 | BP-probit | –1.76 | 0.025 | 1.024 | 12 | 67.18 (55.78–77.08) | 5.72 (0.67–25.58) | < 5.0 |
| Flutamide | 0.019558 | Probit | –0.61 | 2.01 | — | 12 | 2.02 (1.71–2.40) | 0.41 (0.28–0.60) | < 0.5 |
| Procymidon | 0.358141 | BP-logit | –5.09 | 1.21 | 0.1 | 12 | 33.91 (24.81–45.74) | 7.51 (1.0–10.76) | 10.0 |
| Mixture | | bw-Weibull | –19.66 | 16.83 | –0.8 | 13 | 20.76 (17.76–23.52) | 8.21 (6.67–10.42) | < 7.87 |
| **AGD index** | | | | | | | | |
| Vinlozolin | 0.622301 | Logit | –6.80 | 3.59 | — | 1 | 78.65 (67.43–83.32) | 9.21 (1.15–29.08) | 5.0 |
| Flutamide | 0.019558 | Weibull | –1.38 | 1.85 | — | 1 | 5.34 (2.84–4.37) | 0.34 (0.19–0.57) | < 0.5 |
| Procymidon | 0.358141 | bw-Weibull | –6.31 | 2.087 | –0.20 | 1 | 69.38 (56.69–86.38) | 1.84 (1.42–22.47) | 10.0 |
| Mixture | | Glogit II | –9.24 | 7.21 | 0.29 | 1 | 39.77 (32.51–49.48) | 4.68 (6.79–20.73) | 19.67 |
functions (Table 2) and used to calculate the expected responses of a mixture with defined mixture ratio over a large range of responses ("fixed mixture ratio design") (Faust et al. 2001). The choice of doses was based on the concentration range described by the additivity prediction, which is defined for a multi-component mixture of three components as

\[
ED_{x_{\text{mixture}}} = \left( \frac{p_1}{ED_{x_1}} + \frac{p_2}{ED_{x_2}} + \frac{p_3}{ED_{x_3}} \right)^{-1}.
\]

Here, \(ED_{x_1}\), \(ED_{x_2}\), and \(ED_{x_3}\) are the effect doses of vinclozolin, flutamide, and procymidone that on their own produce the same quantitative effect \(x\) as the mixture, and \(p_1\), \(p_2\), and \(p_3\) are the relative proportions of the corresponding individual doses present in the total mixture dose (see Table 2, "Fraction in mixture"). The individual effect doses were derived from the dose–response functions for vinclozolin, flutamide, and procymidone by using their inverse functional form. Equation 1 allows calculation of any effect dose of a mixture under the hypothesis of dose additivity, provided the dose–response functions of all mixture components and the mixture ratio are known. Graphs of predicted mixture dose–response curves (Figure 1) were obtained by calculating numerous \(ED_{x_{\text{mixture}}}\) values, with \(x\) varying from 10 to 90% for the normalized AGD index and from 1 to 11 for nipples. The statistical uncertainty for the predicted mixture–effect doses \(ED_{x_{\text{mixture}}}\) was determined by using the bootstrap method (Efron and Tibshirani 1993) and expressed as 95% confidence intervals (CIs) for the predicted mean estimate. Differences between predicted and observed effect doses were deemed statistically significant when the 95% confidence belts of the prediction did not overlap with those of the experimentally observed mixture effects.

**Results**

**Pregnancy and litter data.** No clinical signs of general toxicity were observed during the daily observations. The maternal body weight gain from GD7 to PND1 was significantly decreased (12.8 ± 14.6 g compared with 24.3 ± 9.4 g in the control group) in dams receiving the highest dose of vinclozolin (160 mg/kg/day), but none of the other doses of vinclozolin provoked this effect. Pregnancy length, litter sizes, birth weight of male and female offspring, and sex ratios in the litters remained unaltered in all vinclozolin-dosed groups when compared to controls. None of the tested doses of flutamide induced reductions of maternal weight gain, or other signs of maternal toxicity. In the range-finding study with procymidone (Table 1, study 2), litter sizes were markedly decreased at the highest dose of 200 mg/kg/day. The dose–response study (Table 1, study 3) using 150 mg/kg/day as the highest dose did not show effects on pregnancy length, litter sizes, birth weights, or sex ratios in the litters. Maternal body weight gain from GD7 to PND1 was decreased in the dams exposed to 25 mg/kg/day procymidone and higher, but no clear dose–response relationship was apparent with these weight gain changes.

In dams exposed to the two highest doses of the mixture of vinclozolin, flutamide, and procymidone (Table 1, study 4), maternal body weight gain from GD7 to PND1 was decreased. Among the groups of pregnant rats that were dosed with the single agents in parallel with the mixture experiment (Table 1, study 4), those receiving the higher dose of procymidone (61.8 mg/kg/day) also had diminished weight gain. Decreased litter sizes were observed at the high dose of flutamide (3.86 mg/kg/day). As this was not found in the previous dose–response study at the similar dose level of 4 mg/kg/day, or at the higher doses of 8 and 16 mg/kg/day, this is considered a random finding unrelated to exposure to flutamide. None of the mixture doses caused significant effects on pregnancy length, litter sizes, birth weights, and sex ratios in the litters.

**Effects of vinclozolin, flutamide, and procymidone on AGD and NR.** All chemicals produced dose-dependent changes in AGD index and NR and the resulting dose–response curves were observed to be quite steep. The entire effect range from control levels to maximal responses could be covered by dose changes of only two orders of magnitude (Figure 2). While vinclozolin and procymidone were of similar potency, flutamide was effective at approximately 10-fold lower doses.

Compared with the AGD index, NR was generally the more sensitive end point. At the lowest tested doses of 5 and 0.5 mg/kg/day, respectively, vinclozolin and flutamide induced statistically significant changes in NR, whereas the respective AGD indices did not differ significantly from those of controls at these doses. NOAELs could therefore not be defined for vinclozolin and flutamide. For procymidone a NOAEL of 10 mg/kg/day was estimated.

To gain an impression of variability among studies, selected dose levels of all three chemicals were retested in parallel with the mixture experiment. The reproducibility of effects observed in the earlier studies (Table 1, studies 1–3) was generally good. At the lower doses, the animals in the repeat studies were slightly less responsive in terms of changes in AGD index, but the NR effects tended to be a little higher upon retesting (Figure 2; Table 3). Table 2 summarizes key parameters characterizing the dose–response relationships of each single substance. Generally, our data are in broad agreement with results published by others (Foster and McIntyre 2002; Gray et al. 1999; Hellwig et al. 2000; Hib and Ponzo 1995; Hotchkiss et al. 2002; McIntyre et al. 2001; Miyata et al. 2002; Ostby et al. 1999; Shimamura et al. 2002). However, because of the unprecedented level of detail in our dose–response analyses, more in-depth comparisons are not possible.

**Combination effects of vinclozolin, flutamide, and procymidone.** The mixture of vinclozolin, flutamide, and procymidone produced dose-dependent changes in AGD index and NR (Figure 1). The NOAEL for changes in AGD index was 19.67 mg/kg/day, but the lowest tested mixture dose of 7.87 mg/kg/day induced statistically significant changes in NR (Table 2). Therefore, the overall mixture NOAEL is lower than 7.87 mg/kg/day.

The dose–response data for the single agents, pooled from all studies (Figure 2; Table 2), were used to compute predicted dose–additive combination effects covering the entire range of effects (Figure 1, green curves). For both end points, the anticipated combination effects fell within the range of the effects that were observed experimentally.

Numerical comparisons between predicted and observed AGD index (Table 3) revealed fairly good agreement. Despite the long period that had elapsed between the recording of the effects of the individual mixture components...
and the mixture experiment itself, the predicted effect doses in the median and high effect ranges differed by only a factor of 1.3 from those experimentally observed. Whether the anticipated combination effects were calculated using the data from the concurrent studies or using the pooled data sets including the historical data had little influence on the quality of the prediction. The joint effects of vinclozolin, flutamide, and procymidone on reductions of AGD in male rats were essentially dose additive.

In contrast, the deviations between prediction and observation were generally larger for NR than for AGD index, with observed NR responses exceeding the predicted mixture effects (Table 3; Figure 1). The effect doses predicted on the basis of the pooled single agent data were higher than the observed mixture–effect doses in the median- and high-effect range (6 and 10 retained nipples/areolas). This was not the case for the low-effect range (1 retained nipple/areola). Predictions based on the responses seen with single agents run in parallel with the mixture study (Table 1, study 4) produced lower effect doses in the median- and high-effect range, in better agreement with the observed results.

**Mixture effects at low doses of individual mixture components.** Because the doses of the single chemicals present in the mixture were quite low, we assessed whether there were significant combination effects when all components were present at doses that individually did not induce observable effects. At a dose of 39.37 mg/kg/day, the mixture induced a marked effect on the AGD index (around 50% reduction). This mixture contained 24.5 mg/kg/day vinclozolin, 0.77 mg/kg/day flutamide and 14.1 mg/kg/day procymidone, and individually these doses did not induce significant reductions in the AGD index (Figure 3). With NR as the end point, the single-chemical effects were small but statistically significant at these doses, whereas the combined exposure induced a marked effect.

**Discussion**

Previous work with anti-androgens has focused mainly on events surrounding AR binding and activation and has shown that combinations of these chemicals are able to act together in an additive fashion (Birkhoj et al. 2004; Nellemann et al. 2003). These studies have prepared the ground for addressing the question as to whether there are also joint effects with responses further removed from receptor binding and activation, such as those related to male sexual differentiation. For the first time, we have addressed this question by using the fixed mixture ratio approach for studying combination effects on the disruption of male sexual development.

With reductions of AGD as the end point, the experimentally observed mixture effects were in good agreement with the dose additivity expectation calculated on the basis of the individual dose–response relationships for vinclozolin, flutamide, and procymidone.

Although the predicted mixture effects for NR also fell within the limits of the lowest experimentally recorded responses, the
NR responses in the median and high end indicated that the mixture was more potent than predicted. The movement away from the anticipated combination effects can be partly attributed to the fact that the single agent responses seen concurrently with the mixture study were slightly higher than previously recorded, particularly in the high-effect range. When the mixture–effect prediction was based solely on the data from the concurrently run single agent studies, the differences between anticipated and observed effect doses for NR became smaller. This could indicate that the animals used for the mixture experiment showed subtle differences in their responses to the anti-androgens compared with the rats used for the earlier dose–response studies. The reason such differences should have become apparent only in terms of altered NR, but not in relation to AGD, may lie partly in the greater sensitivity of NR as an anti-androgenic end point. However, other as yet unrecognized factors may also have played a role. Seen in this light, we hesitate to interpret the joint effects of the mixture on NR as weakly synergistic, although the numerical discrepancies between observed and anticipated additive effects would support such a conclusion. Much larger studies would be required to resolve conclusively whether vinclozolin, flutamide, and procymidone exhibit a weak synergism with respect to NR. Nevertheless, in view of the complexity of the events leading to alterations in AGD and NR, and considering the experimental challenges in recording such effects reliably and reproducibly over a long period, we were surprised that the combined effects of the three anti-androgens could be predicted quite accurately. We therefore conclude that the dose addition approach provides an excellent basis for prediction of the joint effects of multicomponent mixtures of similarly acting anti-androgens.

Although the primary aim of our work was to assess the predictability of mixture effects of anti-androgens, the results of our study also allow assessments of the question as to whether there are joint effects when all mixture components are present at doses that individually do not induce detectable effects. This phenomenon, somewhat provocatively dubbed “something from nothing” (Silva et al. 2002), has been observed with multicomponent mixtures of estrogentic agents in reporter-based assays (Rajapakse et al. 2002; Silva et al. 2002; Tinwell and Ashby 2004). The something from nothing phenomenon also applies to alterations in the AGD of male rats exposed to anti-androgens during development. In this case a combination of 24.5 mg/kg/day vinclozolin, 0.77 mg/kg/day flutamide and 14.1 mg/kg/day procymidone induced half-maximal AGD alterations, but the effects induced by each chemical on its own did not reach statistical significance when compared with effects in untreated controls. However, whether the doses of the chemicals present in the mixture were indeed equivalent to nothing in the sense of zero effect levels is debatable. Regression analysis of the dose–response data for the three chemicals (Figure 2) showed that the effects associated with these doses were between 5 and 10% of a biologically possible maximal effect. In addition, in the earlier dose–response study, vinclozolin actually induced a significant effect on AGD at a lower dose than the 24.5 mg/kg/day present in the mixture. Generally, these results show that lack of statistical significance cannot be equated with an absence of biological effects.

Because of the apparently greater sensitivity of NR as an anti-androgenic end point, the something from nothing effect could not be evaluated with a combination of every mixture component can be replaced totally or in part by an equal fraction of an equi-effective dose of another, it does not matter whether the individual doses are also effective on their own. “Something from nothing” effects should occur even when individual toxicants are present at doses below effect thresholds, provided sufficiently large numbers of components sum up to a suitably high total-effect dose.

The results shown in Figure 3 support the idea that the “something from nothing” phenomenon also applies to alterations in the AGD of male rats exposed to anti-androgens during development. In this case a combination of 24.5 mg/kg/day vinclozolin, 0.77 mg/kg/day flutamide and 14.1 mg/kg/day procymidone induced half-maximal AGD alterations, but the effects induced by each chemical on its own did not reach statistical significance when compared with effects in untreated controls. However, whether the doses of the chemicals present in the mixture were indeed equivalent to nothing in the sense of zero effect levels is debatable. Regression analysis of the dose–response data for the three chemicals (Figure 2) showed that the effects associated with these doses were between 5 and 10% of a biologically possible maximal effect. In addition, in the earlier dose–response study, vinclozolin actually induced a significant effect on AGD at a lower dose than the 24.5 mg/kg/day present in the mixture. Generally, these results show that lack of statistical significance cannot be equated with an absence of biological effects.

Because of the apparently greater sensitivity of NR as an anti-androgenic end point, the something from nothing effect could not be evaluated with a combination of 24.5 mg/kg/day vinclozolin, 0.77 mg/kg/day flutamide and 14.1 mg/kg/day procymidone, because the individual doses induced NR that clearly reached statistical significance (Figure 3). The results, however, illustrate something not too dissimilar from the something from nothing phenomenon, which could be called “marked effects from small effects”: the mixture-induced NR approaching complete feminization of the males, whereas the individual doses caused only modest effects. In general, our findings do not contradict theoretical expectations and are consistent with the earlier observations made with mixtures of estrogenic chemicals (Brian et al. 2005; Rajapakse et al. 2002; Silva et al. 2002; Tinwell and Ashby 2004). The something from nothing phenomenon would most probably have been demonstrated also with NR as the end point, had lower doses been employed or had more mixture components been combined.

In conclusion, our results show that combinations of similarly acting anti-androgens are able to produce developmental effects in male offspring of rats. These effects can be predicted fairly accurately on the basis of information about the potency of the individual mixture components by using the dose addition concept. There are indications that anti-androgens act together to produce marked joint effects when combined at doses that individually produce small, statistically insignificant responses. The significance of these findings for human and environmental risk assessment cannot be overstated; doses of endocrine-active chemicals, which appear to exert only small effects when judged on their own, may induce marked responses when they act in concert with numerous, possibly unrecognized, similarly acting agents.

![Figure 3. Mixture effects on AGD (A) and NR (B) at low doses of individual mixture components. Results shown are group mean ± 95% confidence belt for control males and females (gray), individual doses of 24.5 mg/kg/day vinclozolin (VZ), 0.77 mg/kg/day flutamide (FLUT), and 14.1 mg/kg procymidone (PRO) (blue), the combined mixture dose of 39.37 mg/kg (blue), and the predicted mixture effect (white). Open circles represent litter means. *p < 0.05 compared to control.](image-url)
REFERENCES

Altenburger R, Bödeker W, Faust M, Grimm LH. 2000. Analysis of combination effects in aquatic toxicology. In: Handbook of Hazardous Materials (Corn M, ed). San Diego:Academic Press, 15–27.

Backhaus T, Altenburger R, Bödeker W, Faust M, Schulze M, Grimm LH. 2000. Predictability of the toxicity of a multiple mixture of dissimilarly acting chemicals to Vibrio fischeri. Environ Toxicol Chem 19:2348–2356.

Birchaj M, Nellemann C, Jarfleit K, Jacobsen H, Andersen HR, Dalgaard M, et al. 2004. The combined antiandrogenic effects of five commonly used pesticides. Toxical Appl Pharmacol 210:10–20.

Blount BC, Silva MJ, Caudill SP, Needham LL, Pirkle JL, Sampson JA, et al. 2000. Levels of seven urinary phthalate metabolites in a human reference population. Environ Health Perspect 108:879–882.

Bretz F, Pinheiro JC, Branson M. 2005. Combining multiple studies. Biometrics 61:738–748.

Gray LE Jr, Ostby J, Furr J, Wolf CJ, Lambright C, Parks L, et al. 2001. Effects of environmental antiandrogens on reproductive development in experimental animals. Hum Reprod Update 7:248–264.

Guillellet LJ Jr. 2000. Contaminant-induced endocrine disruption in wildlife. Growth Horm IGF Res 10:545–50.

Hewlett PS, Plackett RL. 1959. A unified theory for quantal responses to mixtures of drugs: non-interactive action. Biometrika 15:591–610.

Hib J, Ponzo R. 1995. The abnormal development of male sex organs in the rat using a pure antiandrogen and a 5 alpha-reductase inhibitor during gestation. Acta Physiol Pharmacol The Latinoam 45:27–33.

Ostby J, Kelce WR, Lambright C, Wolf CJ, Mann P, Gray LE Jr. 1999. The fungicide prochloraz induces sexual differentiation in the male rat by acting as an antisteroid receptor antagonist in vivo and in vitro. Toxicol Ind Health 15:80–93.

Payne J, Schulze M, Kortenkamp A. 2001. Mixtures of four organochlorines enhance human breast cancer cell proliferation. Environ Health Perspect 109:391–397.

Raipakas N, Silva E, Kortenkamp A. 2002. Combining xenosterogens at levels below individual no-observed-effect concentrations dramatically enhances steroid hormone action. Environ Health Perspect 110:917–921.

Raipakas N, Silva E, Scholze M, Kortenkamp A. 2004. Deviation from additivity with estrogenic mixtures containing 4-nonylphenol and 4-tert-octylphenol detected in the E-SCREEN assay. Environ Sci Technol 38:6343–6352.

Robert H, Holson JF, Stump DG, Knapp JF, Reynolds V. 1999. Interpreting the toxicologic significance of alterations in anogenital distance: potential for confounding effects of progeny body weights. Reprod Toxicol 13:383–396.

Scholze M, Bödeker W, Faust M, Backhaus T, Altenburger R, Grimm LH. 2001. A general best-fit method for concentration-response curves and the estimation of low-effect concentrations. Environ Toxicol Chem 20:448–457.

Schwarz G. 1978. Estimating the dimension of a model. Ann Stat 6:461–464.

Shimamura M, Kodaira K, Kenichi H, Ishimoto Y, Yamura H, Iguchi T. 2002. Comparison of antianabolic activities of vinclozolin and camphorquinoine in androgen receptor gene transcription assay in vitro and mouse in utero exposure assay in vivo. Toxicology 174:97–107.

Silva E, Raipakas N, Kortenkamp A. 2002. Something from “nothing”—eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. Environ Sci Technol 36:1751–1756.

Simard J, Luthy I, Guay J, Belanger A, Labrie F. 1986. Characteristics of interaction of the antiandrogen flutamide with androgen receptors. Mol Endocrinol 44:261–270.

Swan SH, Main KM, Liu F, Steward SL, Kruse RL, Calafat AM, et al. 2005. Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ Health Perspect 113:1056–1061.

Timwell H, Ashby J. 2004. Sensitivity of the immature rat uterotrophic assay to mixtures of estrogens. Environ Health Perspect 112:575–582.

Vonk E, Chinchilli VM. 1996. Linear and Nonlinear Models for the Analysis of Repeated Measurements. New York:Marcel Dekker.