Appendix B:

Mammalian Studies Describing the Effects of Chemicals That Disrupt the Androgen Signaling Pathways

Contents of the Appendix of Studies Describing the Effects of Chemicals That Disrupt the Androgen Signaling Pathways

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B. ANDROGEN SIGNALING PATHWAY

The androgen signaling pathway shares many molecular and cellular traits with the estrogen signaling pathway. However, there are also some notable differences. Unlike the E pathway which has several ligand activated nuclear receptor (ERβ and ERλ) there is only one wildtype androgen receptor (AR) in mammals. In addition, there are twp physiologically active androgens. While testosterone (T) is the major regulatory steroid in many androgen-dependent tissues, others rely upon the conversion of T to dihydrotestosterone (DHT) by the enzyme 5α reductase. In the A pathway, as with the E pathway, there are tissue-specific cofactors that imbue tissue-specific responses and multiple forms of the androgen response elements (AREs) on different genes. AREs fall into two general classes, one of which is specific for AR and others that also are activated by PR and GR.

Evolutionarily, the ancestor ER and the E pathway arose first, followed by the progesterone receptor (PR), and more recently the AR and corticoid receptor (GR and MR) mediated pathways (Thornton (2001) fig. B.1).

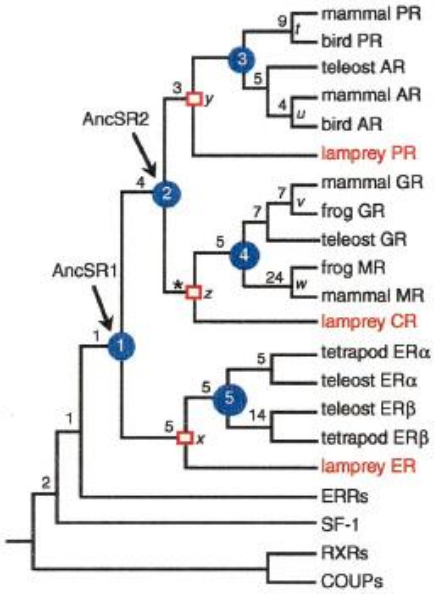


Fig. 1. Phylogeny of the steroid receptor gene family. A reduced version is shown of the single most parsimonious phylogeny of 73 receptor sequences when the relative weight of gene duplications/losses to amino acids $w > 3$. (Length = 3,209 aa changes + 8 duplications + 0 losses. For unreduced phylogeny, see Fig. 7, which is published as supplemental data.) Support for each clade is shown as the number of extra steps required for the labeled node not to appear in the most parsimonious tree (19); all support values are insensitive to w except *, shown for $w = 10$. Blue circles indicate gene duplications within the steroid receptor family; red squares mark the lamprey–gnathostome divergence; and unmarked nodes represent other speciation events. Ancestral steroid receptors are indicated. Italicized node labels correspond to Fig. 3. Tree length = 3,209 aa changes, eight duplications, zero losses; consistency index = 0.628; retention index = 0.870.

Figure B.1 reproduced from Thornton (2001).

Structurally and ancestrally, the AR, and PR are more closely related to one another and then to the GR than they are to the ER. For this reason, chemicals that bind and activate or inhibit AR also often disrupt PR and possibly GR but not ER pathway functions and the ability of different ligands to cross react with the AR, GR and/or PR varies greatly from chemical to chemical often depending upon subtle differences in the chemicals' structures. For example, altrenogest (allyltrenbolone) is a progestogen structurally related to veterinary steroid and PIE trenbolone (TB) and the antiandrogenic pesticide vinclozolin (VIN) (Laws et al., 1996) and the potent synthetic androgens trenbolone and tetrahydrogestrinone (THG) bind both AR and PR (Death *et al*., 2004). However, some chemicals, like bisphenol A (BPA) and bisphenol B are weakly estrogenic and weakly antiandrogenic *in vitro* and in the fathead minnow assay but not *in vivo* when administered orally to rats.

It is clear that the tested substances that bind AR act as antiandrogens and not as androgens; this is by contrast with environmental contaminants that bind ER, which primarily act as agonists, not antagonists.

The literature reviewed in the following section is not as extensive as is the literature for the E pathway, but there are several chemicals with a very robust data base that provides some unique insights on the prevalence of NMRDCs and the conditions under which they occur. Those chemicals are the following:

- AR antagonists Flutamide (FLU), VIN and procymidone
- Finasteride, an inhibitor of 5α reductase
- Prochloraz (PCZ) and Linuron**,** pesticides that display AR antagonism and inhibit androgen synthesis
- Phthalate esters**,** toxicants that inhibit the expression fetal genes involved in sex differentiation, are Sertoli cell and testis toxicants in pubertal males and disrupt pregnancy maintenance

As is the case for the estrogen pathway literature, several different research groups have designed studies using oral administration of the test chemical specifically to examine of shape of the dose response curves over a broad dose response range. These studies include multigenerational and transgenerational studies that have included endpoints sensitive to detection of disruption of the androgen signaling pathway.

In addition, this appendix includes discussion of the androgens T and trenbolone, and a few selective androgen receptor modulators (SARMS). These provide some robust examples of the study conditions under which NMDRs may occur and how these responses relate to other affected endpoints. However, the data for SARMs from multigenerational and transgenerational studies, (using oral exposure, low dose dose-response) are not publically available except as summaries.

B.1 Androgen Receptor Antagonists

B.1.a Flutamide (FLU)

FLU is a potent and well studied antiandrogenic drug that has been used in numerous studies *in vivo* as a "model chemical" to examine the shape of the dose response curve and to identify sensitive endpoints. The studies support an evaluation of the dose related effects of FLU across all levels of biological organization, from upstream toxicogenomic measures to malformations in male rat offspring. Generally the phenotypic no observed effect levels (NOELs) were similar to those obtained from the toxicogenomic data, and the lowest effect level was 0.1 mg/kg/d for reduced ventral prostate weight in the Hershberger assay. When administered *in utero*, FLU induced reproductive tract abnormalities at 0.4 to 2 mg/kg/d with offspring exposed to 6 to 10 mg/kg/d being severely malformed. Toxicogenomic alterations were reported by some authors in the 1-6mg/kg/d range, but they were not seen at 0.2 mg/kg/d**.** None of these studies noted any NMDRs on any endpoint, regardless of the timing of exposure or level of biological organization.

B.1.a.1 Hass et al. (2007) and Metzdorff et al. (2007)

Hass et al. (2007) and Metzdorff et al. (2007) published a pair of papers from a study that included data on the dose-related effects of FLU (0.5, 1, 2, 4, 8 or 15 mg/kg/d,n=8 dams per group) on reproductive development of the male offspring up to 16 days of age. They also studied vinclozolin, procymidone (discussed later) and a mixture of these three AR antagonists. In the mixture study, FLU was also administered by itself at 0.77 and 3.86 mg/kg/d. Dams were dosed from gestation day (GD) 7 to 21 and then from postnatal day (PND) 1 to 16, when the animals were necropsied. The authors examined the following: anogenital distance (AGD); female-like nipple retention; reproductive organ weights and histopathology;, expression of four genes in the ventral prostate; and the gross anatomy of the reproductive tract. For FLU, pups displayed only mild dysgenesis of the external genitalia at the low doses of (0.77 mg/kg/d); this was based upon a dysgenesis scores of 1 (mild), 2 (moderate) and 3 (severe). Animals with a moderate dysgenesis to severe effects were reported at 3.86 mg FLU/kg/d.

Dose- related effects were noted in all the treated groups with nipple retention being the most sensitive endpoint followed by neonatal AGD in the male offspring**.** None of the effects in the current study displayed an NMDR; according to the authors, 0.5 mg/kg/d was a lowest observed effect level (LOEL) (based upon nipple retention in male rat offspring), and a NOEL was not established**.**

Note: There are about 7 publications on the effects of FLU in the male rat that all used the same dose range (oral treatment at doses of 0.4, 2, or 10 mg/kg/day). The intent of the series was to determine the shape of the dose response curve in the low dose region, to identify sensitive endpoints and to integrate standard toxicological adverse effects with more upstream genomic and biochemical endpoints

The next four papers all exposed pregnant rats to FLU at 0.4, 2, or 10 mg/kg/day. Taken together, they support a comprehensive evaluation of the effects of FLU including malformations, reproductive function, pubertal development, and testicular gene and protein expression.

B.1.a.2 Yamasaki et al. (2005)

Pregnant rats were dosed orally with FLU at 0.4, 2, or 10 mg/kg/day from gestational day 6 to PND 20 (12 to 16 dams per dose group), and the effects of FLU exposure on male offspring were examined 10 weeks after birth. In addition, FLU treatment was continued after weaning for one half of the males so that the effects of FLU could be compared to the offspring that were no treated after weaning. FLU-treatment caused the following: neonatal loss (at 10 mg/kg/d); reduced AGD in male offspring at PND 4 (at 2 and 10 mg/kg/d); cleft phallus (at 2 and 10 mg/kg/d); small testes (at 2 and 10 mg/kg/d); and vaginal pouch (one noted at 10 mg/kg/d). In addition the age of preputial separation (PPS - puberty in males) was delayed (at 2 and 10 mg/kg/d) with continuous exposure. This was not observed in males untreated after weaning whose dams were treated with 2 mg/kg/d. Some males not treated after weaning in the 10 mg/kg/d never completed PPS due to presence of genital malformations. Continuous exposure to FLU also caused increases in serum luteinizing hormone (LH) and T (only at 10 mg/kg/d). Continuous treatment with FLU also was more effective in reducing androgen-dependent tissue weights than was FLU treatment only during gestation and lactation. The NOEL for these organ weights was lowered from 2 to 0.4 when treatment was continued after puberty. As expected, continuous FLU treatment did not alter the incidence of malformations as compared to offspring exposed only during gestation and lactation. The authors noted that “the present data demonstrated that the endocrine-mediated effects on rats were more appreciable in offspring treated with FLUT after weaning than in offspring untreated after weaning”. In summary, 0.4 was identified as a NOAEL, and 2 mg/kg/d was the LOAEL. No NMDRs were noted in the current FLU study.

B.1.a.3 Bozec et al. (2004)

In the Bozec et al. (2004) study, FLU was administered to pregnant rats daily by oral gavage from GD 10 to GD 21 or 22 at doses of 0, 0.4, 2, or 10 mg/kg/d (n=15 dams per dose group). F1 male rat offspring were necropsied at 90 days of age, and testes (bilateral descended only) were examined. Testes were examined histologically for Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) staining, and apoptotic germ cells and Sertoli cell nuclei were examined and categorized by tubular staging. FLU induced cryptorchid testes in 33% of the rats at 10 mg/kg/d whereas males in the lower dose groups had normal testis descent. Cell death was increased in a dose related manner in the seminiferous tubules of FLU-treated animals, being seen primarily in tubular stage VI-VII. The effects of *in utero* exposure to FLU, if any, on Bcl-w and Bcl-2, Bak, Bax and Bid mRNA and protein levels in adult rat testes were all monotonic dose-related effects. No NMDR were noted in any of the proteins or mRNA levels.

B.1.a.4 Maire et al. (2005) - Alteration of Transforming Growth Factor

The study by Maire et al. (2005) is a follow up to the observations of Bozec et al. (2004) above. In the Maire et al. (2005) study, FLU was administered to pregnant rats daily by oral gavage from GD 11 to GD 21 or 22 at doses of 0, 0.4, 2, or 10 mg/kg/d. F1 male rat offspring were necropsied at 90 days of age, and testes (bilateral descended only) were examined (n=10 males per group) for FLU-induced alterations of TGFβ-ligands, TGF-β receptors mRNA, and Smad mRNA and protein levels; as only control and high dose groups for the proteins were reported, they are not discussed here. Ten of 12 mRNA expression levels were altered. All of the mRNA expression levels of c-Jun, c-*jun* phosphorylation, and Fas-L were elevated in the F1 adult rat testis. Some these mRNAs were altered at the lowest dosage level but there were no NMDRs.

B.1.a.5 Benbrahim-Tallaa et al. (2008)

FLU was administered to pregnant rats daily by oral gavage from GD 10 to GD 21 or 22 at doses of 0, 0.4, 2, or 10 mg/kg/d (n=15 dams per dose group). mRNA was measured in male offspring by rtPCR on nine testis Sertoli cell genes, apoptosis related caspase protein levels, serum LH, T, estradiol and follicle stimulating hormone (FSH), testis weight, and AR mRNA and AR protein were measured in the testis. The authors concluded that Sertoli cells exposed to FLU *in utero* were protecting the germ cells from apoptosis. Not all the dose response data are shown in the Benbrahim-Tallaa et al. (2008) paper, but no NMDRs were noted in the data that are presented.

B.1.a.6 Miyata et al. (2002)

Miyata et al. (2002) administered FLU (0, 0.15, 0.6, 2.5, 10, 100 mg/kg/d) to pregnant rats from GD 14 to PND3 and examined the postnatal development of the male offspring after birth up to 60 days of age. Fertility, fecundity, pup weight AGD, nipple retention, testis descent and urogenital malformations were determined. Some animals were necropsied at 4 days of age while the rest were necropsied at 60 days of age, organ weights taken, and hormonal and histopathological evaluations conducted.

AGD was decreased at 2.5 mg/kg and above, and the following effects were observed at 10 mg/kg and above: female-like retained nipples, hypospadias, vaginal pouch, penis malformations, undescended testes, and decreased organ weights (prostate, seminal vesicles, levator ani muscle plus bulbocavernosus muscle, testis. Histopathological defects included atrophy and inflammatory alterations in several reproductive tract tissues at 10 mg/kg and above, and body weight was decreased at 100 mg/kg/d. None of these effects displayed a NMRDC.

The authors concluded that the most sensitive measure was AGD, whereby reduction was observed at 2.5 mg/kg and that 0.6 mg/kg/d was a clear NOEL.

The limitation of this study is that is used a small number of dams/litters per dose group (4-6), and the statistical analysis did not account for litter effects. It is included in this appendix as it

complements the more robust transgenerational studies with FLU and it used a slightly lower dosage level.

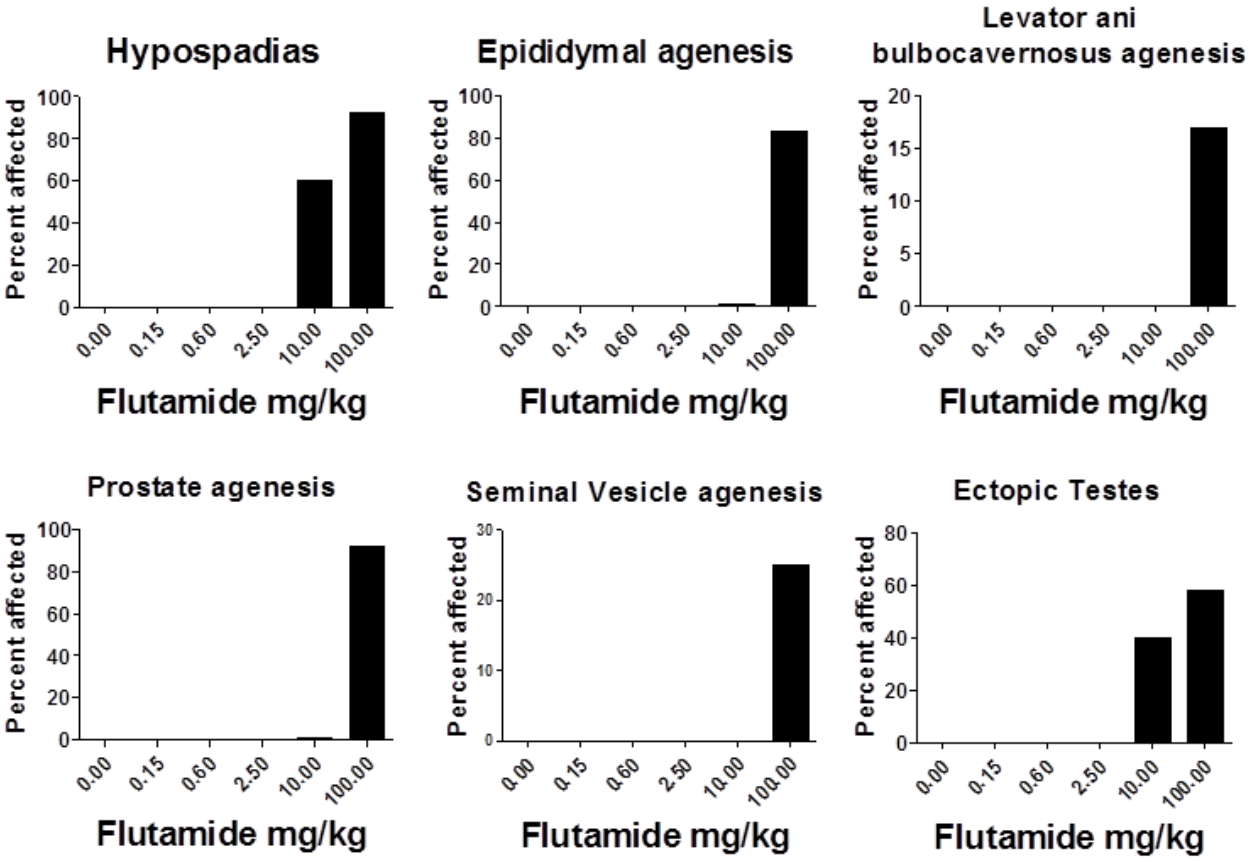


Figure B.2 from Miyata *et al.* (2002).

B.1.a.7 Mcintyre et al. (2001) –

Pregnant rats were dosed by oral gavage with 0, 6.25, 12.5, 25, or 50 mg FLU/kg/day from gestation days 12 to 21. F1 male offspring were studied throughout the postnatal period, and AGD, areola/nipple retention, cryptorchidism, reproductive organ weights, and malformation incidence were examined. *In utero* FLU exposure had the following statistically significant effects: decreased the AGD at birth; increased female-like and induced reproductive nipple retention in infant male rats; and induced reproductive tract malformations including undescended testes, hypospadias, prostate agenesis, and epididymal agenesis and decreased the weights of the seminal vesicles, levator ani bulbo-cavernosus (LABC) muscles, testes, and epididymides in a dose-dependent manner. Interestingly, the relative sensitivites of the endpoints affected by FLU are nearly identical to the sensitivity to disruption to other AR antagonists like VINand procymidone. As shown in figure B.3 below, none of these effects displayed an NMDR, 6.25 mg/kg/d was a LOAEL, and no NOAEL was determined.

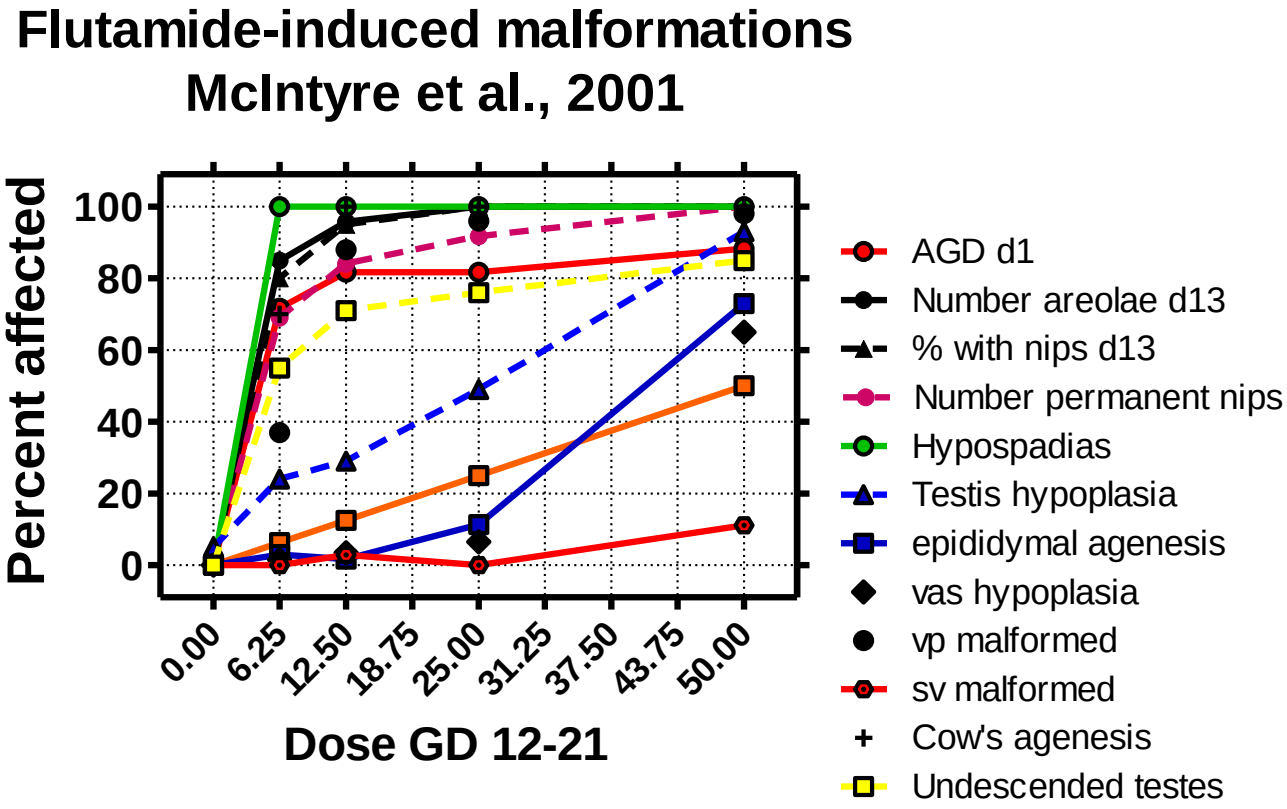


Figure B.3 from Mcintyre et al. (2001).

B.1.a.8 Ludwig et al. (2011)

This study was conducted to compare conventional measures of toxicity (serum T levels, testis histopathology and testis, epididymal, seminal vesicle, pituitary, prostate, adrenal, liver and body weights) with testis gene expression (using both microarrays and reverse transcriptase PCR) and protein expression. Young adult male rats were dosed with FLU for 28 days at 0.2, 1, 6 or 30 mg/kg/day.

Seminal vesicle weight was reduced at 1, 6 and 30 mg/kg/d; epididymal, prostate weights were reduced, and serum T were increased at 6 and 30 mg/kg/d; and testis histopathology (Leydig cell [LC] hyperplasia) was seen at 30 mg/kg/d. Testis and body weights were not affected at these dosage levels. All toxicogenomic assessments (testis gene expression and selected proteins levels) were altered at 1, 6 and 30 mg/kg/d, but no effects were noted at 0.2 mg FLU/kg/d. Furthermore, pathway analysis with Ingenuity software indicated that the six pathways affected by FLU all showed dose related changes with no effects a 0.2 mg/kg/d. In summary, the NOEL/LOEL for standard toxicological and toxicogenomic measures were 0.2/1.0 mg/kg/d, and none of the effects, at any level of biological organization, displayed an NMDR.

B.1.a.9 Rouquié et al. (2009) and Friry-Santini et al. (2007)

In these two studies, young adult male rats were dosed with FLU for 28 days at 0, 0.04, 0.2, 1, or 6 (Rouquié et al., 2009), and 0, 6, 30 or 150 mg/kg/day (Friry-Santini et al., 2007); results of both studies are summarized in the 2009 paper. They determined the following: degree of testis LC hyperplasia; expression of testis genes by microarray analysis(2007 study); and PCR of genes related to lipid and steroid hormone synthesis, cell proliferation and cell death, as well sa few genes involved in amino acid metabolism and carbohydrate metabolism and glycolysis. The results are generally consistent with the effects of FLU in the study by Ludwig et al. (2011) except that the reductions in seminal vesicle weight and toxicogenomic changes were seen only at 6 m FLU/kg/d and above rather than at 1 mg/kg/d as Ludwig had reported. In addition, serum T (at 30 and 150 mg/kg/d) and serum LH (at 6 and above) were elevated as compared to controls. In summary, no NMDRs were detected for any endpoint, histopathological, organ weights, or gene or protein expression levels.

B.1.a.10 Owens et al. (2006)FLU

FLU, along with several other chemicals, was administered by oral gavage in the OECD Hershberger Assay validation; this was a multi-lab (seven different laboratories) exercise done to determine if the protocol developed for this purpose could be used to identify antiandrogenic chemicals reliably (Gray et al., 2005a). FLU was administered to castrate-immature-androgen treated male rats at 0, 0.1, 0.3, 1, 3 or 10 mg/kg/d (n=6 rats/group/laboratory) for 10 days (Owens *et al.,* 2006. Subsequently, the animals were necropsied, and organ weights were weighed; organs included five androgen-dependent tissues and the liver, kidneys and adrenal glands. FLU induced dose-related linear reductions in the androgen-dependent tissues with most effects being statistically significant at the lowest FLU dose of 0.1 mg/kg/d; hence a NOEL was not determined. The effects appear to be linear (in the low dose range, but no NMDRs were induced by FLU in this sensitive screening assay.

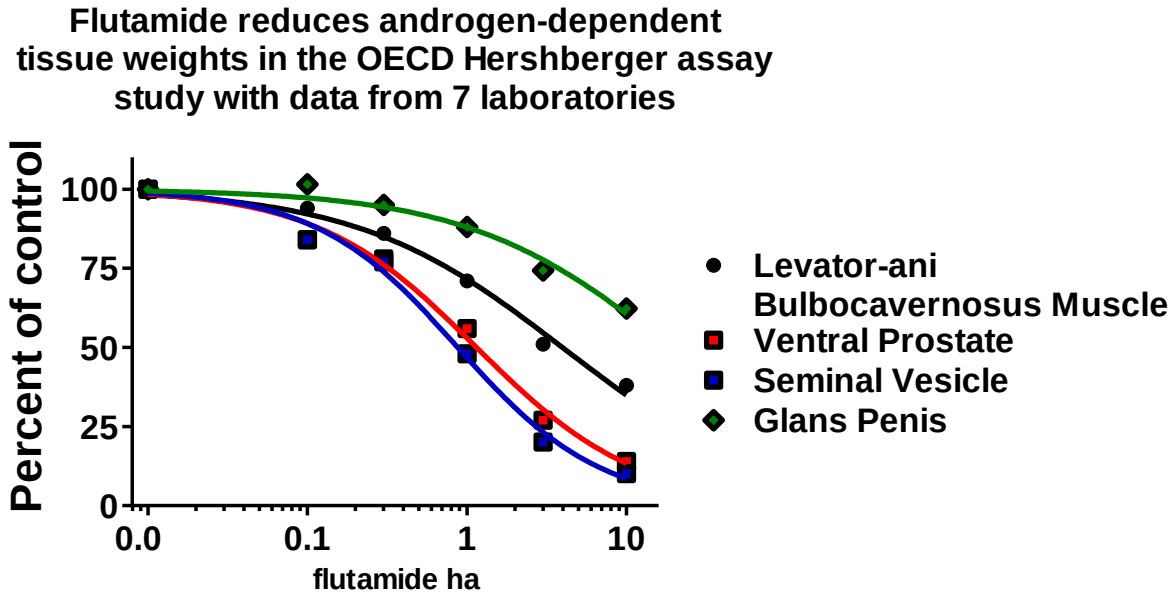


Figure B.4 Adapted from Owens et al. (2006).

B.1.a.11 Kelce et al. (1994), Wong et al. (1995) and Wilson et al. (2002)

FLU and other AR antagonists can display NMDRs and induce Androgen-mediated gene expression *in vitro*. *In vitro*, AR antagonists like FLU and VIN bind AR and inhibit AR-induced gene expression; FLU is about 35 fold more potent *in vivo* (in the Hershberger assay) than is VIN. *In vitro*, the active metabolites of FLU (hydroxyflutamide) and VIN (M2) also display androgenic activity at higher concentrations (Wong et al., 1995; Kelce et al., 1994). In some *in vitro* assays, this mixed antagonist / agonist activity results in NMDRs (Wilson et al., 2002) with the level of gene expression declining and then increasing as the concentration is increased (Fig. B.5 from Wilson et al. (2002)). However, the *in vivo* dose response data for FLU (above) and VIN (below) provide no evidence that the androgenic activity seen *in vitro* is expressed *in vivo*. It addition, several other AR antagonists also are androgenic *in vitro*, and for some of these chemicals, the concentration that produces AR antagonism of androgen in the media also induces AR-dependent gene expression when androgen is not present in the media. All of these, however, appear to act antiandrogens *in vivo*.

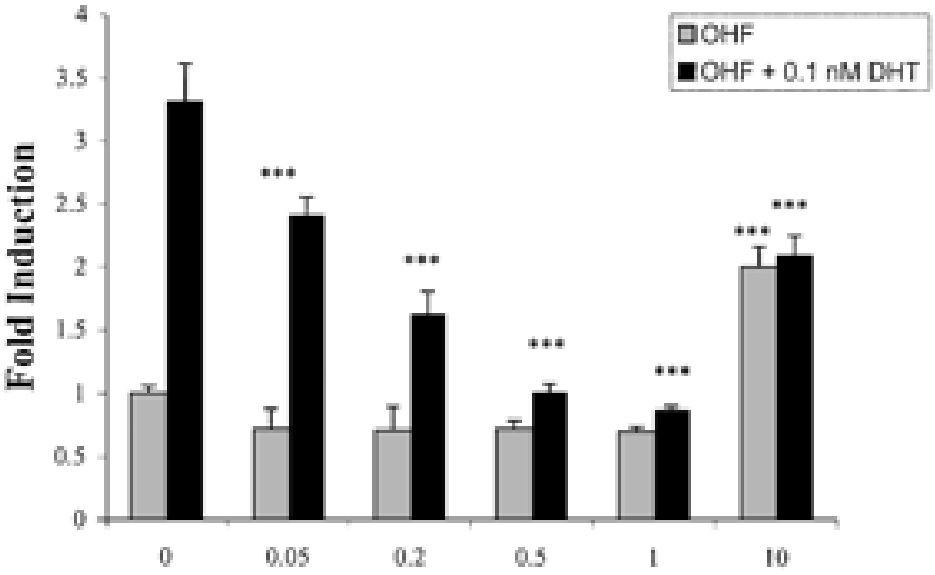
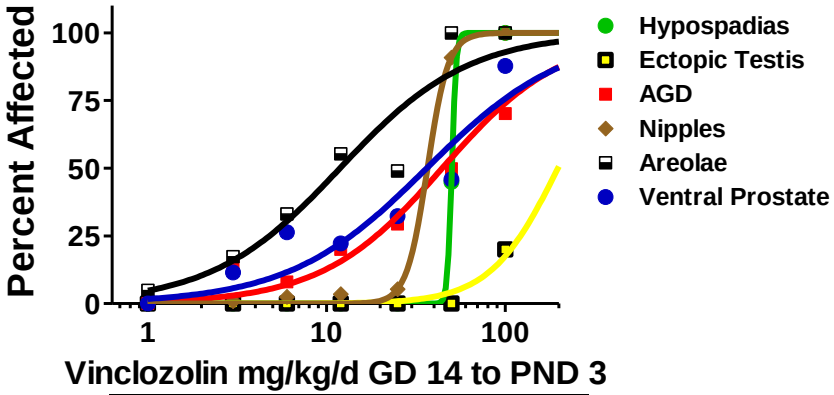


Figure B.5 reproduced from Wilson et al. (2002).
Y axis is level of gene expression; units in x-axis is micromolar.

B.1.b Vinclozolin (VIN)

B.1.b.1 Ostby et al. (1999b)

In the current study VIN was administered at 0, 3.125, 6.25, 12.5, 25, 50, or 100 mg/kg/d by oral gavage to rats from day 14 of pregnancy until day 3 of lactation, and the reproductive development of all the male offspring from every litter was monitored through adulthood. AGD was significantly reduced at doses of 3.125 mg VIN/kg/d and above, and the incidence of female-like nipples/areolas was increased. Ventral prostate weight in one year old male offspring was reduced in all treatment groups, being statistically significant at 6.25, 25, 50, and 100 mg/kg/d. Permanent nipples were detected in of the males at 3.125mg/kg/d, 3.6% at 6.25- , 3.9%, at 12.5 8.5%, at 25- 91%, at 50-and 100 % at 100 mg/kg/d respectively. VIN treatment at 50 and 100 mg/kg/d induced reproductive tract malformations and reduced ejaculated sperm numbers and fertility in male offspring. Even though all of the effects of VIN likely result from the same initial event – AR binding and antagonism of endogenous androgens -- the different endpoints displayed a wide variety of dose–response curves and ED50's. The dose response data for several of the functional endpoints appeared to be linear whereas other endpoints clearly displayed a steep threshold with increasing dosage levels of VIN (see figure B.6below). Similar to FLU, VIN-treatment *in utero* causes LNT dose response curves for sensitive endpoints like AGD and nipple retention, whereas malformations like hypospadias and ectopic testes consistently display steep thresholds. It appears that the shape of the dose response curve is consistent from chemical to chemical for each tissue and not by the mechanism of toxicity. In summary, no NMDRs were noted in the current study.



	ED50	HillSlope
Areolae	11.91	1.208
Ventral Prostate	36.39	1.125
Nipples	36.72	7.451
AGD	44.46	1.29
Hypospadias	50.28	36.39
Ectopic Testis	197.4	2.28

Figure B.6 from Ostby et al. (1999b).

B.1.b.2 Hellwig et al. (2000)

Hellwig et al. (2000) administered VIN by oral gavage to LE and Wistar rats from day 14 of pregnancy until day 3 of lactation, and the reproductive development of all the male offspring from every litter was monitored through adulthood. In the study with LE rats, VIN was administered at 0, 1, 3, 6, 12, or 200 mg/kg/d whereas the Wistar rat study used doses of 0, 3, 12 or 200 mg/kg/d. VIN at 200 mg/kg/d increased the numbers of stillborn pups in both rat strains at the higher dosage levels. In general the effects were similar to those seen by Ostby et al. (1999b) except that the NOELs for AGD and nipple retention were higher than reported by Ostby et al. (1999b). In the Wistar strain, terminal body weight (at 3 mg/kg/d), testes weight (at 12 mg/kg/d), epididymal weight (at 3 and 12 mg/kg/d) and coagulating gland weight (at 3 mg/kg/d) displayed slight, but statistically significant, NMDRs. However, none of these NMDRs were repeated in the LE strain, and, it is not clear that the statistical analysis of the organ weight data was adjusted for litter effects. Not adjusting for litter effects affects statistical significance as the error df is increased, and the SE is often decreased. In summary, NMDRs were seen on body and organ weights in one rat strain but not in the other or in the study by Ostby et al. (1999b) in the LE rat.

B.1.b.3 Matsuura et al. (2005)

The Matsuura et al. (2005) study examined the effects on male and female rats of VIN administration at 0, 40, 200, or 1000 ppm over two-generations (equivalent to 0, 2.3-6, 11-29 and 57-150 mg/kg/d, respectively; n=24 pairs per dose. These dosage levels were selected from a dose range finding study that administered VIN to male and female rats at 0, 500, 1000, 3000 and 6000 ppm for 3 weeks prior to mating up to lactational day 12. In the range finding study body weight gains were suppressed in the two high dose groups, fertility was reduced in the high dose group; AGD was reduced, and retained nipples were displayed in males at 500 ppm and above.

In the main study the investigators measured the following: AGD at birth; nipple retention; onset of puberty (vaginal opening [VO] in females and PPS in males); estrous cyclicity; spermatogenesis; sex organ weights; serum hormone levels (TSH, T3, T4, LH, FSH, prolactin in both sexes, estradiol and progesterone in females and T and DHT in males); and hepatic drug-metabolizing enzyme activities. Male rats exposed to VIN *in utero* displayed the following effects even at the lowest dose of 40 ppm: shortened AGD, nipple retention, delayed puberty, reduced sex organ weights, and altered serum hormone concentrations. In addition histopathological alterations were seen at 200 ppm, and mating was reduced at 1000 ppm. Other effects in males included increased pituitary and testis weights and decreased epididymal and seminal vesicle weights at 1000 ppm and decreased prostate and epididymis weights at 200 and 1000 ppm. Histopathological lesions in males were detected in the liver, adrenals (also seen in females at 40 ppm) and pituitary at 200 and 1000 ppm; lesions were seen in the testes (LC hyperplasia), seminal vesicle atrophy and ovaries at 1000 ppm in P0 and F1 rats. In addition, serum LH, FSH, testosterone, and DHT were increased in P0 and F1 males, and body weight gain was reduced in both sexes at 1000 ppm. Decreases in T3 and/or T4 were observed in both sexes and generations at 1000 ppm and in F0 females at 200 ppm. VIN also induced

hepatic drug-metabolizing enzymes in both sexes and generations at 1000 ppm. Male-mediated infertility in F1 males was induced at 1000 ppm as a consequence of the penile malformations. In contrast to the effects in F1 males, VIN did not affect estrous cyclicity, mating, fertility, pregnancy, parturition, or nursing behavior in either P0 or F1 females. In summary, VIN induced subtle alterations (nipple retention in F2 males) at the lowest dose level of 40 ppm (2.3-6 mg/kg/d); followed by a cascade of reproductive, endocrine and other effects in males and females exposed *in utero* at 200 ppm; finally resulting in male-mediated infertility due to severe reproductive tract malformations at 1000 ppm along with alterations of liver function and serum reproductive and thyroid hormone levels.

This two generation study included measurement of about 200 endpoints, listed by categories below with notation as to observation of NMDRs:

- P0 organ weights male and female: about 25 measures
  - NMDR for liver males but not females at 40 and diestrus uterine weight
- F1 adult organ weights male and female: about 25 measures – No NMDR
- F1/F2 male and female organ weights at weaning: about 25 measures – No NMDR
- P0 and F1 organ histopathology of about 19 measures- No NMDR P0/F1 serum hormones in male and female: about 44 measures- No NMDR
- P0/F1 estrous cycles and sperm measures: about 10 measures- No NMDR
- P0/F1 reproductive and lactational indices: about 40 measure – No NMDR
- F1/F2 male female AGDs, nipple retention, developmental landmarks including puberty: about 22 measures- No NMDR

In summary, this study presented more than 200 dose response curves, each with 3 group comparisons with the control for a total of 600 statistical comparisons. Of these, two presented NMDRs in the P0 generation. As these were not seen in subsequent generations, or both sexes in the same generation (for liver weight) it is reasonable to conclude that these observations are no more than would be expected by chance as a result of conducting such a large number of statistical comparisons.

B.1.b.4 Hass et al. (2007) and Metzdorff et al. (2007)

Hass et al. (2007) and Metzdorff et al. (2007) published a pair of papers from a study that included data on the dose-related effects of VIN and procymidone (at 5, 10, 20, 40, 80 or 160 mg VIN or procymidone/kg/d, n=8 dams per group) on reproductive development of the male offspring up to 16 days of age. They also studied FLU (discussed earlier) and a mixture of these three AR antagonists. In the mixture study, VIN and procymidone also were administered by themselves (at 24.5 and 95.9 mg/kg/d and 14.1 or 61.8 mg/kg/d, respectively). Dams were dosed from GD 7 to 21 and then from PND 1 to 16, when the animals were necropsied. The authors examined AGD, female-like nipple retention, reproductive organ weights and histopathology, expression of four genes in the ventral prostate, and the gross anatomy of the reproductive tract.

The authors reported that VIN and procymidone generally were of equivalent potency and that nipple retention was the most sensitive effect, followed by AGD. The lowest dose of VIN induced nipple retention, whereas 10 mg/kg/d was the LOEL for procymidone and 5 mg/kg/d was a NOEL (Haas *et al*., 2007). Dose- related effects were noted in all the treated groups with nipple retention being the most sensitive endpoint followed by neonatal AGD in the male offspring**.** None of the effects in the current study displayed an NMDR; according to the authors, 5 mg/kg/d was a LOEL for VIN and a NOEL for procymidone (based upon nipple retention in male rat offspring.

B.1.b.5 Christiansen et al. (2009a, b)

Christiansen et al. (2009a, b) administered VIN (0, 5, 10, 20, 40, 80, 160 mg/kg/d), PCZ (5, 10, 25, 50, or 100 mg/kg/d), finasteride (FIN) (0.001, 0.01, 0.1, 1, 10, or 100 mg/kg/d) or DEHP (10, 30, 100, 300, 600, or 900 mg/kg/d) individually or in a mixture to dams from GD 7 to 21 and then from PND 1 to 16, when the animals were necropsied. The authors reported that retained nipples were the most sensitive endpoint, with effects noticeable at the lowest doses; this was followed by reductions in male AGD, prostate and LABC weights, and genital malformations. The only individual chemical data presented (in a supplemental file and not in the paper itself) are for occurrences of genital malformations (VIN, LOAEL 40 mg/kg/d and NOAEL of 80 mg/kg/d; PCZ, LOAEL 100 mg/kg/d and NOAEL of 150 mg/kg/d; FIN LOAEL 0.01 mg/kg/d and NOAEL 0.1 mg/kg/d. There were no e DEHP-related genital malformations even at 600 and 900 mg/kg/d;4.8% of the animals were affected at 300 mg/kg/d, but this was not statistically significant since the n is rather small). The LOAELs in Christiansen et al. (2009a, b) are rather high as compared to other studies that examined male offspring at adulthood as compared examination of the F1 males at 16 days of age. The genital malformations did not display an NMDR for any of the tested chemicals.

B.1.b.6 Flynn et al. (2001)

This study is from an NCTR/NTP project on the long-term continuous administration of VIN in the diet at 0, 10, 150 and 750 ppm (approximately 0, 0.8, 12 and 60 mg/kg/d). The data for effects of VIN on the reproductive system of the F1 generation are currently unavailable.

Flynn et al. (2001) examined the effects of VIN on several non-reproductive sexually dimorphic behaviors including open field and running wheel activity, play behavior, and saccharin- and sodium preference. High dose F1 females drank more saccharin than controls females and were hypoactive in running wheels; by contrast VIN-treatment did not affect play behavior or sodium intake. The number pups per litter, litter sex ratios, and birth weight were not affected by VIN treatment. None of these effects displayed an NMDR.

B.1.b.7 Schneider et al. (2011)

In the Schneider et al. (2011) study, VIN was administered in the diet at levels approximating 0, 4, 20 or 100 mg/kg/d for two weeks prior to mating (P0 females) to four weeks prior to mating (P0 males) through PND 21 (n=25 pairs per group). After weaning, F1 animals were maintained

on the diet through PND 70. A large number of measures were taken and discussed in the paper, but not all the data are presented in a form wherein the shape of the dose response curve can be examined.

The following refers to data in the paper for about 50 responses that are presented and for which the dose response can be evaluated. There were results in 3 treated groups which were compared for each of the 50 control values for a total of 150 individual statistical comparisons.

- The following dose related effects were seen at 20 and 100 mg/kg/d:
  - One day old pup weight
  - F1 male AGD at PNDs 1 and 21
  - Female-like nipples in males at PNDs 13 and 21
  - Delay in puberty in males
  - Increased body weight at puberty
  - Reduced T4 in F1males at PND 71
- The following effects were only seen in the high dose group:
  - F1 male and female weaning weight was reduced
  - The age at VO was accelerated in females
  - F1 female weight was reduced at puberty
  - T4 was reduced in females at PND 71
- The following effects were observed to have an NMDR:
  - Percent pup survival from PND 4 to 21 was reduced by 11%; however, litter sizes at PND 4 and 21 were not reduced
  - Two of six measured antibody titers were reduced and displayed NMDRs

In summary, two measures of immune function displayed NMDR

B.1.b.8 Owens et al. (2007)

VIN, along with several other chemicals, was administered by oral gavage in the OECD Hershberger Assay validation this was a multi-lab (seven different laboratories) exercise done to determine if the protocol developed for this purpose could be used to identify antiandrogenic chemicals reliably (Gray et al., 2005a).

VIN was administered to castrate-immature, androgen-treated male rats at 0, 3, 10, 30 or 100 mg/kg/d (n=6 rats/group/laboratory) for 10 days (Owens et al., 2007). Subsequently, the animals were necropsied, and organs were weighed; organs included five androgen-dependent tissues as well as liver, kidneys and adrenal glands. VIN induced low-dose, linear reductions in the androgen-dependent tissues; the ventral prostate was statistically significantly reduced at the lowest dose by about 8% (3 mg/kg/d – a LOEL with no NOEL) whereas all other androgen-dependent tissues were reduced at 10 mg VIN/kg/d and adrenal gland weights were increased at 30 mg/kg/d**.** There were no NMDRs in this study.

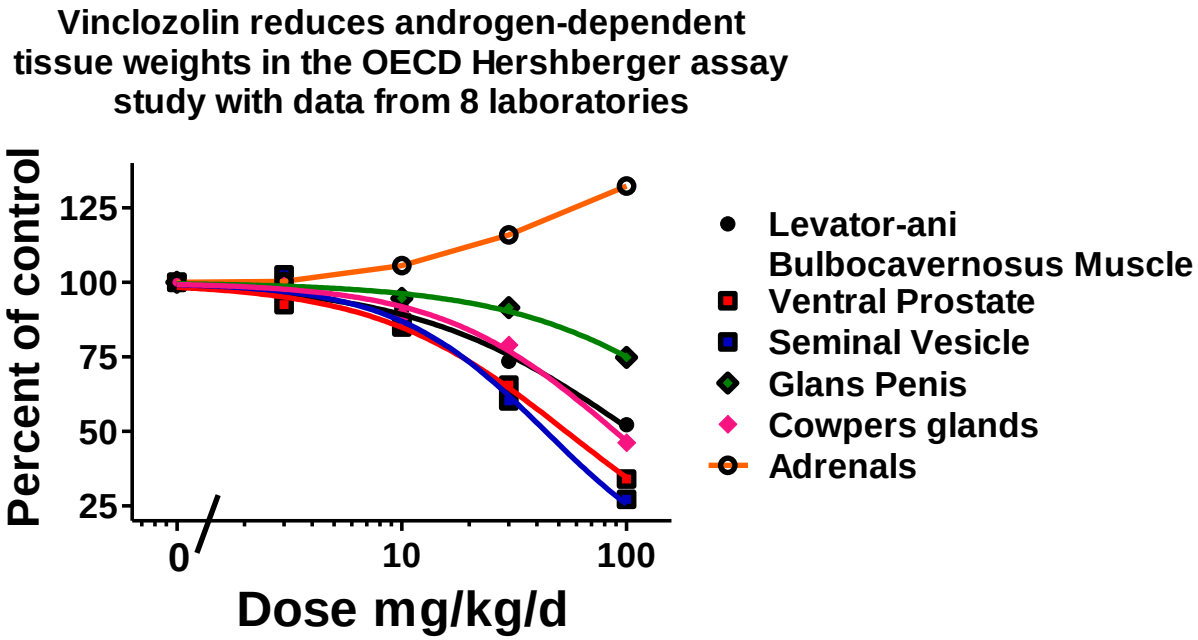


Figure B.7 from Owens et al. (2007).

B.1.c Procymidone

Procymidone is a dicarboximide fungicide like VIN, which displays AR antagonism *in vitro* and *in vivo*. There is a reasonably comprehensive published data set on procymidone including *in vitro* AR binding and gene expression assays, short-term *in vivo* assays and some genomic data and multigenerational studies. The dose range used in these studies ranges from low mg/kg/d to hundreds of mg/kg/d. Most of the studies used in regulatory agency risk assessments, including several with relatively low procymidone exposure levels are summarized in the assessment documents, but the dose response data are not available for public review. The summaries of unpublished studies indicate adverse developmental reproductive effects in the 10-15 mg/kg/d range with the severity of effects increasing with dosage level.

An example quote from a published summary is below.

A developmental toxicity study in rats by oral gavage at dose levels of 0, 3.5, 12.5, 125 and 500 mg/kg/day. Maternal toxicity was observed at 125 mg/kg/day and above as increased clinical observations, lower body weight gain, decreased food consumption and efficiency. Developmental toxicity was noted at 12.5 mg/kg/day and above as reduced AGDin males. Reproductive toxicity was at 12.5 mg/kg/day and above as decreased AGDin the male pups at postpartum day 1 and 21, an increase in the number of male rats with undescended testes, increased incidences of hypospadias with severity of the hypospadias increased with increasing dose, distended preputial gland, decreased testis and prostate weights. Female pups were relatively unaffected (Federal Register Volume 70, Number 163 (Wednesday, August 24, 2005), pages 49597-49599, http://www.gpo.gov/fdsys/pkg/FR-2005-08-24/pdf/05-16685.pdf).

Procymidone and VIN appear to induce nearly identical toxicity profiles on the reproductive system, ventral prostate gene expression and liver and adrenal weights. They both bind AR and inhibit androgen-induced gene expression *in vitro* with similar affinities; they inhibit androgen dependent sex accessory gland weights in the castrate-testosterone-treated male rat; they produce an identical genomic profile in the ventral prostate of the castrate-androgen-treated male rat; and they produce an identical syndrome of malformations in male rat offspring after *in utero* exposure. When a binary mixture of procymidone and VIN is administered *in utero* the mixture produces additive effects on sexual differentiation.

B.1.c.1 Owens et al. (2007)

In the current study, conducted as part of the OECD Hershberger Assay inter-laboratory validation program, procymidone was administered orally to castrate-immature-testosterone-treated immature male rats for ten days at 0, 3, 10, 30 or 100 mg/kg/d (n = 24 per group). Procymidone statistically significantly reduced the weights of several androgen-dependent tissues at 10, 30 and 100 mg/kg/d in a dose related manner (see figure below). The NOEL from this study of 3 mg/kg/d is very similar to that identified as the NOAEL for AGD from a developmental study with *in utero* exposure. All effects were dose related, and no NMDRs were detected.

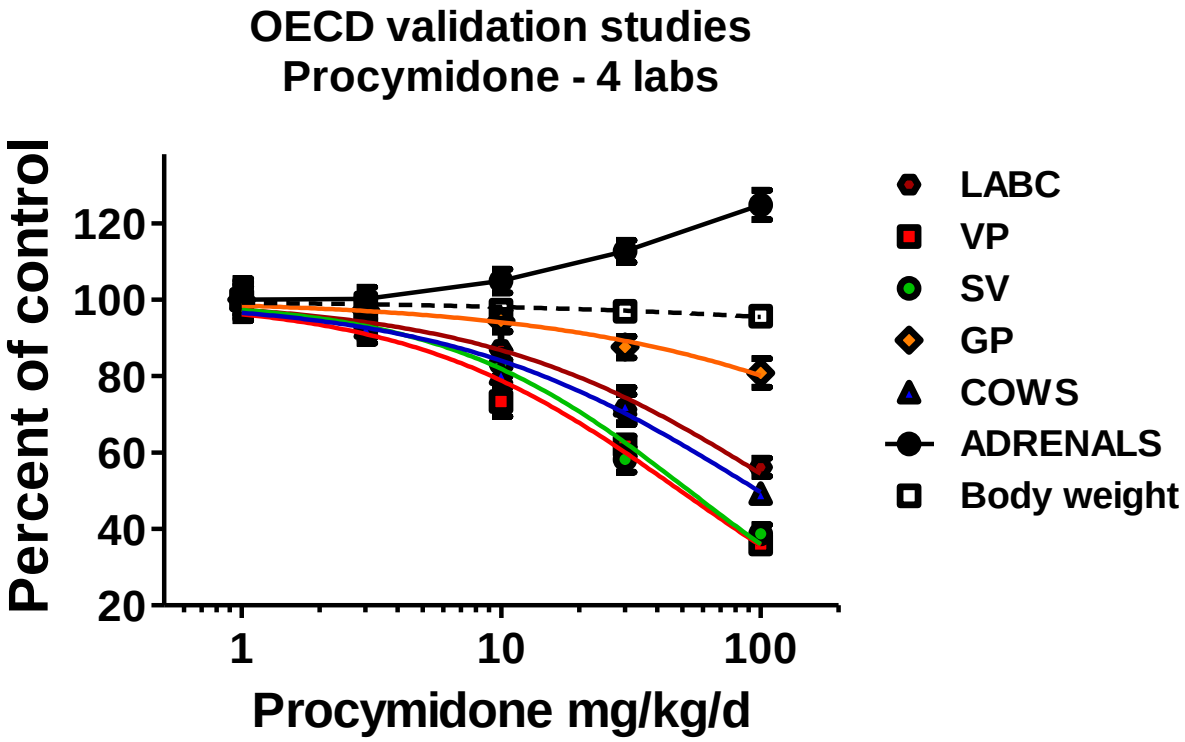


Figure B.8 from Owens et al. (2007).

B.1.c.2 Ostby et al. (1999a)

Maternal procymidone exposure at 0, 25, 50, 100, or 200 mg/kg/d during gestation and early lactation (GD 14 to PND day 3) altered reproductive development of male offspring at all dosage levels tested. Male offspring exhibited shortened AGD at 25 mg/kg/d and above. , At higher dosage levels the following effects were reported: induced permanent nipples;, reduced weight of several androgen-dependent tissues including the levator ani and bulbocavernosus muscles, prostate, seminal vesicles, Cowper's gland and glans penis; and malformations (hypospadias, cleft phallus, exposed os penis, vaginal pouch, hydronephrosis, occasional hydroureter, epididymal granulomas, and ectopic, undescended testes). In addition, at 50 mg/kg/d and above, perinatal procymidone treatment had a marked effect on the histology of the lateral and ventral prostatic and seminal vesicular tissues of the offspring. These effects consisted of fibrosis, cellular infiltration, and epithelial hyperplasia. This constellation of effects is similar to that produced by perinatal exposure to VIN. However, procymidone appears to be slightly less potent (by a factor of about two) in inducing malformations than VIN. All effects were dose related, and no NMDRs were detected.

B.1.c.3 Hotchkiss et al. (2010)

In the Hotchkiss et al. (2010) study, pregnant rats were administered procymidone at 0, 25, 50, 100, 150 or 250 mg/kg/d or dibutyl phthalate (DBP) at 0, 250, 500, 750 and 1000 mg/kg/d from GD 14 to 18. In addition, pregnant rats were administered a binary mixture of procymidone and DBP orally on GD 14–18. The mixture as administered as percentages of a stock solution, which contained 150 mg/kg procymidone and 1125 mg/kg DBP, and the percentages used were 0, 4.17, 8.33, 16.7, 33.3, 50, 66.7, and 83.3%. The mixture ratio was designed such that each chemical would contribute equally to the effects if they behaved in a dose additive manner.

There were no NMDRs in either of the individual chemical studies. In the in the individual chemical and mixture studies, AGD and retained nipples displayed apparent linear whereas some of the malformations, like hypospadias displayed threshold curves. None of the endpoints in three single chemical studies displayed a statistically significant NMDR (see Fig. B.9 below).

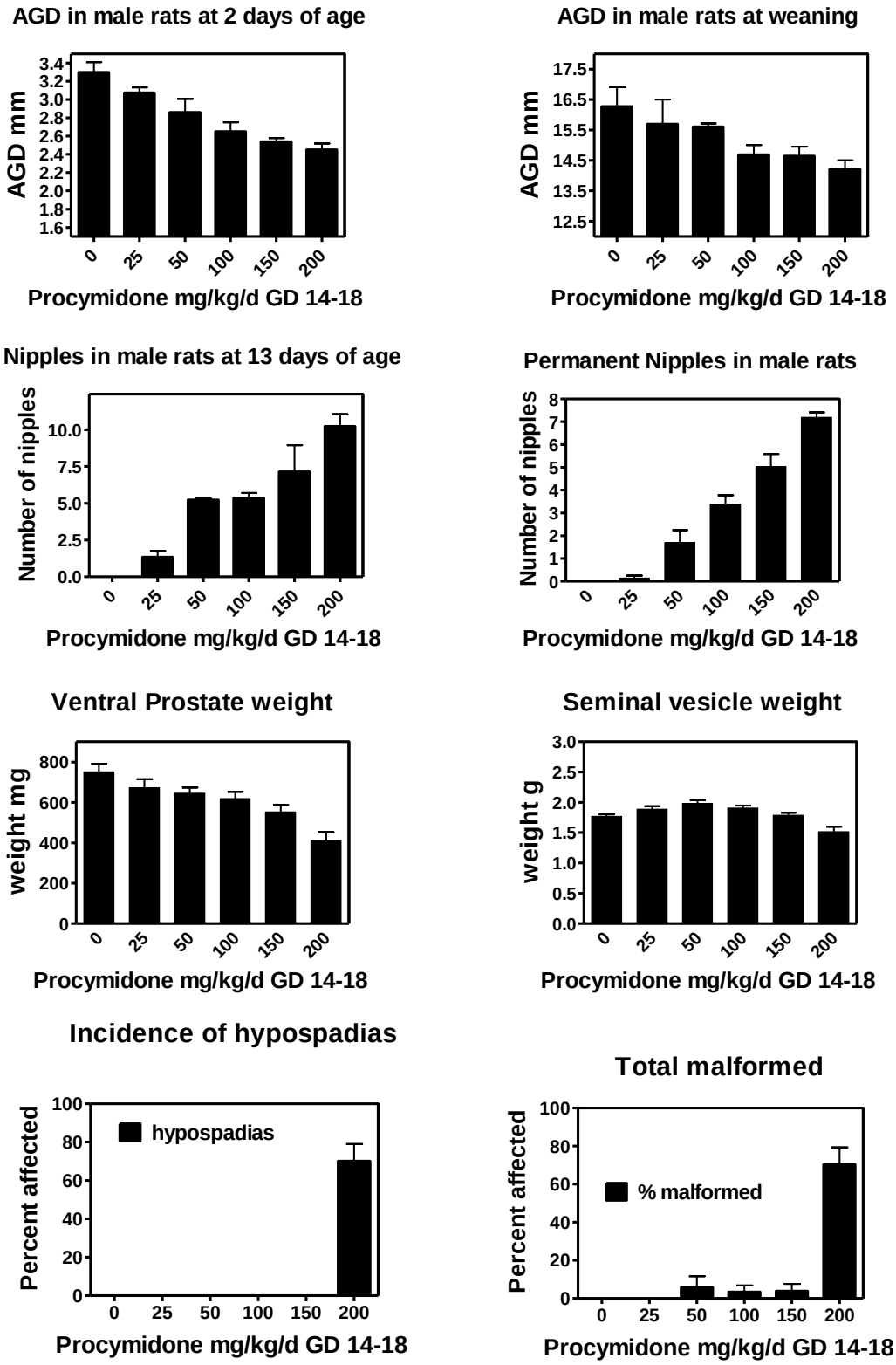


Figure B.9 from Hotchkiss et al. (2010).

In the mixture study, however, glans penis weight displayed an NMDR. In the example below, (Fig B.10) glans penis weight reaches a nadir in the mixture group exposed *in utero* to 50 % of the highest dose of DBP) plus procymidone whereas males exposed to the highest dose of the mixture display organ weights greater than (not less than), control values. This results from the fact that in the mid-dose group the glans was reduced in size, but not malformed, whereas almost all of the tissues were too malformed to weigh in the high dose group with the exception of 1 or two males that had relatively normal organ weights. This observation of NMDR is not uncommon with chemicals that induce malformations or histopathological alterations.

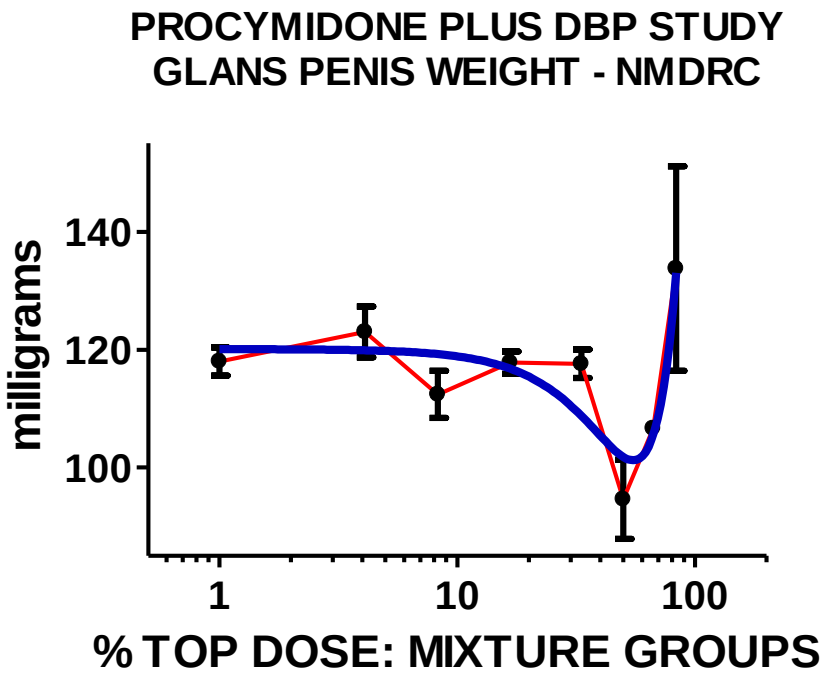


Figure B.10 from Hotchkiss et al. (2010).

B.1.c.4 Metzdorff et al. (2007)

Procymidone was administered orally to pregnant Wistar rats from GD 7 to PND 16 at 0 (16 control dams), 5, 10, 25, 50, 100, or 150 mg/kg/day (eight per dose group). At 16 days of age male offspring were examined for genital dysgenesis (subjective score from 0 to 3); organ weights (left and right testis, epididymides, ventral prostate, seminal vesicle, LABC, Cowper's gland, liver, kidney and adrenals); and prostate gene expression. There were dose-related reductions in reproductive organ weights, with some affected at the lowest dose tested of 5 mg/kg/d. An NMDR was noted (see Table 1 of Metzdorff et al. (2007). This was for left and right testis weights, increased at 10 mg/kg/d but reduced at 100 and 150 mg/kg/d; however the weights differed from control value by only 1.3 and 0.9 mg with standard errors of about 1.2 mg. The prostate-binding protein subunit C3 mRNA data that are presented in full did not display a NMDR. Pups in the lower dose groups display mild genital dysgenesis while moderate to severe dysgenesis was only seen in the high dose groups.

B.1.c.5 Hass et al. (2007)

Hass et al. (2007) reported the effects of procymidone on AGD and nipple retention in the male rat offspring from the above study. The dose response curves for these two endpoints (Figure 2 E and F from Hass et al. (2007) display monotonic responses.

B.1.d Fenitrothion

Fenitrothion is one of many organophosphate (OP) pesticides that bind AR; it displays antiandrogenic activity *in vitro* (Tamura et al., 2003; Tamura et al., 2001) and *in vivo* (Turner et al., 2002; Tamura et al., 2001). Fenitrothion is far more potent *in vitro* than linuron with potency nearly equivalent to the drug FLU. *In vivo* studies, however, indicate that fenitrothion is not nearly as potent, relative to FLU and other antiandrogenic pesticides as one would expect from the *in vitro* results.

B.1.d.1 Tamura et al. (2001) and Sunami et al. (2000)

In vivo, seven days of oral fenitrothion treatment inhibited T-induced growth of androgen-dependent tissues in the young castrate male rat at dose levels of 15 and 30 mg/kg/d. , This endocrine activity occurs at dosage levels above those used by regulatory agencies as NOAELs for inhibition of brain and/or serum acetylcholinesterase. Tamura et al. (2001) noted that the reduction in androgenicity was accompanied by reduced motor activity levels, overt signs of neurotoxicity and about a 75% reduction in brain acetylcholinesterase activity. When Sunami et al. (2000) administered this pesticide for 5 days at lower dosage levels of 0, 0.75, 1.5 or 3 mg/kg/d, they did not detect any effects on the androgen dependent tissue weights, whereas cholinesterase activities in the brain and erythrocytes were significantly suppressed at 3 mg/kg/d by 77-81% and 66-67% of control levels, respectively. None of the effects reported in these two studies displayed an NMDR.

B.1.d.2 Turner et al. (2002)

Turner et al. (2002) administered fenitrothion to pregnant rats by gavage at 0, 5, 10, 15, 20, or 25 mg/kg/day (n = 6–11/group) from GD12 to 21. Dams displayed muscle tremors and decreases in body weight gain, and litter sizes were reduced at 20 and 25 mg/kg/d. Male offspring in the 25 mg/kg/d dose group displayed shortened AGDs at birth and retained female-like areolae/nipples at 13 days of age. There were, however, no indications of abnormal phenotypes or development of androgen-dependent tissues on PND 100. None of the effects in this study displayed an NMDR.

B.2 Inhibition of Androgen Synthesis –

B.2.a Phthalates

Some phthalate esters administered to pregnant rats *in utero* cause male reproductive tract abnormalities, fetal loss, abortions and skeletal malformations. The reproductive effects in the fetal male rats arise from abnormal testicular androgen and insl3 hormone synthesis whereas these chemicals induce pregnancy loss apparently by reducing maternal ovarian progesterone synthesis. In young pubertal males, these same phthalates disrupt Sertoli cell function, hormone synthesis and induce testicular atrophy in a wide range of mammalian species.

The list of phthalate esters that induce these effects continues to grow as more research is done on the class and now includes these: di-2-ethylhexyl phthalate (DEHP) DBP, butyl benzyl phthalate (BBP), diisobutyl phthalate (DIBP), dipentyl phthalate (DPeP), dicyclohexyl phthalate (DCHP), dihexyl phthalate (DHP), diisononyl phthalate DINP and methylethyl hexyl phthalate (MEHP). Of these DPeP is the most potent, and DINP is only weakly positive for induction of the above effect. Studies also demonstrate that some other phthalates do not produce these reproductive effects at any dosage level. There are numerous robust multigenerational/transgenerational and pubertal studies of these active phthalates. This provides a relatively comprehensive data base for examination of the shape of the dose response curves over a broad range of doses. There have been reported a few examples of apparent low-dose NMDRs curves.

The literature also provides data on an important issue for low dose studies; that is, ubiquitous chemicals, like some of the phthalates, are commonly found in rodent diets and beddings. For example, the control dietary value determined by measurement of background contamination levels reported by Blystone et al. (2010) is 1.5 ppm; this is equivalent to about 0.12 mg/kg/d, a value exceeding the levels of DEHP administered in several of the low-dose studies (Do et al., 2012; Grande et al., 2007; Andrade et al., 2006b; Andrade et al., 2006a; Grande et al., 2006). Similarly, [1]Kondo et al. (2010) also found DBP and DEHP in each of 12 untreated rodent diets tested (0.14 to 1.41 ppm) as well as in all 13 beddings examined (0.02 to 7.6 ppm). Phthalates also were found in cereals, fat and oil and other products in levels up to 10 ppm by Wormuth et al. (2006) and 58 ppm by Jarosova et al. (2009). As a result, the tissues of control animals may contain ppm levels of phthalate metabolites (Jarosova et al., 2009). Since several of the phthalate esters disrupt development in rats via a common mechanism of toxicity the following section has been organized by species (rat then mouse), developmental period (*in utero* and lactation then pubertal) and then by chemical (DEHP then DBP).

[1]From Kondo et al. (2010) "We analyzed commercial animal diets and beddings, and found that the levels of phthalates varied from sample to sample; the concentrations of five phthalates were 141–1,410 ng/g for diets and 20.5–7,560 ng/g for beddings." These values were converted to µg/rat/d or mouse as follows. Calculations for top background level: rat is getting 1.4 µg/g diet consumed; a pregnant rat eats about 20 g food/d resulting in 20 x 1.4 µg/g diet=28 µg/rat/d; at rat weighs about 0.3 kg; 28 µg/rat/d divided by 0.333 kg = 84.7 µg/kg body weight/d; a pregnant mouse eats about 5 g per day resulting in 5 x 1.4 µg/g diet=7.05 µg/mouse/d. a mouse weighs about 0.033 kg resulting in 7.05 µg eaten/d = divided by 0.033 kg = 213.6 µg/kg body weight/d**.** The low dose is ten fold lower than the high dose for the rat and mouse, being 8.47 and 21.3 µg/kg body weight/d, respectively.

B.2.a DEHP - *In utero* and lactational studies:

B.2.a.1 DEHP –

Blystone et al. (2010) administered DHP in the diet at 1.5 (control), 10, 30, 100, 300, 1000, 7500, and 10,000 ppm DEHP; this was equivalent to doses of 0.12, 0.78, 2.4, 7.9, 23, 77, 592, and 775 mg/kg/day for the P0 animals; equivalent to 0.09, 0.48, 1.4, 4.9, 14, 48, 391 and 543 mg/kg/day for the F1 animals; and equivalent to 0.1, 0.47, 1.4, 4.8, 14, 46, and 359 mg/kg/day for the F2 animals.

In thist study, male pups were evaluated for gross reproductive tract malformations associated with the ‘‘phthalate syndrome (PS).’’ DEHP treatment had minimal effects on P0 males, whereas DEHP treatment induced significant increases in F1 and F2 total PS malformations; these effects were observed in the testis, epididymides, seminal vesicle, and prostate in the 7500-ppm dose group and in the F1 10,000-ppm dose group. The 10,000–ppm exposed F1 males did not produce an F2 generation. The NOAEL for F1 and F2 PS malformations was 100 ppm (4.8 mg/kg/day), which was close to the 5% response benchmark dose lower confidence limit of 142 ppm. Summary tables of the study results for mating and extra, non-mating animals are listed on pages 416-423 of (IHCP, 2008).

Blystone et al. (2010) reported an NMDR with increased fertility versus control in the F3c and higher dose groups at 10 and 30 ppm DEHP in the diet; this was due to a low control fertility rate. This effect was not seen in any of the other eight mating periods at these dosage levels, and increased fertility is not considered an adverse effect. With this exception there were no NMDR in any generation**.**

B.2.a.2 Gray et al. (2009)

In order to define the dose-response relationship between DEHP and the Phthalate Syndrome (PS) of reproductive alterations in F1 male rats, Gray et al. (2009) dosed rat dams by gavage from GD 8 to day 17 of lactation with 0, 11, 33, 100, or 300 mg/kg/day DEHP (71–93 males per dose from 12 to 14 litters/group). Half of the male offspring were also exposed to DEHP via gavage from 18 days of age to necropsy at 65 days of age (PUB cohort; n=16–20/dose). Remaining males were not exposed after postnatal day 17 (*in utero*-lactational [IUL] cohort) and were necropsied after reaching full maturity. AGD, sperm counts and reproductive organ weights were reduced in F1 males in the 300 mg/kg/day group, and they displayed retained nipples. In contrast to Christiansen *et al.* (2008), these effects were not statistically significant at lower dosage levels. In the IUL cohort, seminal vesicle weight was also reduced at 100 mg/kg/day. In contrast, serum T and estradiol levels were unaffected in either the PUB or IUL cohorts at necropsy. A statistically significant percentage of F1 males displayed one or more P S lesions at 11 mg/kg/day DEHP and above; one true hermaphroditic male was found in the 100 mg/kg/d dose group.

The authors reported that they were able to detect effects in the lower dose groups because all the males in each litter were examined rather than only one male per litter. Power calculations

demonstrated that using multiple males/litter versus one male/litter enhanced the detection of the effects of DEHP. The results at 11 mg/kg/day confirm those from a NTP multigenerational study which reported no observed adverse effect levels (NOAEL) or lowest observed adverse effect levels (LOAEL) of 5 and 10 mg/kg/day DEHP, respectively, via the diet.

Of the more than 50 measures taken in the study, treatment with DEHP produced non-significant, inverted U-shaped NMDR dose responses on necropsy body and liver weights as the males from the 11 mg/kg/day dose group were heavier than control males., , These effects, however, were not statistically significant by ANOVA or Dunnett's *post hoc* test.

B.2.a.3 Grande et al. (2007); Grande et al. (2006) and Andrade *et al*., 2006 a,b,c

This research team published three papers on the effects of DEHP on the Wistar male rat offspring and two papers on female rat offspring. Animals were exposed by oral gavage from GD6 to lactation day 21 at doses of 0, 0.015, 0.045, 0.135, 0.405, 1.215, 5, 15, 45, 135 and 405 mg DEHP/kg/d (11 to 16 dams/group). The four of lowest dosage levels equaled or exceeded the daily intake of 0.12 mg/kg/d DEHP in the control diets used by Blystone et al. (2010) and other rodent and domestic animal diets (Kondo et al., 2010; Jarosova et al., 2009).

Grande et al. (2006)

All maternal and offspring lactational indices, litter sizes and body weights (of 10 measures) were unaffected by DEHP treatment at any dose level (Grande et al., 2006). Although pup birth weight was not affected by DEHP treatment at birth**,** body weights of females necropsied at PND 1 (2 per litter) displayed an NMDR, being increased at 0.045, 1.125 and 5 mg/kg/d, and liver weights were increased in the two highest dosage groups. The effects on body and liver weights seen on PND 1 were not present in females necropsied at weaning on PND 22. AGD and nipple retention in F1 females on PND 22 were not affected by DEHP treatment. The authors detected a dose related approximately 2 day delay in VO in the 15-405 mg/kg/d dose groups, but the age at first estrus was not affected. Several effects including "vaginal opening, and first estrous" were reported as NMDRs in the review by Vandenberg et al. (2012)(Table 7). This is not consistent with Grande et al. (2006) analyses of the data (Fig. B.11 below).

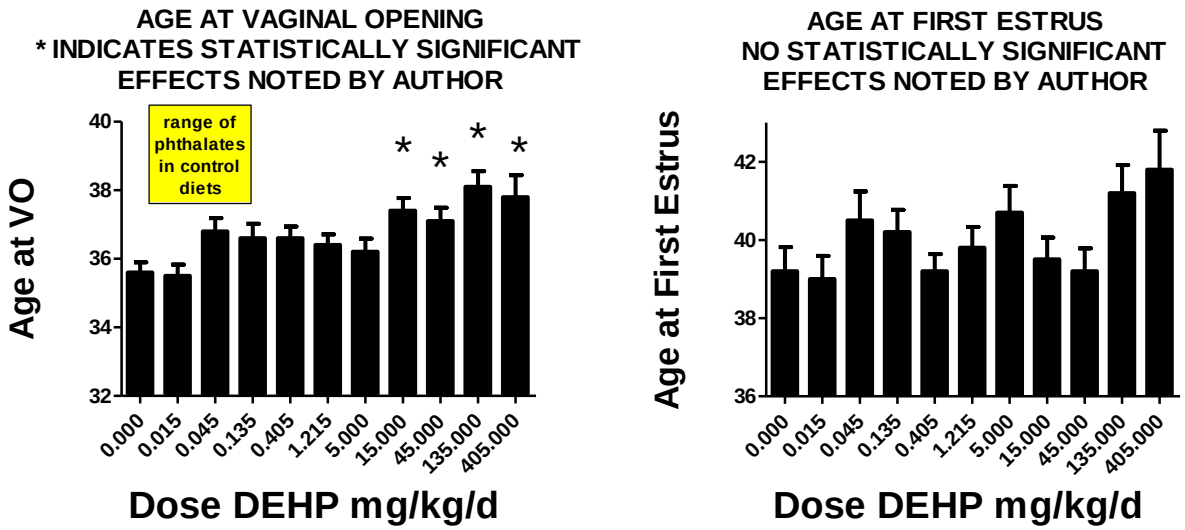


Figure B.11 from Grande et al. (2006).

At weaning on PND 22 dams were necropsied and organ weights taken; liver and kidney weights were increased at 405 mg/kg/d. Maternal kidney weight displayed an NMDR in that it was also increased in dams treated with DEHP at 0.045 mg/kg/d.

B2.a.4 Grande et al. (2007)

In the second paper, which examined the effects of DEHP on F1 female rats in adulthood, Grande et al. (2007) did not find any effects of treatment on organ weights (liver, kidney, spleen, thymus, thyroid, ovary and uterus), nor were there effects on body weights, estrous cyclicity or serum estradiol or progesterone levels. Although uterine and vaginal histology were not affected, DEHP treatment at 405 mg/kg/d increased the number of atretic ovarian follicles from 8 in controls to 16 in the high dose group. There were no NMDRs noted in the data from this paper.

B.2.a.5 Andrade et al. (2006b); Andrade et al. (2006c); Andrade et al. (2006a)

Andrade et al. (2006c) reported that similar to F1 females Grande et al. (2006), body weights of males necropsied at PND 1 (2 per litter) displayed an NMDR, being increased at 0.045, 1.125 and 5 mg/kg/d. Liver weights were increased in the two highest dosage groups. Also similar to their female siblings, the effects of DEHP on body and liver weights at PND 1 were not present in males necropsied at weaning on PND 22.

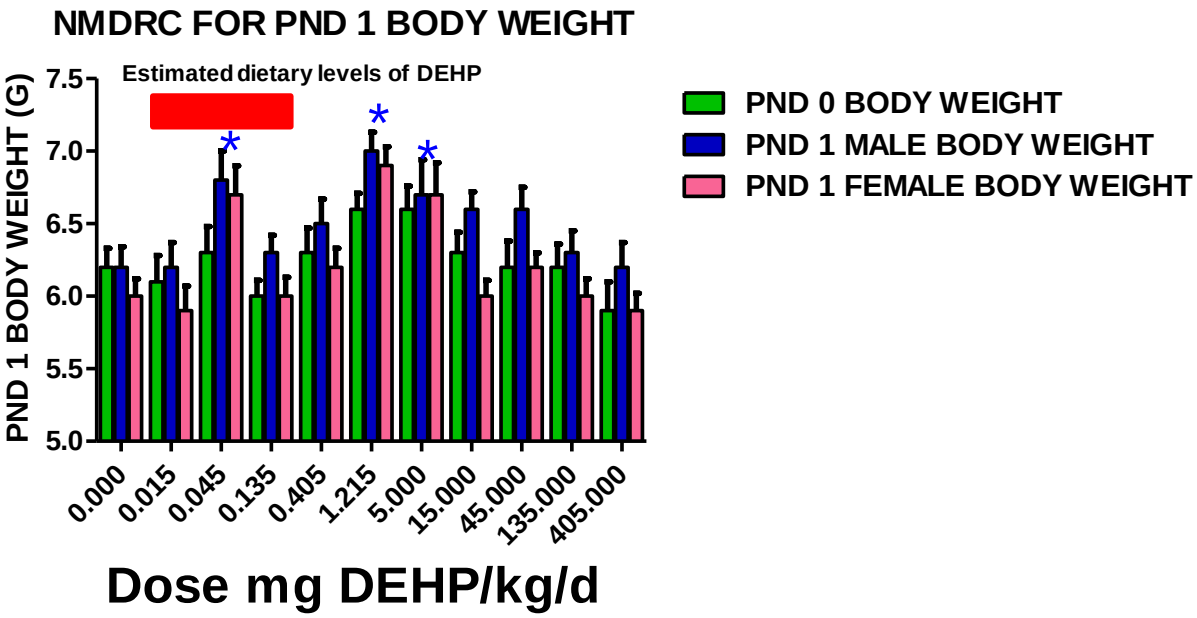


Figure B.12 adapted from Andrade et al. (2006c).

They reported that nipple retention and reduced AGD at PND 22 were seen only in males exposed to the highest dose (405 mg/kg/day); this is in contrast to the low dose effects of nipple retention and reduced AGD at PND2 for 3 mg/kg/d and above reported by Christiansen *et al*. (2008 (2008)). In addition, Andrade et al. (2006c) reported an NMDR for AGD at PND22, being increased in the 0.015 mg/kg/d DEHP group versus controls and decreased at 405 mg DEHP/kg/d.

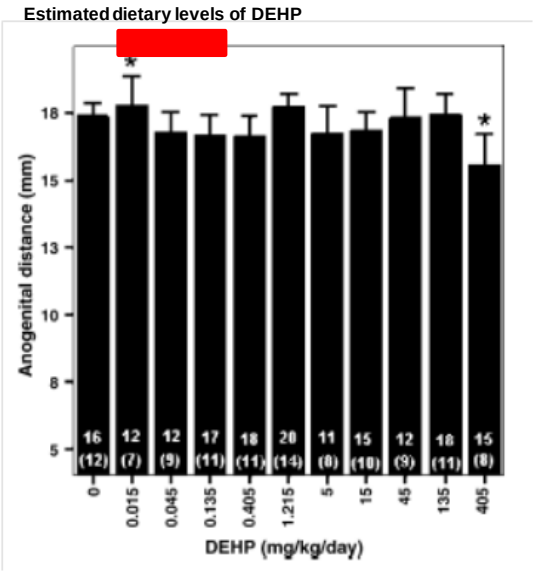


Figure B-13 adapted from Andrade et al. (2006c).

Histopathological examination of the testis on PND 1 and 22 revealed changes at 135 and 405 mg DEHP/kg/day. The most prominent finding on PND 1 was the presence of multinucleated gonocytes. On PND 22 signs of reduced germ cell differentiation in seminiferous tubules of exposed animals were observed.

An NMDR effect on testis weight was reported by the authors as statistically significantly increased at 5, 15, 45 and 135 mg/kg/day on PND22. This effect differed qualitatively from effects of exposure to higher doses in the current study; it also differs from testis weight data from other studies in prepubertal male rats exposed to DEHP in this dose range (Christiansen et al., 2008), Noriega et al. (2009), for example).

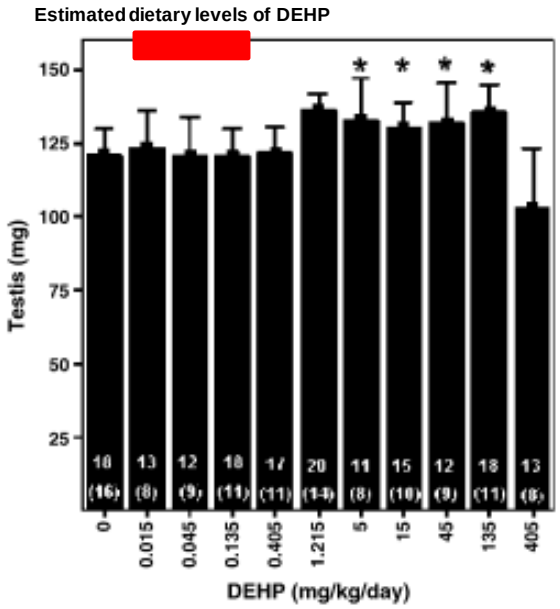


Figure B.14 adapted from Andrade et al. (2006c).

Histopathological examination of the testis on PND 1 and 22 revealed changes at 135 and 405 mg DEHP/kg/day. The most prominent finding on PND 1 was the presence of multinucleated gonocytes. On PND 22 signs of reduced germ cell differentiation in seminiferous tubules of exposed animals were observed. A dose-related monotonic delay in PPS was observed in animals exposed to 15 mg DEHP/kg/day and higher doses. The lack of an NMDR on the age of PPS is consistent with the observations of Noriega et al. (2009). Neither Andrade et al. (2006c) nor Noriega et al. (2009) were unable to replicate the NMDR reported for this developmental event by Ge et al. (2007).

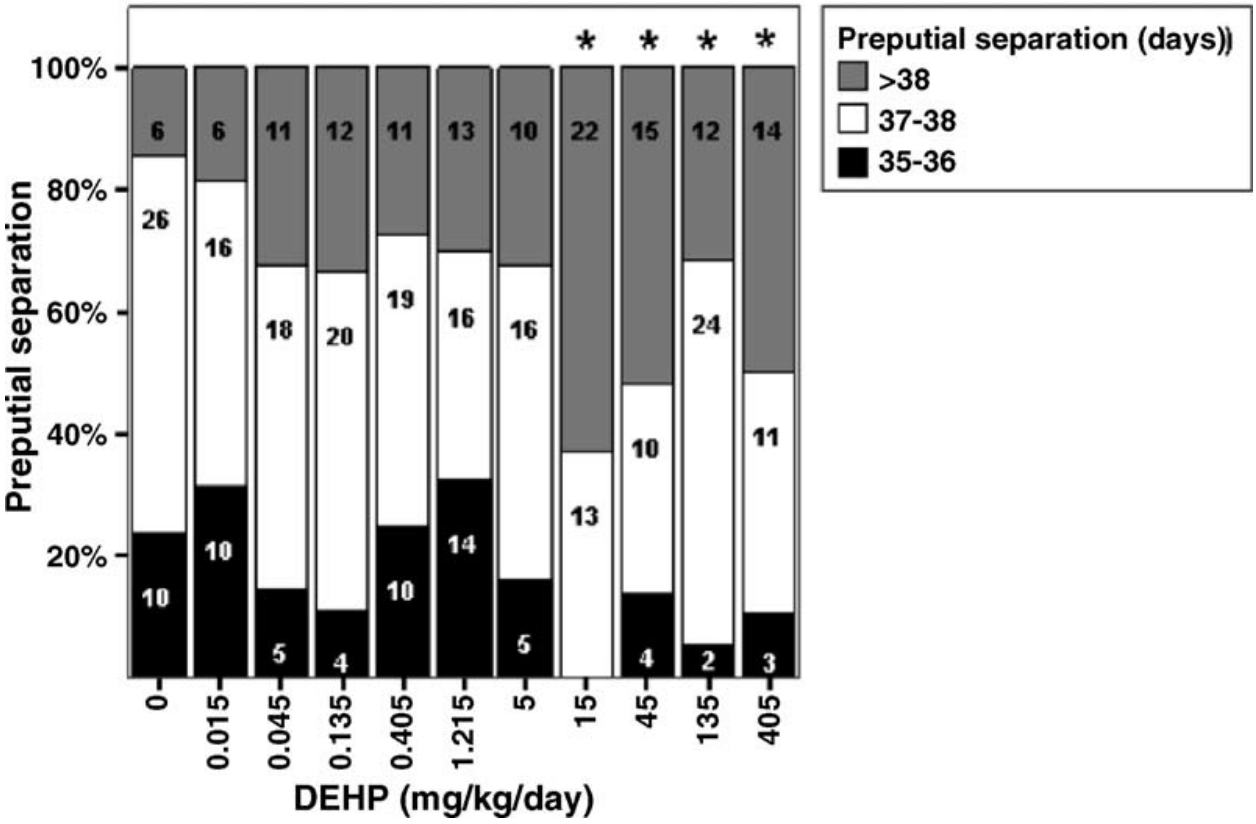


Figure B.15 from Andrade et al. (2006c).

B.2.a.6 Andrade et al. (2006b); Andrade et al. (2006a)

In the second paper (Andrade et al., 2006b) published by this team on the effects of perinatal DEHP on the male offspring they reported an NMDR effect on brain aromatase activity (measured *ex vivo*) in F1 male rat offspring at PND1. This effect was not seen in males at PND22 or F1 female siblings at PND1 or PND2.

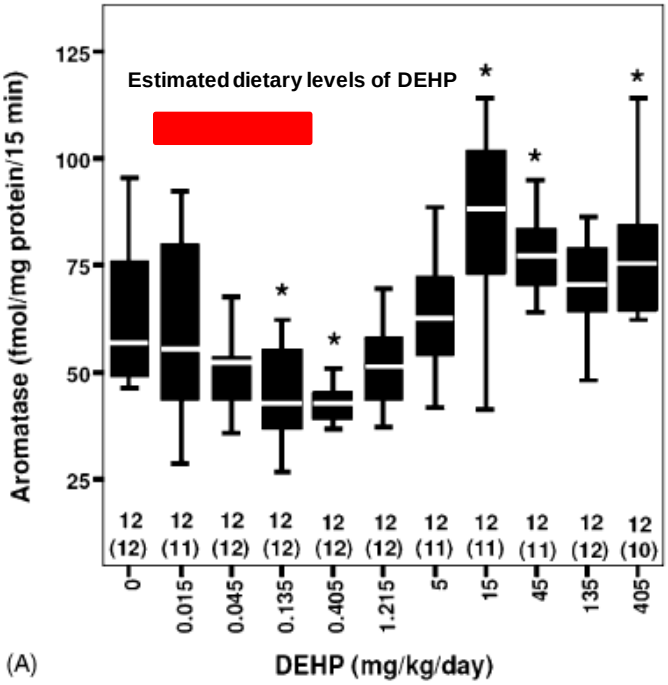


Figure B.16 adapted from Andrade et al. (2006b).

A reduction in aromatase activity in the brain is potentially biologically significant. The higher levels of brain aromatase seen in male rats versus female rats at birth are involved in sexual differentiation, and inhibition of this enzyme could reduce the ability of DEHP-treated males to display normal male sexual behavior and/or other sexually dimorphic behaviors. The authors tested this hypothesis in the animals (Andrade et al., 2006a), and male sex behavior was not affected. Andrade et al. (2006a) made the following statement:

> "It has been suggested that DEHP may impair male copulatory behavior in rats by interfering with the sexual differentiation of the central nervous system (Moore et al., 2001). Recently, we investigated the effects of *in utero* and *in utero* and lactational DEHP exposure on rat brain aromatase enzyme activity (Andrade et al., 2006b), which is believed to play a critical role in the masculinization of the central nervous system (Lephart, 1996). The most prominent effects in males occurred on postnatal day 1 where a biphasic response with low-dose inhibition and high-dose stimulation of aromatase activity was observed. In the present study, we investigated whether the same exposure paradigm would affect the masculine sexual behaviour of the adult male offspring. When DEHP and control males were paired with receptive females no adverse changes in copulatory behavior were detected. Taken together these results indicate that the observed changes in brain aromatase activity in newborn males are not associated with impairment of male sexual behaviour later in life."

In the third paper, Andrade et al. (2006a) measured over 30 endpoints including these: terminal organ and body weights; testis sperm counts and sperm morphology; T levels; and several indices of male mating behavior. Some of these endpoints displayed NMDRs. The DEHP effects on sperm counts in the two high dose groups were not seen in another study from this group using a similar dose range (100 and 500 mg/kg/d) (Dalsenter et al., 2006).
NMDR were displayed by the following endpoints; however, some of the lowest treatment groups appear to be at background dietary DEHP exposure levels reported by Kondo et al. (2010):

- Serum T had 3 peaks and 3 valleys, being elevated at 0.045, 0.405 and 405 mg/kg/d
- Sperm morphology was altered at 0.45 and 0.135 mg/kg/d
- Mean diameter of seminiferous tubules (1 of 6 measures of testis morphometry) was reduced at 15 mg/kg/d

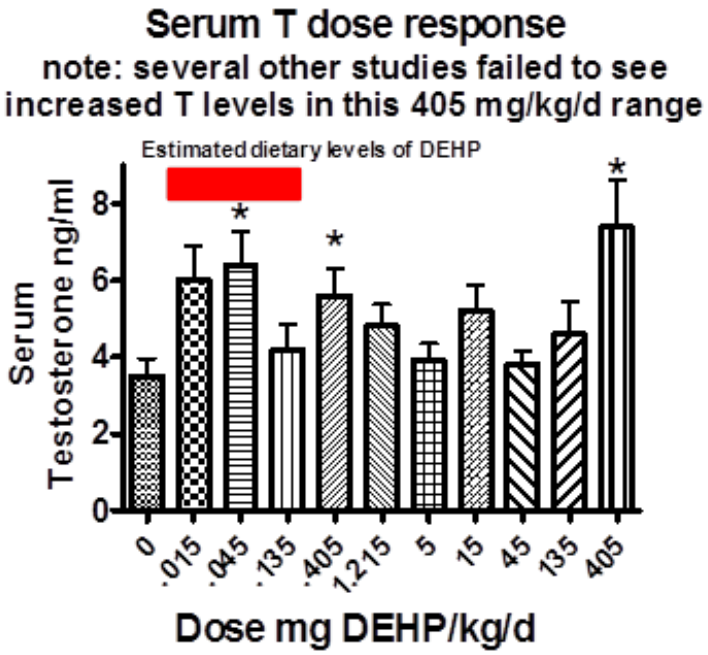


Figure B.17 adapted from Andrade et al. (2006a).

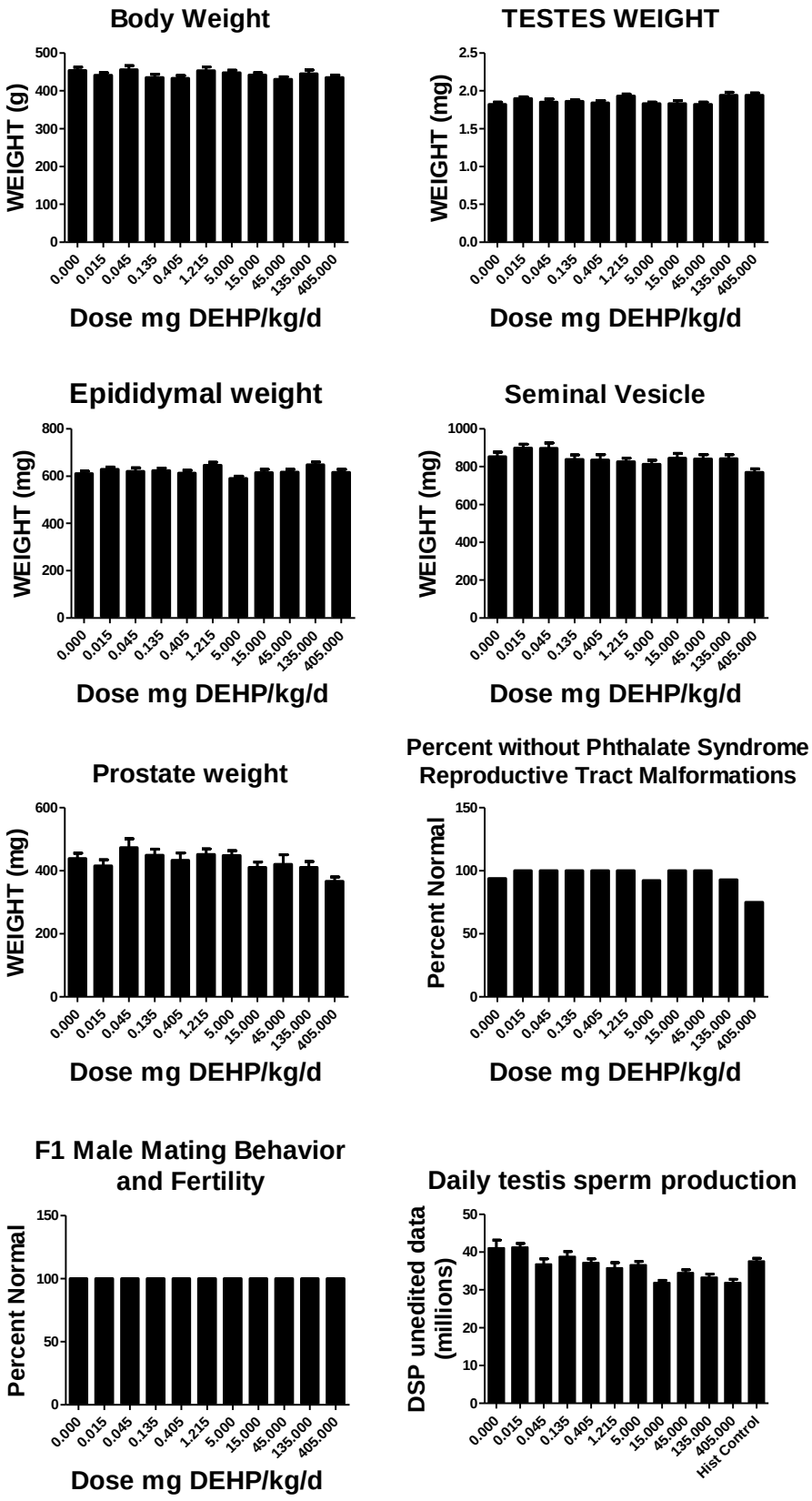


Figure B.18 from Andrade et al. (2006a).

B.2.a.7 Dalsenter et al. (2006)

Dalsenter et al. (2006) evaluated the effects of DEHP on reproductive function and sexual behavior of F1 male rat offspring rats after *in utero* and lactational oral gavage treatment of dams with 0, 20, 100 or 500 mg/kg/d. No effects were noted on the dams, litter size, birth weight, or neonatal viability. Postimplantation loss, however, was increased at 500 mg/kg/d, and weaning weight displayed an NMDR being increased only at 100 mg/kg/d. When males were necropsied at 65 or 155 days of age, their testis and epididymal weights were unaffected whereas ventral prostate and seminal vesicle weights, and sperm counts were reduced, and sexual behaviors were altered in the high dose group. This last group of effects showed no NMDR.

B.2.a.8 Christiansen et al. (2010)

Christiansen et al. (2010) administered DEHP from GD7 to postnatal day 16 with doses of 0, 3, 10, 30, 100, 300, 600 and 900 mg/kg/d by oral gavage (n=6 to 30 dams per group). The study was conducted in two blocks. At 10 mg DEHP/kg/d male, AGD was decreased, the incidence of nipple retention was increased, weights of androgen-dependent tissues were reduced, and external genitalia dysgenesis was observed. Higher doses of DEHP induced histopathological effects on the testes and reduced both testis weight and expression of androgen-regulated genes in the prostate. The study did not report the incidence of internal malformations, which would have been expected in the high dose DEHP groups (>100 mg/kg/d). NMDRs were seen in the current study, but there were generally not reproducible from block to block as described below:

- F1 male but not female weaning weight was increased only at 100 mg/kg/d in block 1 but not block 2
- Postimplantation loss was increased only at 10 mg/kg/d, in block 2 but not block 1
- "Mild" genital dysgenesis (on a score of 0 to 3, mild=1) was increased in 16 day old males in all dose groups except 30 mg/kg/d. No other study has noted malformations of the external genitalia at this dose in adult F1 animals
- PCB C3 gene expression displayed an NMDR in block 2 but not 1, and ODC gene expression was affected in block 2 but not block 1
- Right but not left testis weight was reduced in block 1 but not 2 at 100 mg/kg/d; this was unaffected at 300 in this group
- LABC weight was decreased in block 1 in all treated groups except 600 mg/kg/d. In block 2, 10 and 30 mg/kg/d slightly reduced LABC weight, but 100 mg/kg/d did not (this higher dose group was affected in block 1)
- Adrenal weight was reduced in block 1 at 10, 100 and 900 mg/kg/d but not in other dose groups. The effects at 10 and 100 mg/kg/d were not replicated in block 2
- Many of these NMDR were no longer apparent when the two blocks were pooled and analyzed together.

B.2.b *In utero* and lactational studies: DBP

B.2.b.1 Mylchreest et al. (1999)Mylchreest *et al*. (2000)

Mylchreest *et al*., (2000) dosed pregnant rats with DBP by gavage at 0, 0.5, 5, 50, 100 (19-20/group), or 500 mg/kg/day (11/group) from GD12 to 21. F1 male AGD was decreased at 500 mg, and retained nipples were present in 31 and 90% of male pups at 100 and 500 mg/kg/day, respectively. PPS was not delayed by DBP treatment in males with normal external genitalia, but cleft penis with hypospadias as well as epididymal, vas deferens, seminal vesicle and ventral prostate agenesis was observed in males at 500 mg/kg/day. F1 males also displayed reduced weights of the testis, epididymis, dorsolateral and ventral prostate, seminal vesicle and LABC muscles at 500 mg/kg/day. The testes of high dose group males also displayed seminiferous tubular degeneration and interstitial cell hyperplasia and adenoma. They reported that the NOAEL and LOAEL (lowest-observed-adverse-effect level) were 50 and 100 mg/kg/day, respectively. None of the >35 endpoints in this study displayed an NMDR.

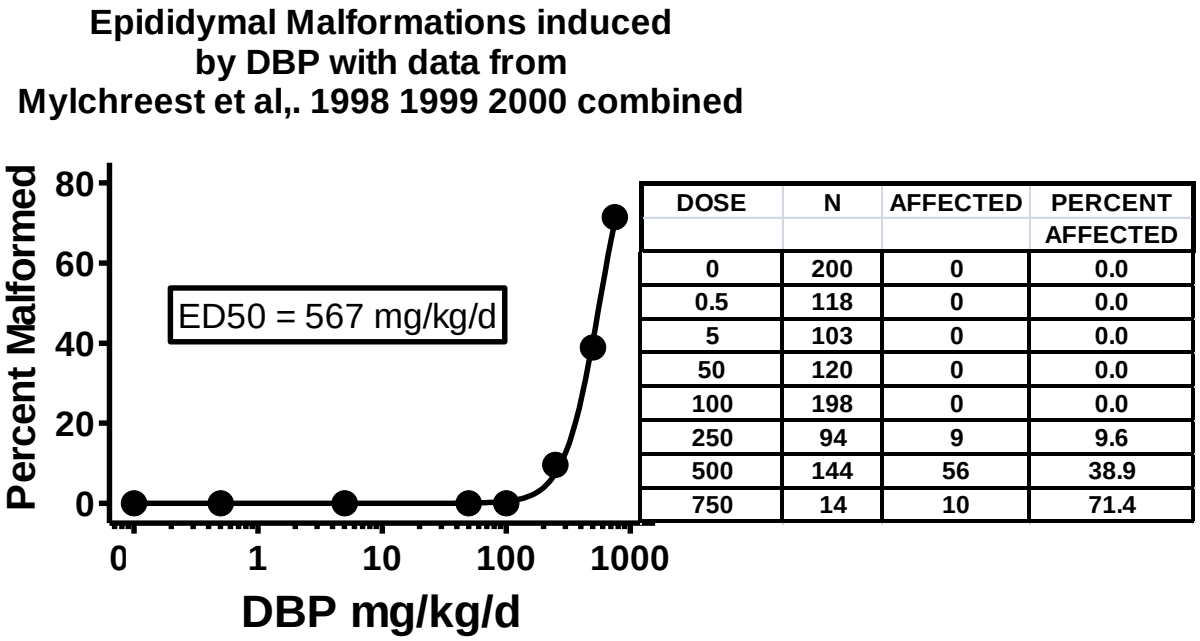
B.2.b.2 Mylchreest *et al*., 1999

Mylchreest et al. (1999) exposed pregnant rats by oral gavage to DBP at 0, 100, 250, or 500 mg/kg/day (10/group) from GD 12 to 21. In F1 males, 500 mg DBP/kg/day induced the following effects: hypospadias, cryptorchidism; agenesis of the prostate, epididymis, and vas deferens; degeneration of the seminiferous epithelium; and interstitial cell hyperplasia of the testis. DBP at 250 and 500 mg/kg/day also caused retained nipples and decreased AGD in male offspring. Interstitial cell adenoma occurred at 500 mg DBP/kg/day in a few males. PPS was delayed in all dose groups being statistically significant only at 100 and 500, but not 250 mg DBP/kg/day. The delay in PPS reported for DBP at 100 mg/kg/d was not seen at this dose in the paper discussed above (Mylchreest et al., 2000). None of the >30 endpoints in this study displayed an NMDR.

B.2.b.3 Mylchreest et al. (1998)

Mylchreest et al. (1998) dosed pregnant rats (10/group) with DBP at 0, 250, 500, or 750 mg/kg/day by oral gavage from GD 3 until PND 20. At 750 mg/kg/day, the number of live pups per litter at birth was decreased, but maternal weight was unaffected. AGD was decreased at birth in males at 500 and 750 mg/kg/day. Epididymal agenesis was displayed in 9, 50, and 71% of F1 males at 100 days of age at 250, 500, and 750 mg/kg/day, respectively; this was associated with testicular atrophy and widespread germ cell loss. Hypospadias occurred in 3, 21, and 43% of males, and ectopic or absent testes were reported in 3, 6, and 29% of males at 250, 500, and 750 mg/kg/day, respectively. Absence of prostate gland and seminal vesicles as well as small testes and seminal vesicles were noted at 500 and 750 mg/kg/day. VO and estrous cyclicity were not affected in the F1 female offspring, although low incidences of reproductive tract malformations (vaginal agenesis) were observed at 500 and 750 mg/kg/day. None of the >40 endpoints in this study displayed an NMDR.

Since epididymal agenesis is the most common severe malformation seen in adult F1 male rat offspring after *in utero* phthalate exposure, the data from these three studies by Mylchreest et al. (2000); Mylchreest et al. (1999); Mylchreest et al. (1998) were pooled to describe the dose response relationship between *in utero* DBP and epididymal agenesis in F1 male rat offspring.



DOSE	N	AFFECTED	PERCENT AFFECTED
0	200	0	0.0
0.5	118	0	0.0
5	103	0	0.0
50	120	0	0.0
100	198	0	0.0
250	94	9	9.6
500	144	56	38.9
750	14	10	71.4

Figure B.19 adapted from Mylchreest *et al.* (1998, 1999Mylchreest et al. (2000).

B.2.b.4 Zhang et al. (2004)

In the Zhang et al. (2004) study, DBP was administered at 0, 50, 250, or 500 mg/kg/d by oral gavage from GD1 to PND21, and the male offspring were examined through adulthood. DBP had no affect on the dams; however, birth weight (at 250 and 500 mg/kg/d), number of live pups per litter (500 mg/kg/d), body weight gain (250 and 500 mg/kg/d) and male GD (250 and 500) were reduced. Severe damage to the reproductive system of mature F1 male rats was observed in the group treated with 250 mg/kg BW/day and higher and included these measures: testicular atrophy; underdeveloped or absent epididymis; undescended testes; obvious decline of epididymal sperm parameters; total sperm heads per g testis; and decrease of organ/body weight ratio of epididymis and prostate. These results showed that the male reproductive system was the main target organ of DBP exposure. The NOAEL (no observable adverse effect level) for developmental toxicity of DBP was established based on pup body weight and male reproductive lesions at 50 mg/kg BW/day. There were 20 endpoints measured with 60 potential statistical comparisons with the control group. Of these, prostate weight displayed an NMDR being reduced only at 250 mg/kg/d, a dose at which several other endpoints also were significantly affected in a monotonic manner. Thus this NMDR would not affect determination of the NOAEL in the study.

B.2.b.5 Lee et al. (2004)

Pregnant rats were treated with DBP in the diet at 0, 20, 200, 2000 or 10,000 ppm from GD15 to PND21, when the offspring were weaned and treatment terminated (n=6-8 dams per group). Male and female pubertal onset was measured, and estrous cycles were evaluated at two periods. Animals were necropsied at weaning, (11 and 21 weeks of age) and reproductive organs were weighed and evaluated histologically. Statistical analysis did not account for litter effects on the measures taken on the offspring after weaning. Organ weight data were analyzed relative to body weight, which adds some uncertainty in the analysis of the data; for example, weanling male offspring in the high DBP dose group have statistically significantly larger relative brain weights versus controls, likely due to the fact that body weights were smaller and brain size was not reduced.

This paper reports several "low-dose" effects and multiple NMDRs:

- Number of live births was reduced, and body weights increased in the low dose group only at PND2
- Age at puberty in males occurred 1.3 days earlier at 200 ppm by comparison to controls but not at higher doses
- Pituitary weight in males at week 11 was increased at 20, 200 and 2000 ppm, but not in females (where the weight is decreased at 10,000) or in males at other ages
- Prostate weight was increased at week 11 in the 200 ppm group but not at 20 weeks (at which time point the weight was slightly reduced)
- Slight to mild reductions in spermatocyte development in the testis were noted in weanling males in the low dose groups (monotonic response), but there we no low dose testicular abnormalities at 11 or 20 weeks of age
- NMDRs for slight to mild histological alterations in male mammary gland were noted in the low and middle dose groups and the 11 and 20 week necropsies. No mammary gland effects were noted at weaning or in female offspring

In summary, several NMDRs and low dose effects were noted in the current study. However, the low-dose testicular alterations reported here were not seen by several other investigators using DBP in this dose range.

B.2.b.6 Mahood et al. (2007) -

In the Mahood et al. (2007) study pregnant Wistar rats were gavaged daily from GD 13.5 to either GD20.5 (fetal samples) or GD21.5 (postnatal tissue) with 0, 4, 20, 100, or 500 mg/kg DBP. The male fetuses and F1 adult male offspring were examined. The authors found that fetal testicular T levels were reduced, and abnormal LC aggregation and the occurrence of MNG in the 20 mg DBP /kg/d and above (not statistically significant at 20 mg/kg). In addition, fetal testis weight was reduced at 100 at 500 mg/kg/d (statistically significant mg/kg at 500 mg/kg). In the F1 adult males, fertility was reduced in all dose groups (statistically significant only at 500 mg/kg/d), and males in the high dose group displayed undescended testis and reduced testis weight. Histological examination of the testes in the F1 adult males revealed an increased incidence of focal dysgenesis at 500 mg/kg/d, but not at lower dosage levels. All of the effects displayed monotonic responses with one exception: the frequency of mild LC clusters in GD 21.5 testes (see below). This NMDR arises as the severity of the testis alterations progresses from medium at 100 mg/kg/d to large at 500 mg/kg/d.

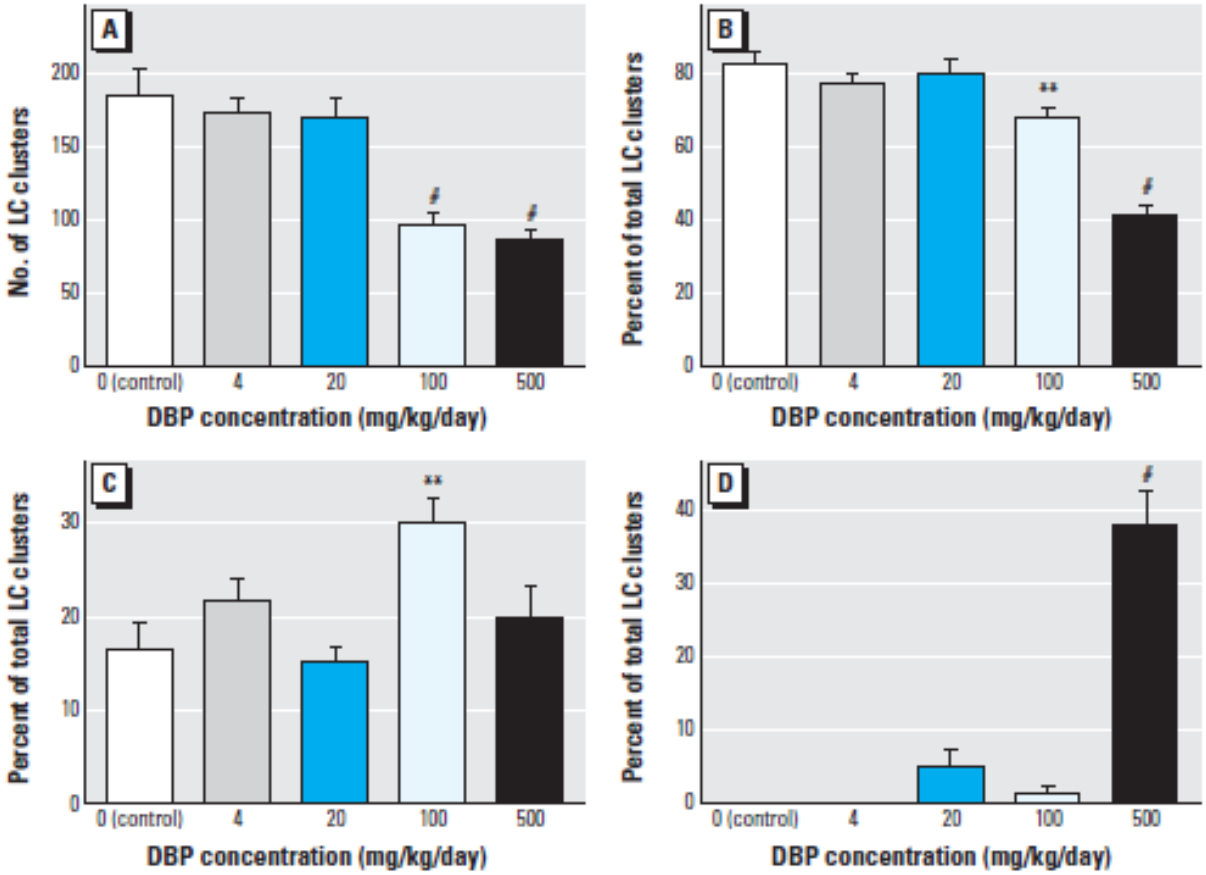


Figure 6. Number and distribution of LC clusters in testes collected on GD21.5 from male rats exposed *in utero* (GD13.5–GD20.5) to corn oil (control) or to DBP. The number of LC clusters per testis section (*A*) and the percentage occurrence of small (*B*), medium (*C*), and large (*D*) LC clusters are shown for each treatment group. Values shown are litter mean ± SE for five to six litters per treatment group. Small clusters account for ≤ 5% of the total LC cluster area per testis, medium clusters for 5.1–14.9%, and large clusters for ≥ 15%. $^{**}p < 0.01$. $^{\#}p < 0.001$ compared with respective control values.

Figure B.20 reproduced from Mahood et al. (2007).

B.2.b.7 Lehmann et al. (2004)

Lehmann et al. (2004) administered DBP by oral gavage to pregnant rats on GD 12 to 19 at 0, 0.1, 1.0, 10, 50, 100, or 500 mg/kg/day. Fetal testes were isolated on GD19, and changes in gene and protein expression and testicular T concentration were measured. DBP induced dose-dependent reductions in testis T levels and the expression of mRNA and proteins associated with steroid transport and steroid hormone synthesis (SR-B1, StAR, P450scc, CYP17, 3β-HSD, c-Kit and Insl3). Of the mRNAs, SR-B1, 3β-HSD and c-Kit displayed NMDRs being significantly reduced at the lowest dosage levels (0.1 and 1 mg/kg/d) but not at 10 mg/kg/d. All the other endpoints (4 protein levels and T) displayed monotonic responses being statistically significant at 50 or higher mg/kg/d levels. Hence, the NOEL for the effects of DBP on SR-B1, 3β-HSD, and c-Kit all display NMDRs**.**

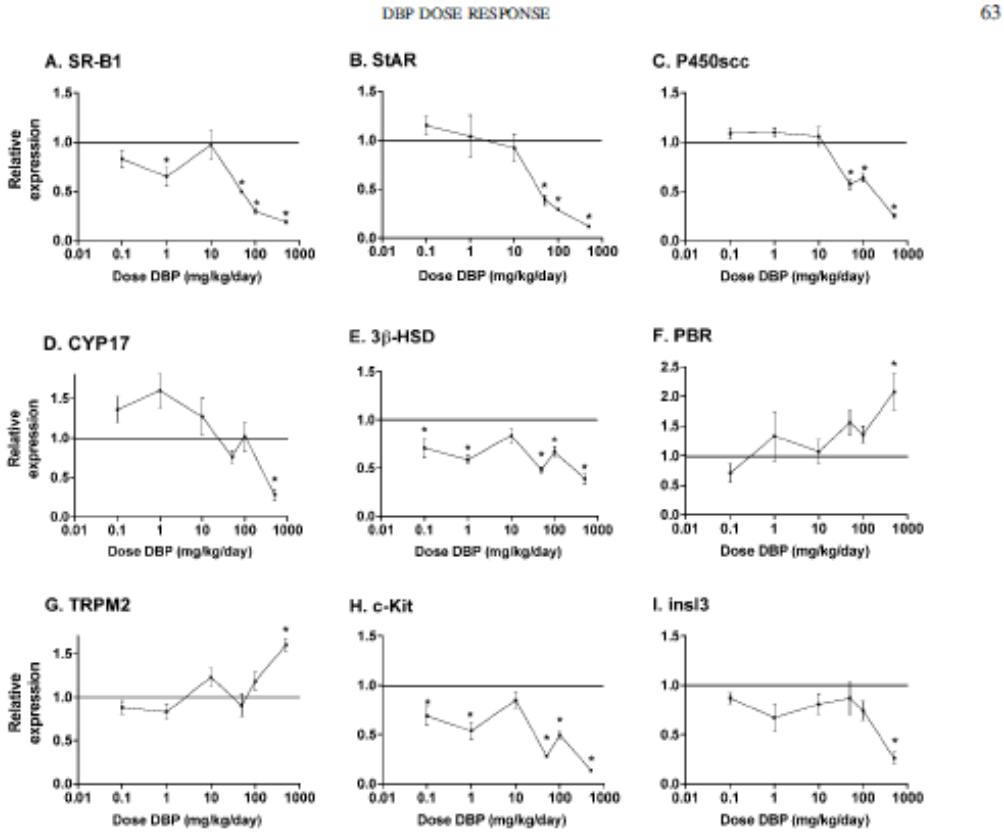


FIG. 1. Real-time quantitative RT-PCR analyses of testicular mRNA collected on gestation day (GD) 19 from control and DBP-exposed fetuses. Gene expression values from DBP-exposed testes are expressed relative to control values and represent the average ± SEM from five separate rat fetuses from different dams per treatment group. (A) Scavenger receptor B-1 (SR-B1); (B) steroid acute regulatory protein (StAR); (C) cytochrome P450 side-chain cleavage (P450scc); (D) CYP17; (E) 3β-hydroxysteroid dehydrogenase (3β-HSD); (F) peripheral benzodiazepine receptor (PBR); (G) testosterone-repressed prostate message-2 (TRPM-2); (H) c-Kit; and (I) insulin-like factor 3 (Insl3). *$p < 0.05$.

Figure B. 21 reproduced from Lehmann et al. (2004).

Attempts to replicate the low dose NMDR effects of DBP on SR-B1 and 3β-HSD were unsuccessful, whereas some of the reductions reported at the higher dosage levels were replicated (Gray personal communication).

B.2.b.8 Wyde et al. (2005) –

Wyde et al. (2005) dosed pregnant rats by oral gavage with DBP at levels of 10, 50, or 500 mg/kg/day from GD 12 to 19, and maternal and fetal liver samples were collected on day 19 for analyses. The authors found increases in protein and mRNA levels of CYP 2B1, CYP3A1, and CYP 4A1 in maternal and fetal liver in the 500-mg dose group. DBP also caused an increase in the mRNA of hepatic estrogen sulfotransferase and UDP-glucuronosyltransferase 2B1 in the dams but not in the fetuses. None of the genomic or proteomic measures displayed an NMDR in the current study and alterations were not observed at dose levels below 500 mg/kg/d.

B.2.b.9 Hannas et al. (2012); Hannas et al. (2011)

In these two studies, phthalates (dipentyl-, dihexyl-, diisobutyl-, diisononyl-, and diheptyl-phthalate were administered orally to pregnant rats on GD 14-18 (5 dose levels of each chemical), and fetal testicular T production and gene expression were measured in GD 18 male fetuses. Each of these studies reported dose-related reductions in T production and gene expression in the testis. Neither hormone production nor the affected genes displayed NMDR.

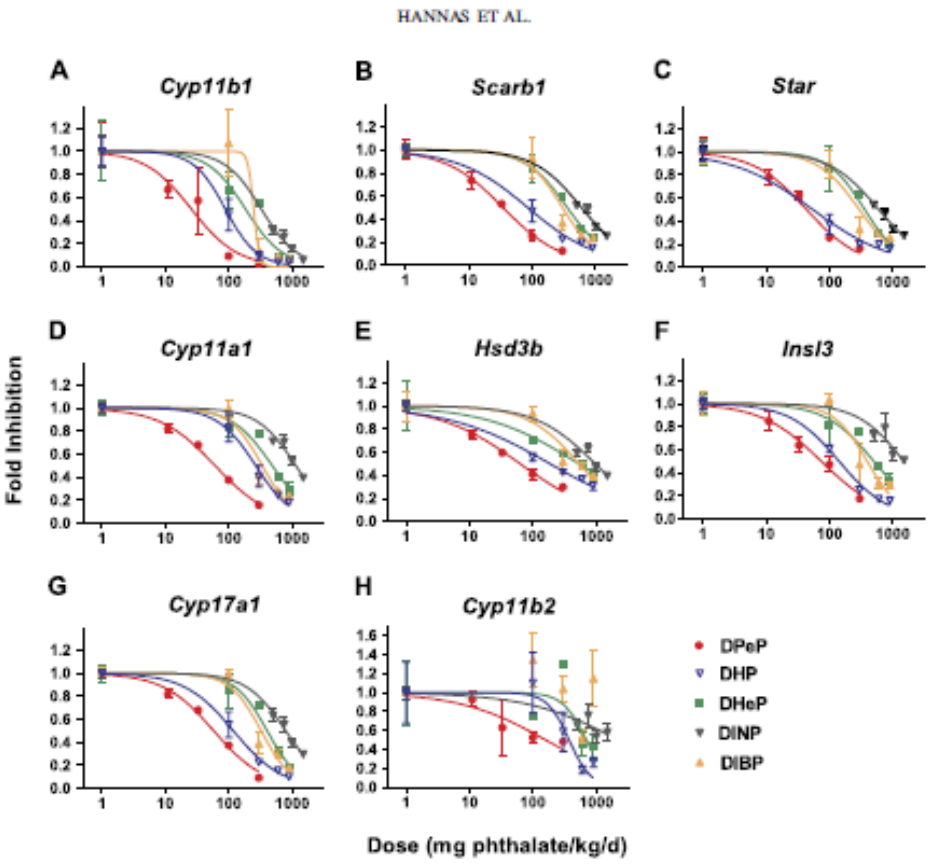


FIG. 4. Dose-response curves for fold inhibition of fetal testis RNA expression levels for (A) *Cyp11b1*, (B) *Scarb1*, (C) *Star*, (D) *Cyp11a1*, (E) *Hsd3b*, (F) *Insl3*, (G) *Cyp17a1*, and (H) *Cyp1 1b2* on GD 18 following *in utero* exposure (GD 14–18) to DPeP, DIBP, DHP, DHeP, or DINP. Each data point represents the mean (± SEM) of pooled litters ($n = 3$–4 l). The $2^{-\Delta\Delta C}{}_T$ method was used to analyze data and change in gene expression levels are reported as fold change.

Figure B.22 reproduced from Hannas et al. (2012).

B.2.c Peripubertal exposure effects of DEHP on male rat reproductive development

Phthalate treatment during peripubertal development causes testicular lesions in several mammalian species including the rat, ferret, guinea pig, and some strains of mice and hamsters. The sensitivity of the species varies considerably. The constellation of effects of peripubertal treatment in the young males includes testicular atrophy, seminiferous tubule hypospermatogenesis, reduced sperm counts, infertility, delayed puberty, reduced androgen-dependent tissue weights, and transient alterations in serum androgen levels. Some effects also have been seen in male and female marmosets.

Results from multigenerational studies demonstrate that some of the effects of phthalate treatment, such as delayed puberty, are consistently seen only when the treatment continues after weaning, and through the peripubertal period rather than with *in utero* and lactational exposure alone. Multigenerational studies with pubertal dosing have not reported NMDRs for PPS (Wolf *et al*. 1999).

An examination of the literature on the pubertal effects of DEHP on indicates that two of the studies have reported NMDRs on pubertal measures in young male rats (Vo et al., 2009; Ge et al., 2007). These papers did not report NMDRs for the same endpoints. The NMDRs are not consistent with other data from the same laboratory (Akingbemi et al., 2004; Akingbemi et al., 2001) or other laboratories (Noriega et al., 2009; Andrade et al., 2006c; Wolf et al., 1999).

B.2.c.1 Akingbemi et al. (2001)

Prepubertal rats (n=10/ group) were dosed by oral gavage with DEHP at 0, 1, 10, 100 or 200 mg/kg and day for 14 days during either PND 21-34 or 35-49. No effects were observed on serum LH or T, body weight, testis or seminal vesicle weights. However, *ex vivo* Leydig cell T production and steroidogenic enzyme activities were decreased after exposure to 100 or 200 mg/kg and day (exposure PND 21-34), or 10, 100 or 200 mg/kg and day (exposure PND 35-48). In contrast the reduction in T production induced by DEHP with exposures from PND 21-34 or 35-49, exposure to DEHP from PNDs 21-48 produced dose-related increases in Leydig cell T production**.** Also in contrast to PND 21-34 or 35-49 DEHP treatment (which had no effect on serum T), prepubertal rats were exposed for 28 days (PND 21-48) displayed dose related increases in serum T (about 25%), interstitial fluid T, and serum LH at 10, 100 and 200 mg/kg and day. There were no NMDRs in this study.

B.2.c.2 Akingbemi et al. (2004)

In the second study, the authors examined the effects of longer-term oral dosing with DEHP exposures on Leydig cell T production and serum hormone levels (LH and T). Male prepubertal rats were exposed to DEHP at 0, 10, or 100 mg/kg/d from PND 21–90 or 21-120. Serum T and LH were increased at 100 mg/kg/d at 90 and 120 days of age as well as in the 10 mg/kg/d group at 90 days of age. In contrast, *ex vivo* T production was decreased in the same DEHP groups as

compared to controls. These endpoints did not display NMDRs, but there were only 2 treated dose groups.

B.2.c.3 Ge et al. (2007)

In the Ge et al. (2007) current study (from the same laboratory as the two studies by Akingbemi et al. (2004); Akingbemi et al. (2001) discussed above) rats were dosed by oral gavage with DEHP for 28 days at 0, 10, 500 or 750 mg/kg/d from PND 21 to 49. This study reports a low dose NMDR for the age at puberty in treated males with a 1.8 day acceleration at 10 mg/kg/d, no statistically significant effect at 500 mg/kg/d and a 6.9 day delay at 750 mg/kg/d. Ge et al. (2007) also reported that body and seminal vesicle weights and serum T levels were increased at 10 mg/kg/d. It appears that the authors randomly assigned rats to treatment groups rather than controlling for weaning body weight as recommended in the EPA EDSP test guideline; this may introduce a confounder in the pubertal male assay. Failure to control for this confounder could explain the acceleration in PPS and other effects attributed to this 10 mg/kg/d dosing. In the 2001 study (Akingbemi et al., 2001), this dose did not cause an increase in body weight in similarly exposed males, whereas LH was increased; the latter an effect not seen by Ge et al. (2007). Another difficulty with interpretation is that the two studies from the same laboratory report serum T and LH levels that differ by two fold across the control groups; this is greater than the reported DEHP effects at 10 mg/kg/d.

There are other inconsistencies across studies. Noriega et al. (2009) did not see accelerated puberty or increased serum T levels in males exposed to DEHP at 10 mg/kg/d whereas significant delays in puberty were seen at 300 mg/kg/d and above. Similar low dose studies with DBP (Bao et al., 2011), a phthalate with the same mechanism of toxicity and mode of action as DEHP, did not observe any of the hormonal changes reported by Ge et al. (2007).

When Ge et al. (2007) dosed rats with DEHP for 14 days from PND 21 to 34 none of the low dose effects from exposure from PND 21-49 were noted at 10 mg/kg/d. These authors reported that MEHP *in vitro* produced an NMDR on Leydig cell T production, increased above baseline at 10^{-4} and 10^{-3} M and then decreased at 10^{-2} M; these high test concentrations are of uncertain relevance to experimental animal or environmental exposures.

In summary, the NMDR effects of DEHP on PPS and body and reproductive organ weights at 10 mg/kg/d were not seen by Noriega et al. (2009) (discussion follows) in either of two rat strains studied. There are inconsistencies across studies for some of the low dose effects.

B.2.c.4 Noriega et al. (2009) –

The Noriega et al. (2009) study was designed to determine if the dose response to DEHP in the pubertal male rat was nonmonotonic, as hypothesized. They exposed immature male LE and SD male rats to 0, 10, 100, 300 or 900 mg DEHP/kg/d (10 rats per group per strain) from weaning at PND 23 until necropsy at 56 or 98 days of age. These dosage levels were selected to encompass the dose levels used by Akingbemi et al. (2001) as well as a high dose (900 mg DEHP/ kg/day) expected to delay PPS. The Akingbemi et al. (2001) study reported that T was occasionally increased after DEHP treatment and, therefore, would accelerate PPS. Noriega et al. (2009) reported the following measures; age at puberty; weights of testes, epididymal, seminal vesicle, ventral prostate, LABC, Cowper's glands, liver, kidney, glans penis and adrenal glands; epididymal sperm counts; *ex vivo* testicular T production; serum T and serum LH; and testicular and epididymal histopathology. There were approximately 20 measures in two strains at two ages or about 80 dose response curves; as measures were for reported for 4 treated group values, there were about 320 *post hoc* statistical tests.

None of the NMDRs or low dose effects reported by Ge et al. (2007) or Akingbemi et al. (2001) were seen in the Noriega et al. (2009). Puberty was not accelerated, and serum T was not increased at 10 or 100 mg/kg/d. Exposure to high dosage levels of some phthalates delayed the onset of puberty and reduced androgen-dependent tissue weights in both LE and SD male rats treated with 300 and 900 mg DEHP/kg/day. These effects were generally of greater magnitude in LE than in SD rats. In contrast, alterations in testis histopathology (300 and 900 mg/kg/day) were more severe in SD than in LE rats. Treatment with DEHP generally reduced serum T and increased serum LH levels; this demonstrates that the reduction in T was due to the effect of DEHP on the testis and not via an inhibition of LH from hypothalamic-pituitary axis. T production *ex vivo* was consistently reduced in males at the time of puberty and shortly thereafter in contrast to the effects seen by Akingbemi et al. (2001).

There was no evidence of an NMDR to DEHP during puberty on any the reproductive or endocrine endpoints reported by Noriega et al. (2009).

B.2.c.5 Poon et al. (1997)

Poon et al. (1997) executed a 90 day study with DEHP according to OECD Guidelines under GLP for 90 day studies in SD male rats. Young male and female rats (32-37 days old, 10/group) were given DEHP in the diet at 0, 5, 50, 500 or 5,000 ppm (equivalent to about 0, 0.4, 3.7, 37.6 or 375 mg/k/d) for 13 weeks.

DEHP increased liver weights and induced histopathological alterations in the liver and thyroid of both male and female rats, and it induced seminiferous tubular atrophy in the testes of male rats at 5,000 ppm. Sertoli cell vacuolation was seen in the testes of treated males in all dose groups, with the incidence and severity increasing as the dose of DEHP increased. They concluded that the NOEL was 3.7 mg/kg/d for effects in the male. All of these effects displayed monotonic dose related increases whereas two of six serum biochemistry indices displayed an NMDR in female but not male rats.

B.2.d Peripubertal exposure effects of DBP on male rat reproductive development

B.2.d.1 Bao et al. (2011)

In the Bao et al. (2011) study, five week old male SD rats were orally administered DBP at 0, 0.1, 1.0, 10, 100 or 500 mg/kg/d (20 rats per group) for 30 days. Reproductive organ weights, testicular histopathology and serum hormonal levels were measured at necropsy. In addition, proteomic analysis was performed to identify proteins affected by DBP treatment; however, these analyses were not conducted in the 100 and 500 mg/kg/d groups.

Reproductive organ weights were affected only at 500 mg/kg/d, and histopathology alterations were seen only in the two high dose groups. Spermatid numbers were decreased and spermatocyte and spermatogonia numbers were increased in a dose related manner at 100 and 500 mg/kg/d.

Several low dose and nonmonotonic effects were noted in the current study:

- Serum E2 was increased at 0.1 and 500 mg/kg/d but not in other dose groups
- Serum LH was increased in all groups, but the increase was not statistically significant at 1.0 mg/kg/d
- Serum FSH was increased (monotonically) and was statistically significant at 1 mg/kg/d and above
- Of the twenty proteins reported to be affected in the low dose groups (10 mg/kg/d and below) the dose response data are only shown for 4 of these, one of which (vimentin) displayed an NMDR (n=3 per group)

In summary, reproductive organ weights and histopathology of the testis displayed monotonic dose related responses to DBP treatment (see Fig. B.23), whereas serum E2 and one of the proteins displayed NMDRs. In addition, peripubertal DBP-treatment did not display NMDR effects on serum T at low doses, in contrast to some, but not all, of the above reports of DEHP administered during puberty.

Effects of di-n-butyl phthalate on male rat reproduction following pubertal exposure

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POTENTIAL NMDRCs

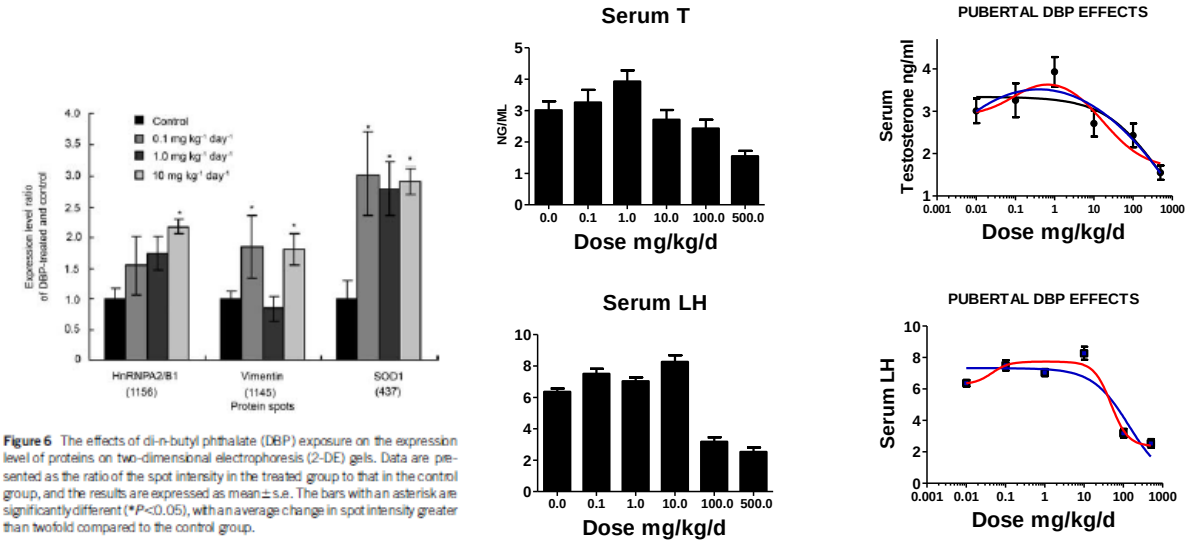

Figure 6 The effects of di-n-butyl phthalate (DBP) exposure on the expression level of proteins on two-dimensional electrophoresis (2-DE) gels. Data are presented as the ratio of the spot intensity in the treated group to that in the control group, and the results are expressed as mean ± s.e. The bars with an asterisk are significantly different (*P<0.05), with an average change in spot intensity greater than twofold compared to the control group.

Figure B.23 reproduced and adapted from Bao et al. (2011).

B.2.d.2 Vo et al. (2009)

In the Vo et al. (2009) study, DEHP was administered at 0, 10, 100 or 500 mg/kg/d via oral gavage from PNDs 21 to 35 (4 males per dose group). At necropsy, four testes per group were used for total RNA isolation, which was assessed using microarrays, and four testes were used for histopathology assessments. In addition, serum LH and T were measured using ELISA kits.

Terminal body weights were not reduced in any dose group whereas testis (high dose only) and prostate weights (all dose groups) were reduced in a dose related manner. Serum T was reduced similarly in all DEHP-treated groups. NMDRs were seen for epididymal weight (which was reduced only at 10 mg/kg/d) and for seminal vesicle weight, (which was reduced at 10 and 500 mg/kg/d but not 100 mg/kg/d). The assessment of mRNA levels in the testes examined by microarray only for controls and treated males from the 100 mg/kg/d dose group; the expression of mRNA for eight testicular genes were assessed by rtPCR.

The results of DEHP in this study on were cited by Vandenberg *et al.*, 2012 as cites this study as providing examples of NMDRs in animal studies on what they list as "seminal vesicle weight, epididymal weight, testicular expression of steroidogenesis genes". However, three steroidogenesis genes measured (StAR, CYP11a1 and HSD3β1) do not appear to be altered by DEHP treatment at any dose level (see Vo et al. (2009) Fig 4, a,b,c). Two of five other genes

assessed in the testis, identified by Vandenberg et al. (2012) as “TP, DEHP or FLU markers”, do display NMDRs.

In summary, NMDRs were noted in the Vo et al. (2009) study on two reproductive organ weights and on mRNA expression levels for two testis genes. However, the small sample sizes used in the current study (n=4/group) and the lack of concordance of the organ weight effects reported here with several other more robust studies introduces uncertainty about the biological significance of these results.

B.2.e *In utero* and lactational exposure effects of DEHP on male mouse reproductive development

NMDRs have been described for some endpoints at low-dose dose levels for a few endpoints after *in utero* DEHP exposure in mice. However, there is uncertainty in interpreting results of some of these studies, as they report administered DEHP at dose levels the published background levels of this ubiquitous contaminant in rodent diets. In addition, the effects are small and are not necessarily adverse. At present there is debate in the scientific community about ability of phthalates, including DEHP, to demasculinize fetal mice as is seen in the fetal rat. The link between the changes seen in the fetal mouse and postnatal reproductive outcomes in not established.

B.2.e.1 Pocar et al. (2012)

DEHP was administered in the diet during pregnancy and lactation at 0, 0.2857, 28.57, and 2857.0 mg/kg food (about 0, 0.05, 5, and 500 mg/kg body weight /d; n about 10 per group). The male and female offspring were examined and necropsied at PNDs 21 and 42. There were no viable pups at 500 mg/kg/d, and maternal liver weight was heavier at 5 mg/kg/d. Other maternal and litter variables were not affected in the dams with litters. Male and female offspring weights were reduced in both DEHP-treated groups, and females had significantly less abdominal fat at 21 and 42 days of age. At 42 days of age, male and female AGDs were not affected by DEHP treatment. Ovarian weights were increased (both treated groups,) and seminal vesicles and cauda epididymal sperm counts were reduced (both dose groups). The changes in ovarian weight were associated with a reduction in the percentage of mature oocytes and an increase in the percentage of degenerating oocytes.

DEHP induced an NMDR response on testis weight, being reduced only in the low dose group**.** The analysis of these data does not appear to be adjusted for litter effects, which can alter the statistical significance of the reported effects. Effects on *in vitro* fertilization indicated NMDRs with reductions in the cleavage and blastocyst rates in only the low dose group. It is uncertain how many times the *in vitro* assays were replicated as the analyses appear to be based upon a single pool of oocytes and fertilized ova for each dose group. The expression of several genes related to steroidogenesis was affected in the ovary, testis and pituitary of the offspring in a dose related manner. These data indicate LOELs of 0.05 mg DEHP/kg/d with no NOEL for several effects on the male and female offspring. The observed NMDRs would not affect the determination of a LOEL in this study.

B.2.e.2 Do et al. (2012)

Do et al. (2012) administered DEHP from GD 9 to 18 orally at 0, 0.5, 1.0, 5, 500, 50,000, and 500,000 µg/kg/d and examined maternal and fetal (1M males only) hormones on GD 18. Although the study was conducted in three blocks (incomplete block design) only the pooled data are presented. Data for fetal males from intrauterine positions other than 1M are not presented. The effects are small and none of the effects shown in Table 1 of the paper were statistically significantly altered by DEHP-treatment (all F values from the ANOVA are non-significant; > 0.05). The authors report NMDR for several of these effects. The biological significance of the reported effects (small increases in serum T) is not clear, as T changes dramatically in the dam and fetal male during this stage of pregnancy. There is uncertainty as to the comparison of the low administered doses of DEHP to the background levels in the animals' diet and bedding.

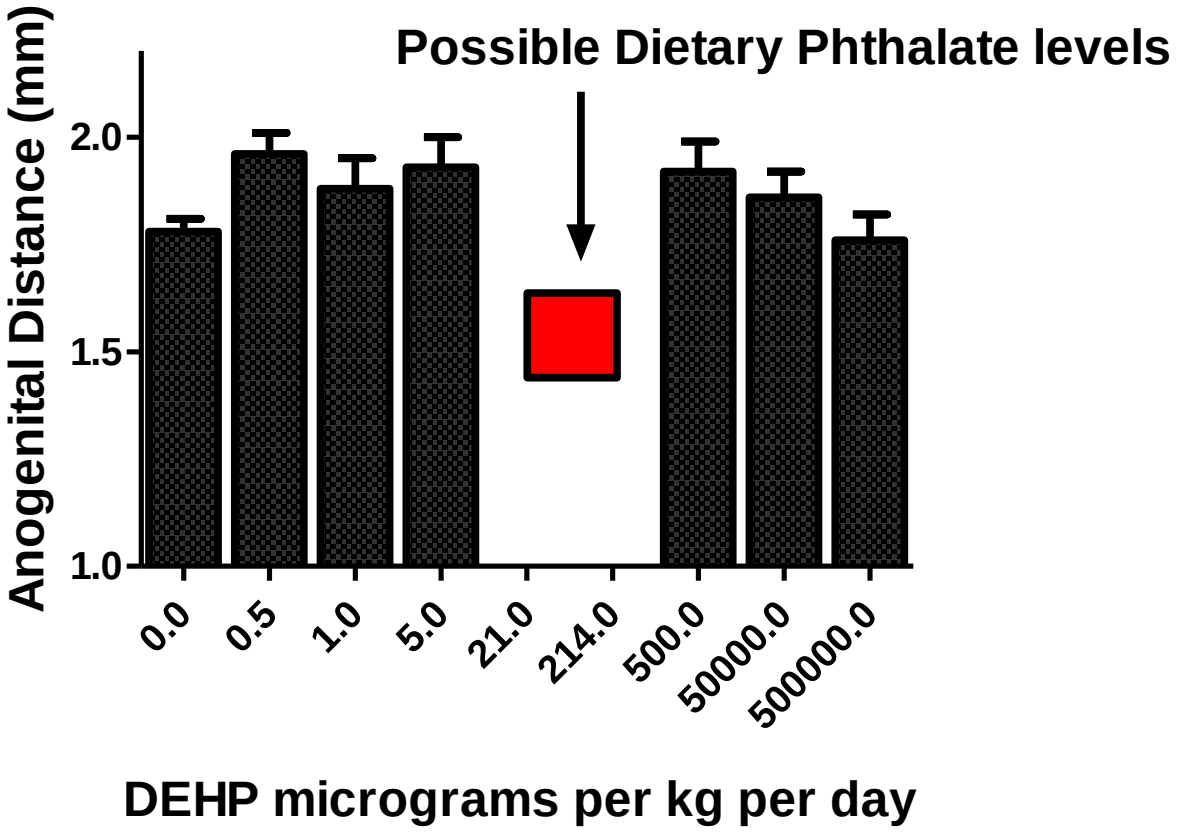

Figure B.24 adapted from Do et al. (2012)

B.3 Inhibition of DHT synthesis

Finasteride blocks T to the more potent androgen DHT. DHT and T are androgens critical for normal sexual differentiation in all mammals, including humans. T is produced primarily in the testes of the fetal male whereas DHT is produced locally in the tissues from T by the enzyme 5α reductase. Several robust studies spanning a broad range of doses have been conducted with the drug Finasteride that inhibits the activity of this enzyme, demasculinizing the fetal male if exposure occurs during sexual differentiation. Finasteride also was administered orally in the OECD Hershberger Assay Validation Study to castrate-immature male rats for ten days. It produced dose-related, linear-changes in androgen dependent tissue weights.

B.3.a Clark et al. (1990)

This project included a summary of the results of 6 studies that examined the ability of Finasteride to demasculinize the external genitalia of the male rat, exposed *in utero* by oral gavage.

R.L. CLARK ET AL.

TABLE 1. Outline of study protocols

Study No.	Treatment period, gestational days	Dosage levels, mg/kg/day	Evaluation on Day 20 of gestation: Litters/ group	Evaluation on Day 20 of gestation: Fetal examinations	Postnatal evaluation, litters/group
1	6–17	10, 30, 100, 300	10	External	0
2	6–17	0.03, 0.1, 0.3, 1, 3	10	External	0
3	6–17	0.006, 0.1, 3, 100	25	External, visceral, skeletal	0
4	6–17	0.1, 3, 100	0	—	15
5	6–20	0.0003, 0.003, 0.03, 0.3	0	—	22
6	−14–20[1]	0.1, 3, 100	20	External, visceral, skeletal	15

[1]Treatment began 2 weeks prior to cohabitation with untreated males. Those females killed on Day 20 of gestation received their last dose on Day 19 of gestation.

Table B.1 reproduced from Clark et al. (1990).

As indicated in the` above table from the paper, the doses of Finasteride spanned over 5 orders of magnitude from 0.006 to 300 mg/kg/d. AGD, nipple retention and incidence of hypospadias were recorded. In the Clark et al. (1990) study, Finasteride did not induce any NMDR responses. AGD was reduced in a robust dose related (linear) manner whereas the incidence of hypospadias (a different response in the same tissues) displayed a clear threshold response. Thus, the shapes of the dose response curves for these two endpoints is consistent with those seen with FLU, VIN, and the phthalates, which demasculinize the male fetus via different mechanisms of toxicity.

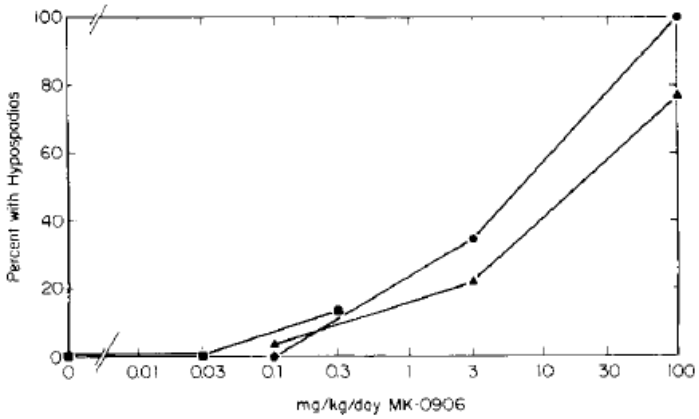

Fig. 5. Dosage response for MK-0906-induced incidence of male hypospadias. Treatment was on gestational Days 6 through 17 (▲, Study 4), Days 6 through 20 (■, Study 5), or from 2 weeks prior to cohabitation until Day 20 of gestation (●, Study 6). Approximately 30 males per group were examined in Studies 4 and 6 and 110 per group in Study 5. No animal had hypospadias at 0.0003, 0.003, and 0.03 mg/kg/day in Study 5.

Figure B.25 Reproduced Clark et al. (1990).

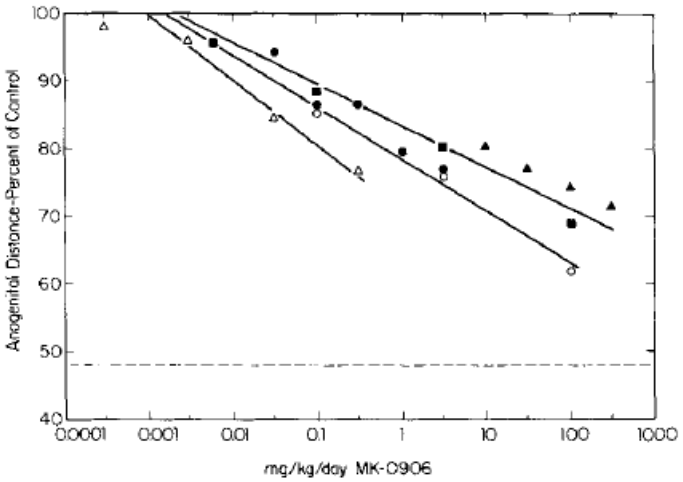

Fig. 2. Dosage response for MK-0906-induced decrease in male anogenital distance. Treatment was on gestational Days 6 through 17 (▲, Study 1; ●, Study 2; ■, Study 3), Days 6 through 20 (△, Study 5), or from 2 weeks prior to cohabitation until Day 20 of gestation (○, Study 6). Measurement of anogenital distance was on gestational Day 20 (Studies 1, 2, and 3) or postnatal Day 0 (Studies 5 and 6). The decreases at the lowest dosage levels (0.0003, 0.003, and 0.006 mg/kg/day) were statistically significant ($P \leq 0.05$).

Figure B.26 reproduced from Clark et al. (1990).

B.3.b Clark et al. (1993)

In the Clark et al. (1993) study, rats were exposed to Finasteride *in utero* by oral gavage at 0, 0.003, 0.03, or 3 mg/kg/d from GD15 to 21 and the males examined after birth. All effects displayed monotonic, dose related changes. There we no effects on maternal or litter indices or female pup AGD. Male AGD was reduced at 0.03 and 3 mg/kg/d, with the high dose effect being permanent. Nipple retention was seen in 3% and 29% of the males at 11 days of age whereas a delay in puberty (PPS) and hypospadias were only seen at 3 mg/kg/d. These results again demonstrate that AGD in males at birth and nipple retention are sensitive indicators of disruption of the androgen signaling pathway; both being more sensitive than are the age at puberty or reproductive malformations.

B.3.c Bowman et al. (2003)

In the Bowman et al. (2003) study, rats were gavaged with either vehicle or finasteride at 0.01, 0.1, 1.0, 10, or 100 mg/kg/d (5-6 dams/group) on GD12 to 21, and all male offspring were monitored until necropsy on PND 90. Maternal weight gain displayed an NMDR, being reduced at 0.1 and 100 mg/kg/d, but the low dose effect was not associated with any other maternal or neonatal alterations. Litter sizes and pup weight were not affected at any dosage level. AGD at birth was reduced, and the numbers of retained nipples increased in all treated groups. These effects were permanent except the effects at 0.01 mg/kg/d, which were not altered at 90 days of age. Gross malformations of the penis, testis and androgen-dependent tissues were seen at 10 and 100 mg/kg/d. Testis descent was altered at 10 and 100 mg/kg/d and in 1 male in the 1 mg/kg/d dose group. Ventral and dorsolateral prostate eights were reduced at 10 and 100 mg/kg/d, and LABC weights were reduced at 1, 10 and 100 mg/kg/d. Dose response model fits to the data in this study confirmed the apparent linear response of AGD and nipple retention to disruption and the steep threshold responses of malformations like hypospadias, undescended testis and agenesis of sex accessory tissues (Figure 6 in Bowman et al. (2003)). In summary, no NMDRs were seen in the current study for any reproductive effect in the male offspring.

B.4 Hypothesized alteration of the androgen signaling pathway

Semicarbazide (SEM) is a metabolite of the banned antibiotic nitrofurazone and a breakdown product of azodicarbonamide, used as a blowing agent in plastic gaskets (The EFSA Journal (2005). SEM is known to inhibit enzymes, such as lysyl oxidase, semicarbazide-sensitive amine oxidase and glutamic acid decarboxylase. SEM acts as osteolathyrogen, and induces osteochondral and vascular lesions in young rats due to impaired cross-linking reactions of collagen and elastin (Takahashi et al., 2009). In addition, teratogenic effects such as induction of cleft palate and aortic aneurysms have also been reported. This chemical has been described as an example of an EDC that induced an NMDR on the age at puberty in male rats. The literature described provides little support for this endocrine disruption by SEM. The reported NMDR is at a dose level that produces severe reduction in weight gain during dosing and is higher than doses of SEM that produce lesions of the joints and vascular system.

B.4.a Steffek et al. (1972)

SEM was included in a study on lathyrogenic agents by Steffek et al. (1972), who administered SEM at 5, 10, 25, 50 or 100 mg/day on days 10-who or 12-15 of gestation and examined the fetuses for cleft palate one day before term. At the highest dose, 3 out of 9 pregnant rats died. The incidence of resorptions was 3, 0, 3, 38 and 56% and the incidence of cleft palate in surviving fetuses was 0, 0, 43, 95 and 100% in 5, 10, 25, 50 or 100 mg/day dose groups respectively. From this study a no-effect level of 10 mg/day (equivalent to around 30-40 mg/kg/d) for cleft palate and intrauterine death was apparent.

B.4.b Takahashi et al. (2009)

SEM was administered in the diet to 6 week old male and female rats for 90 days at 0, 250, 500 or 1000 ppm. The authors found that the lower dose effects of SEM were observed in the bones, cartilages and aorta. Histopathological examination, revealed “disarrangement, fissures and deformation of the cartilages in both sexes” at 250 ppm and above. The authors concluded that the NOAEL in their study was less than 250 ppm in both sexes (equivalent about 20 mg/kg/d).

The study included a large number of endpoints, a few of which displayed NMDRs at doses above the NOAEL:

- One of the >40 hematology measures
- Food consumption in male rats
- None of the >40 serum biochemistry measures
- None of the histopathological effects
- None of the >20 organ weights

B.4.c Maranghi et al. (2010); Maranghi et al. (2009)

In the investigations reported by Maranghi et al. (2010); Maranghi et al. (2009), SEM was administered at 0, 40, 75 and 150 mg/d to 23 day old male and female rats (10/sex/group) by oral gavage for 28 days. The top two doses induced mortality, and all dose levels reduced the weight gain in males. VO was delayed in females in the high dose group, and the authors reported that puberty in males was accelerated at 40 and 75 mg/kg/d but delayed at 150 mg/kg/d. Rats displayed several adverse effects in all the dose groups including among other effects histopathological alterations in the cartilage, reduced organ weights, behavioral alterations. Given all the lesions seen with SEM in the lowest dose level, taken together with what is known about the mechanism of toxicity for the bone and vascular lesions, it doesn't appear that SEM is an EDC. NMDR effect on puberty was observed at high dosage levels.

B.5 Pesticides that disrupt the Androgen signaling pathway via multiple mechanisms of toxicity --Prochloraz and Linuron

It has been shown that a number of environmental chemicals disrupt the androgenic signaling pathway in an antiandrogenic manner via multiple mechanisms of action. Pesticides such as linuron and prochloraz display dual mechanisms of endocrine disruption, acting as AR antagonists and as inhibitors of fetal T synthesis. In contrast to the phthalates, discussed above, prochloraz and linuron reduce fetal T production without inhibiting mRNA expression of steroidogenic enzymes or the insl3 hormone. Although the chemicals that disrupt androgen signaling in the fetal male rat produce some malformations in common in male rat offspring, the specific profiles of effects are pathognomonic for each chemical.

B.5.a Prochloraz (PCZ)

B.5.a.1 Blystone et al. (2007b)

Pregnant SD rats were dosed by gavage with PCZ at 0, 7.8, 15.6, 31.3, 62.5, or 125 mg/kg/d from GD 14 to 18. On GD 18, the effects of PCZ on fetal steroidogenesis were assessed by measuring hormone production from fetal testes. Fetal progesterone and 17α-hydroxyprogesterone production levels were increased significantly at every dose, whereas T levels were significantly decreased at only the two high doses. These results suggest that PCZ affects the conversion of progesterone to T through the direct inhibition of CYP17. Testis mRNA levels for CYP17, CYP11a and StAR were not affected at any dosage level. In addition, maternal weight gain was reduced at 31.3 mg/kg/d and above without any affect on fetal viability. No NMDR were noted in the current study, 7.8 mg/kg/d was a LOEL, and no NOEL was determined.

B.5.a.2 Blystone et al. (2007a)

The Blystone et al. (2007a) study was designed to determine the following: 1) if PCZ delayed puberty and reduced T production in a pubertal male rat assay; and 2) if PCZ acted as an AR antagonist *in vivo* in castrate-immature-androgen-treated young male rats in the Hershberger assay. Two experiments were conducted with pubertal male rats. In the first experiment, SD weanling male rats were dosed by gavage with PCZ at 0, 31.3, 62.5, or 125 mg/kg/day from 23 to 42 or 51 days of age. There was a significant delay in PPS (PPS) at 125 mg/kg/day PCZ, and several of the androgen-dependent organ weights were decreased statistically significantly. At both ages, serum T levels and *ex vivo* T release from the testis were statistically significantly decreased whereas serum progesterone and 17*α*-hydroxyprogesterone levels were statistically significantly increased at dose levels below those that affected PPS or reproductive organ weights. The hormone results suggested that PCZ was inhibiting CYP17 activity. None of the effects displayed an NMDR. The dose response curves, however, varied among the groups necropsied at 42 and 51 days of age, as did the LOEL for organ weight reductions, being 31.3 mg/kg at 42 days and 62.5 at 51 days of age. In the second pubertal study were dosed by gavage with PCZ at 0, 3.9, 7.8, 15.6, 31.3, or 62.5 mg/kg/d. Serum T levels and *ex vivo* T production were statistically significantly reduced at 15.6 mg/kg/d whereas *ex vivo* androstenedione production was statistically significantly reduced at 7.8 mg/kg/d with 3.9 mg/kg/d being and NOEL. Two related endpoints did show NMDR in this study, as body weight at necropsy and glans penis weights were increased at 3.9 mg/kg/d.

In order to determine if PCZ displayed AR antagonism *in vivo* independent of its effects on T synthesis, castrated immature male rats were dosed with androgen and 0, 15.6, 31.3, 62.5, or 125 mg/kg/day PCZ for 10–11 days (Hershberger assay). In this assay, androgen-sensitive seminal vesicle and LABC weights were statistically significantly decreased at 125 mg/kg/d. Liver weight was increased at 31.3 mg/kg/d and above, and serum LH was decreased at 125 mg/kg/d. These effects were all dose related. In this study, glans penis weight displayed an NMDR being reduced only at 15.6 mg/kg/d; this response was in the opposite direction from the NMRDC seen in the above pubertal study**.**

B.5.a.3 Christiansen et al. (2009b)

In the current study PCZ was administered at 0, 5, 10, 25, 50, 100 or 150 mg/kg/d by oral gavage to pregnant rats from GD7 to PND16 in three independent studies (6 to 20 dams per group). On PND 16 male offspring were examined for genital malformations. Only the high dose group displayed any genital dysgenesis, which was present in 9.5% (of 21 males from 6 litters). No other dose response data for PCZ are presented in the paper from these three studies.

B.5.a.4 Noriega et al. (2005)

Noriega et al. (2005) conducted two studies in which PCZ was administered by oral gavage to pregnant rats from GD 14 to 18, the critical period of sexual differentiation of the male rat reproductive tract.

In the pilot study, PCZ was administered at 62.5, 125, 250 and 500 mg/kg/d. The highest dose group reduced maternal weight gain during dosing, and there were no live births in this dose group. The onset of parturition was delayed in all PCZ-treated groups. Delays in delivery were associated with stillbirths at 125 and 250 mg/kg. AGD was reduced in male rat offspring at 62.5 and 250 mg/kg. Infant males displayed areolas/nipples on PND 13 at frequencies of 40%, 71%, and 100% in the 62.5, 125, and 250 mg/kg groups, respectively. When necropsied as adults, the male offspring displayed reduced weight of the levator ani plus bulbocavernosus muscles in a dose-related fashion. At necropsy, nipple retention in male offspring was observed at frequencies of 10%, 14%, and 100% in the 62.5, 125, and 250 mg/kg groups, respectively. One-third of males in the 250 mg/kg-BW per day treatment group showed incomplete PPS, and the same percentage displayed a group of phallus abnormalities that included cleft phallus and hypospadias.

In the main study, PCZ was administered at 31.25, 62.5, 125, and 250 mg/kg/d as in the pilot study. Time to parturition increased significantly with dose, with dams in the 250 mg/kg group initiating delivery more than 24 h after dams in other treatment groups. Dams at 125 mg/kg per day or greater completed parturition more than 28 h after controls, all of which delivered by time zero (morning of postcoital day 23). Mean times to complete parturition were 2.5, 6.7, 22.2, and 28.4 hr after controls for 31.25, 62.5, 125, and 250 mg groups, respectively. In addition, five of eight dams treated with 125 mg/kg delivered at least one stillborn pup (mean rate being 12%). In the 250 mg/kg group, one dam died during prolonged delivery, two dams did not deliver any live pups, and the five remaining dams 32% of the pups were stillborn .F1 adult males also displayed reduced body, epididymal, and ventral prostate weights at 125 and 250 mg/kg/d, reduced testis and seminal vesicle weights at 250 mg/kg/d and histopathological alterations of the testes at 62.5 mg/kg/d. All the effects in these experiments were dose related, and no NMDRs were seen.

B.5.b Linuron

B.5.b.1 OEHHA (2002)

In 2002 the California Office of Environmental Health Hazard Assessment (OEHHA CA) reviewed the database on linuron in order to establish a maximum allowable dose level (MADL) for this pesticide. However, the raw data, means and standard errors are not available to the public at this time (OEHHA, 2002). Several studies conducted by Haskell Laboratory (Haskell Laboratories, 1984, 1979) were evaluated along with the peer-reviewed publications on the reproductive effects of linuron. The industry dietary studies are among the lowest dose studies conducted to date using doses of linuron as low as 25 ppm (equivalent to 1.25). One study reported that 2 yrs of exposure >125 ppm linuron induced testicular interstitial cell adenomas

and hyperplasia in males and cystic endometrial hyperplasia in females, with a NOEL of 25 ppm. In the multigenerational studies, linuron reduced maternal body weight gain, (parental NOEL 25ppm) and resulted in smaller litters, reduced 24 hour pup survival, and pup weights.

The MADL for linuron exposure was determined by CA OEHHA to be 0.46 mg/day for the oral and inhalation routes of exposure based upon a two-generation reproductive toxicity study (Haskell Laboratory (1990) In this study, linuron was administered in the diet at 0, 12.5, 100, or 625 ppm (20 rats/sex/group) M: 0, 0.84, 6.8, or 44.75 mg/kg/day; F: 0, 1.0, 8.3, or 54.1 mg/kg/day and animals were examined after 147-161 days of feeding. In this study decreased pup weights, decreased litter size (F2) and pup viability were seen in the F2 with a developmental NOEL = 100 ppm in diet of dams (8.3 mg/kg/day).
None of the study summaries indicate that any of the effects displayed a NMDR: however, the means and standard errors are not available for public review.

B.5.b.2 Wolf et al., (1999)

(for review of the alternative reproductive toxicity protocol see Gray et al. (1988))

Protocol to Assess Reproductive Effects of Toxicants in the Rat

Initiate Daily Dosing

Weaning d 21	Puberty	Young Adulthood	Breeding	Necropsy Males	Gestation/ Lactation	Necropsy Females	Offspring
Experiment 1 Methoxychlor (25, 50, 100, or 200 mg/kg/d)	Females d 21–on • Age at Vaginal Opening • Age at First Estrus • Age at Onset of Cyclicity • Daily Vaginal Smears		Day 80–90	Day 100–105		Day 25 Postpartum	0 and 50 mg/kg Continous Breeding 11 Months
Experiment 2 Carbendazim (50, 100, 200, or 400 mg/kg/d)							0, 100, and 200 mg/kg Continous Breeding 7 Months
Experiment 3 Dibutyl Phthalate (250, 500 or 1000 mg/kg/d)	Males d 21–on • Age at Preputial Separation						No Offspring
Experiment 4 Lindane (10 or 20 mg/kg/d)							No Offspring

Fig. 1. The protocol used in the present investigation to compare multiple endpoints of reproductive function in the male and female rat to infertility.

Figure B.27 reproduced from Gray et al. (1988).

In the alternative reproductive toxicity (ART) protocol with linuron, dosing was initiated at weaning for both sexes and continued until necropsy after mating and production of the F1 generation. Linuron was administered orally by gavage at 0, 10, 20 or 40 mg/kg/d. F1 males

and females from the control and high dose group were mated to produce the F2 (not dosed directly), which were observed until the completion of a continuous mating cycle.
In the high dose group, linuron delayed PPS by about 2.8 days and reduced seminal vesicle and cauda epididymal weights. Serum T was reduced in all dose groups (not statistically significant, $p<0.15$). Body, liver, adrenal, pituitary, testis and kidney weights, and serum LH, FSH and prolactin levels were unaffected at any dose. F1 males in the high dose group displayed epididymal agenesis, reduced testis and epididymal weights, reduced sperm counts and reduced fecundity over several mating cycles. No adverse effects were noted in P0 or F1 females. None of the effects in the current study displayed an NMDR.

B.5.b.3 Wilson et al. (2009)

The Wilson et al. (2009) study investigated the impact of *in utero* linuron treatment on fetal testis gene expression and T production. Pregnant SD rats were dose with 0, 12.5, 25, 50 or 75 mg linuron/kg/d by oral gavage from GD13 to 18. Fetal T production, examined on GD 18, was significantly decreased at 50 and 75 mg/kg/d (NOEL 25 mg/kg/d). Unlike the phthalate esters, linuron treatment did not affect fetal testis insl3, cyp17a, cyp11a or StAR mRNA expression. When untreated GD 18 fetal testes were incubated with increasing concentrations of linuron (1–300 micromolar), T production was statistically significantly reduced at 30 micromolar and above, whereas, testis progesterone was unaffected. This indicated that linuron directly inhibited T production in the absence of cytotoxicity. None of the *ex vivo* or *in vitro* measures displayed an NMDR.

B.5.b.4 McIntyre *et al*., 2001

McIntyre *et al*. (2001), administered linuron by oral gavage at 0, 12.5, 25 or 50 mg/kg from GD12-21 and examined F1 male SD rats at PND1, PND21 and PND 100-105 (11 dams per group). Maternal body weights, food consumption, kidney, liver, uterine, ovarian and adrenal weights, and numbers of implantation sites were unaffected by linuron treatment. Litter sizes at birth and numbers of live pups were normal, but survival to weaning was reduced in the 50 mg/kg/d dose group. The authors also observed that AGD was unaffected, whereas male areola/nipple retention was increased in a dose-responsive manner. Malformed testes were seen in 2/56, 8/69 and 5/44 rats in adult offspring in the 12.5, 25, and 50 mg/kg/day dose groups, respectively. In addition, malformed epididymides occurred in 1/56 rats, 8/69 rats, and 2/44 rats in the 12.5, 25, and 50 mg/kg/day dose groups, respectively. Partial agenesis of the epididymides was observed in 3/44 rats only in the 50 mg/kg/day group. Histological lesions were detected in the testes and epididymides of F1 adult males in the two highest dose groups. The authors concluded that "These data indicate that *in utero* exposure to linuron preferentially impairs testosterone-mediated, rather than DHT-mediated, reproductive development. This effect is distinctly different from the effects induced by flutamide, an AR antagonist." None of the effects in the current study displayed an NMDR, the lowest dose used being 12.5 mg/kg/d.

In a follow up mechanistic study, McIntyre repeated the exposure to linuron using only the 50 mg/kg/d dose group **(Mcintyre et al., 2002).** All of the high dose effects seen in the above study were seen in the follow up study, and in addition, AGD at birth was slightly but statistically

significantly decreased. The 8% reduction in AGD was similar to that seen in the earlier study, but was statistically significant here is the authors used a larger number of treated litters (n=20 dams) and male offspring (n=51 to 58). In a second follow up study, McIntyre *et al.* (2002b) necropsied male fetuses or offspring on GD 17, 19, and 21, and PND 7 and 14. Epididymal malformations were not observed in fetuses from linuron-treated dams but were seen in linuron-exposed male offspring on PND 7 and 14. No testicular lesions were observed at any time point.

B.5.b.5 Owens et al. (2007)

(Data are from one laboratory from the interlaboratory study–Kang et al. (2004))

As part of the OECD Hershberger Assay inter-laboratory validation program, linuron was administered orally to castrate-immature-T-treated immature male rats for ten days at 0, 3, 10, 30 or 100 mg/kg/d (n = 24 per group). Linuron statistically significantly reduced the weights of two of five androgen-dependent tissues at 30 and 100 mg/kg/d in a dose related manner (see figure below). Body weight and the three other tissue weights were reduced at 100 mg/kg/d. The LOEL from this study of 30 mg/kg/d is slightly higher than that identified as a LOAEL for a low incidence of epididymal abnormalities from a developmental study with *in utero* exposure (McIntyre *et al*., 2000). Linuron produced less pronounced effects in this assay than do the more potent AR antagonists PRO and vinclozolin.

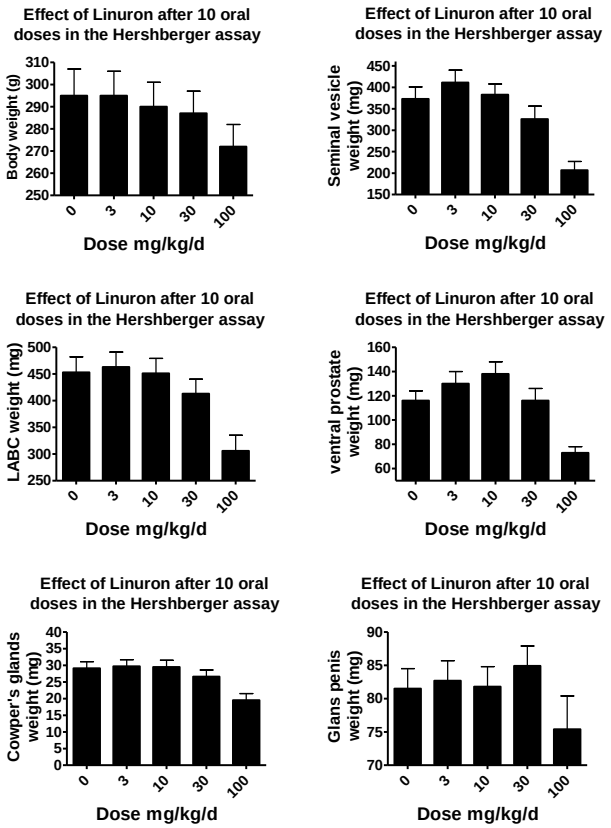

Figure B.28 From Owens et al. (2007).

B.5.b.6 George et al. (2004) - Report prepared for USEPA 'EDSP Pubertal Male rat validation study with linuron'

In the George et al. (2004) study, linuron was administered to weanling male rats by oral gavage at 0, 50, or 100 mg/kg/d following the EPA pubertal male rat test guideline. Several antiandrogenic dose- related effects were noted in each dose group, and body weight was reduced in the highest dose group. No NMDRs were noted; however, this study only used 2 linuron-treated groups and high dosage levels. It was included here for comparison to the effects seen at lower doses *in utero* and at similar dosage levels in the OECD Hershberger Assay.

B.6 Androgen Receptor Agonists

A search of the literature uncovered no published or publically available rodent multigenerational study that administered an androgen agonist in the diet or by oral gavage over a broad dose response range. Most of these data sets have been developed for submission to regulatory agencies for drug approval. The extensive set of low dose dietary and oral gavage studies on the synthetic androgen TB has been reviewed and robust summaries are available on the internet, however, these do not allow one to examine the shapes of the dose response curves. The working group that developed the NMDR State of the Science document inquired about obtaining data on TB, as it is a registered veterinary drug that has been thoroughly scrutinized. The data are not currently accessible.

This class of chemicals provides some examples of robust NMDRs for clearly adverse effects several androgens including T, TB (two papers using rats and one paper with fish) and methyltestosterone (one paper using fish). Some of the most interesting dose response studies with T and SARMS have been done with young adult and aged men.

The NMDRs seen with androgens in the mammalian studies wherein the chemical was administered either by sc injection or by implants; the relevance of this route of administration to human health effects can be debated. When the androgenic effects were compared in the Hershberger Assay between injected versus an orally administered androgen, the effects are similar, but the chemicals administered by the oral route were about 80 fold less potent (Wilson et al., 2002). One of the studies showing an NMDR administered T sc during pregnancy and evaluated the male and female offspring (Wolf et al., 2002). Another administered T in sc implants to adult male rats and monitored the effects on sperm counts and androgen dependent tissues. These studies show that effects that would be considered as adverse occur at androgen levels below those producing an NMDR, indicating that the NMDR would not affect determination of a NOAEL in the studies.

In another study, testosterone propionate was administered to castrate immature male rats by sc at levels that were below those used in peripubertal intact male rats (OECD Hershberger validation study with 16 laboratories). The dose response curves for the five androgen-dependent tissues measured in the Hershberger assay all display apparent linear-no-threshold responses rather than threshold or NMDRs.

In addition to the rodent literature, the effects of androgens have been studied extensively for decades in many vertebrate species, including humans. In this section of the review, a number of experimental studies on the dose-related effects of androgens in humans is presented. It is clear from these studies that few if any robust NMDRs were seen when T was administered to humans over a broad dose response range.

B.6.a Trenbolone (TB)

Extensive dose response studies on TB have been conducted in a number of mammalian species including, rats, domestic animal, and nonhuman primates, These studies are summarized in FDA (2009). The following is the summary for the multigenerational study. It does not indicate that the low dose effects of this chemical display NMDRs, but the data are not available for review:

> "The effect of trenbolone acetate on reproductive function of multiple generations in the rat was studied by Dr. Penny James at Huntington Research Centre, Huntington, England. Over 700 male and female rats were fed in this study to assess the effect of trenbolone acetate on growth and reproductive performance of the rat through two (2) consecutive generations. Trenbolone acetate was fed in the diet at concentrations of 0, 0.5, 3.0 and 18.0 ppm. The test diets were fed to F0 males for 9 weeks and F0 females for 2 two weeks prior to mating and then through to termination of the study. Two F1 generations were selected, reared and mated. One was treated continuously at the same dietary concentrations as the F0 generation and one was removed from exposure to trenbolone acetate at three weeks of age and remained untreated throughout. Treatment with trenbolone acetate at 18 ppm was associated with the following effects: Generally higher weekly body weights effecting F0 and F1 treated males and females, and the females of the F1 untreated generation. A depression in mean body weight gain during gestation affecting both matings of F0 generation. Signs of coarseness of the hair coat and discoloration of the skin affecting F0 and treated F1 females. Clitoral prominence in treated female F1 offspring and to a lesser extent in those where treatment was withdrawn at three weeks of age. Similar effects were observed in treated F2 offspring from treated F1 parents but not in offspring of the untreated F1 generation. The presence of occlusive strands in the vagina and/or precocious incomplete vaginal opening affecting treated F1 pups and F2 pups from the treated F1 generation. A delay in the occurrence of testicular descent affecting F2 pups from the treated F1 generation. A marked reduction in pregnancy rate effecting the second mating the F0 generation and the treated F1 generation. An increase in pre-coital time for the second mating of the F0 generation and the treated F1 generation. A marginal extension of the duration of gestation affecting the F0 generation and the treated F1 generation. A marked increase in the incidence of extended parturition in total litter loss in the treated F1 generation and a significant increase in the percentage of males per litter. Effects on litter parameters, principally lower litter size and litter weight at birth, or 20 day sacrifice at both matings of the F0 and for the treated F1 generation and increased post implantation/pre-birth loss for the F0 and treated F1 generation. At terminal autopsy an increase in the incidence of depressions in the fore stomach epithelium affecting F0 and F1 males was observed. Among organ

weights of adults consistent findings included a significant reduction in seminal vesicle/prostate weight in F0 and F1 treated males and an increase in mean ovary weight among F0 and F1 treated females. Among organ weights of offspring at 6 weeks, F1 and F2 male pups showed a significant decrease in the weight of seminal vesicle/prostate, testes, and epididymides. F1 and F2 female pups showed reduction of adrenal weight. The laboratory examinations relating to the second mating of the F0 generation (teratology phase) revealed a significant reduction in anogenital distance among male fetuses and a marginal increase in the incidence of skeletal variance.

The only apparent effects of treatment with trenbolone acetate at 0.5 ppm were: Higher group mean body weights for males of the F0 generation. A slight but not statistically significant delay in the mean age of vaginal opening of F1 pups and F2 pups of the F1 treated generation; subsequent mating performance and resulting litter parameters were comparable with those of controls. Among organ weights of the offspring at 6 weeks treated F1 and F2 male pups from the treated parents showed a statistically significant decrease in seminal vesicles/prostate weight with the F2 males additionally showing a significant decrease of weight of epididymides. Trenbolone acetate exerted a marked effect at 18 ppm and some effect at 3 ppm when considered in terms of reproductive performance of the two generations of rats examined in this study. At the lowest dose level examined (0.5 ppm) there was a slight delay in the mean, age of vaginal opening and at 6 weeks of age effects were seen on the weight of the epididymides and/or seminal vesicles/prostate. Effects appeared more marked in the F2 pups than had been observed in the F1 pups of a comparable age. However, in terms of overall reproductive performance, trenbolone acetate exerted no effect at 0.5 ppm. The reproductive performance of all groups of F1 animals following withdrawal from treatment showed no marked difference from that of the control group. Because the hormonal no effect level could not be determined in the proceeding multiple generation rat study, a supplementary study on the effect of trenbolone acetate on pregnancy of the rat and development of the offspring was conducted by Dr. Penny James at Huntington Research Centre, Huntington, England. At the initiation of this study trenbolone acetate was fed to 270 male and female rats at levels of 0, 0.1, 0.3, 0.5, 3.0 and 18.0 ppm. The rats received the test diet at the above concentrations for 2 weeks prior to mating and throughout the mating period, gestation and lactation. The adult males were sacrificed as the majority of litters approached weaning. Male pups were sacrificed on day 22 post-partum. All animals were subjected to post mortem examination. The following organs of all male pups were weighed prior to preservation: testes, seminal vesicles with prostate, and epididymides. On day 24 post partum the female pups and the parent females were sacrificed. Treatment with trenbolone acetate

at 18 ppm was associated with: Slightly higher mean weekly body weight of F0 females but lower body weight gain during gestation and slightly lower weight gain of males over the last three weeks of treatment. 22/29 F0 females showed clitoral prominence at autopsy and all F1 female offspring were similarly affected from approximately 3 weeks of age. A statistically significant extension and duration of gestation. 4/29 F0 females showed total litter loss. Effects on litter parameters included reduced litter size, lower litter weight, marginally higher pup mortality and higher mean pup weight.

It is concluded that there is no deleterious effect of trenbolone acetate upon the weights of the testes, seminal vesicles with prostate or epididymides at the dosage level of 0.5 ppm. Therefore the hormonal no effect level for the rat is determined to be a dosage level of 0.5 ppm in the diet. The toxicological no effect level of trenbolone acetate as determined in these rat reproduction studies is not lower than that established as the hormonal no effect level in the Rhesus monkey.

Trenbolone acetate was studied in a preliminary oral toxicity study in Cynomolgus monkeys by Dr. Rodney Sortwell, Huntington Research Centre, Huntington, England. The object of the study was to obtain preliminary information relating to the toxicity of trenbolone acetate when administered by oral gavage to young adult Cynomolgus monkeys. The monkeys were dosed once daily for 8 weeks by oral gavage. Two pairs (1 male and 1 female in each pair) of monkeys received trenbolone acetate at levels of 0.375 mg/kg per day or 1.875 mg/kg per day. These two dosages for a 4 kg. monkey consuming 300 gms of dry diet per day would be equivalent to 5 or 25 ppm trenbolone acetate respectively. A third pair of animals acted as the controls. After a period of 8 weeks the monkeys were killed and subjected to macroscopic examination followed by microscopy of selected tissues. There were no mortalities during the course of the study and there were no treatment related effects on clinical signs, body weight or feed consumption. The males showed decreased prostate weights and increased seminal vesicles and testes weights.

A study to determine the hormonal no effect dose level for trenbolone acetate in the female Rhesus Macaque was conducted by Dr. David Hess at the Oregon Regional Primate Research Center, Beaverton, Oregon. The purpose of the study was to determine the hormonal no effect dosage of trenbolone acetate in a representative female primate. Trenbolone acetate was administered in the diet for 3 estrous cycles or a maximum of 122 days to 3 groups of 6 mature female monkeys at 60, 240 and 960 µg/day. Blood samples were obtained on a daily basis from all animals during a pretreatment menstrual cycle, at 3 day intervals during the first 2 treatment menstrual cycles and daily during the last treatment menstrual cycle or 30

days. Serum concentrations of estradiol, progesterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) were determined by radioimmunoassay. The largest dose resulted in maximum average serum levels of 2.3 ng/ml of 17b - trenbolone and this dose may have inhibited gonadotropin secretion and ovarian function in 3 of 16 reproductive cycles. We conclude that trenbolone had no effect at the mid and low dose although the inhibitory effects of orally administered trenbolone on the reproductive parameters studied in these females were marginal, at least in comparison with progestational compounds. A conservative hormonal no effect level was established at 40 microgram per kg per day (240 microgram per day via the diet)."

B.6.a.1 Wilson et al. (2002)

In vitro, TB binds the AR and is a potent androgen agonist. It also displays agonist activity at relatively low doses (sc or oral) in the Hershberger assay (see figure below) with the TB administered sc being about 80 fold more potent than with oral exposure.

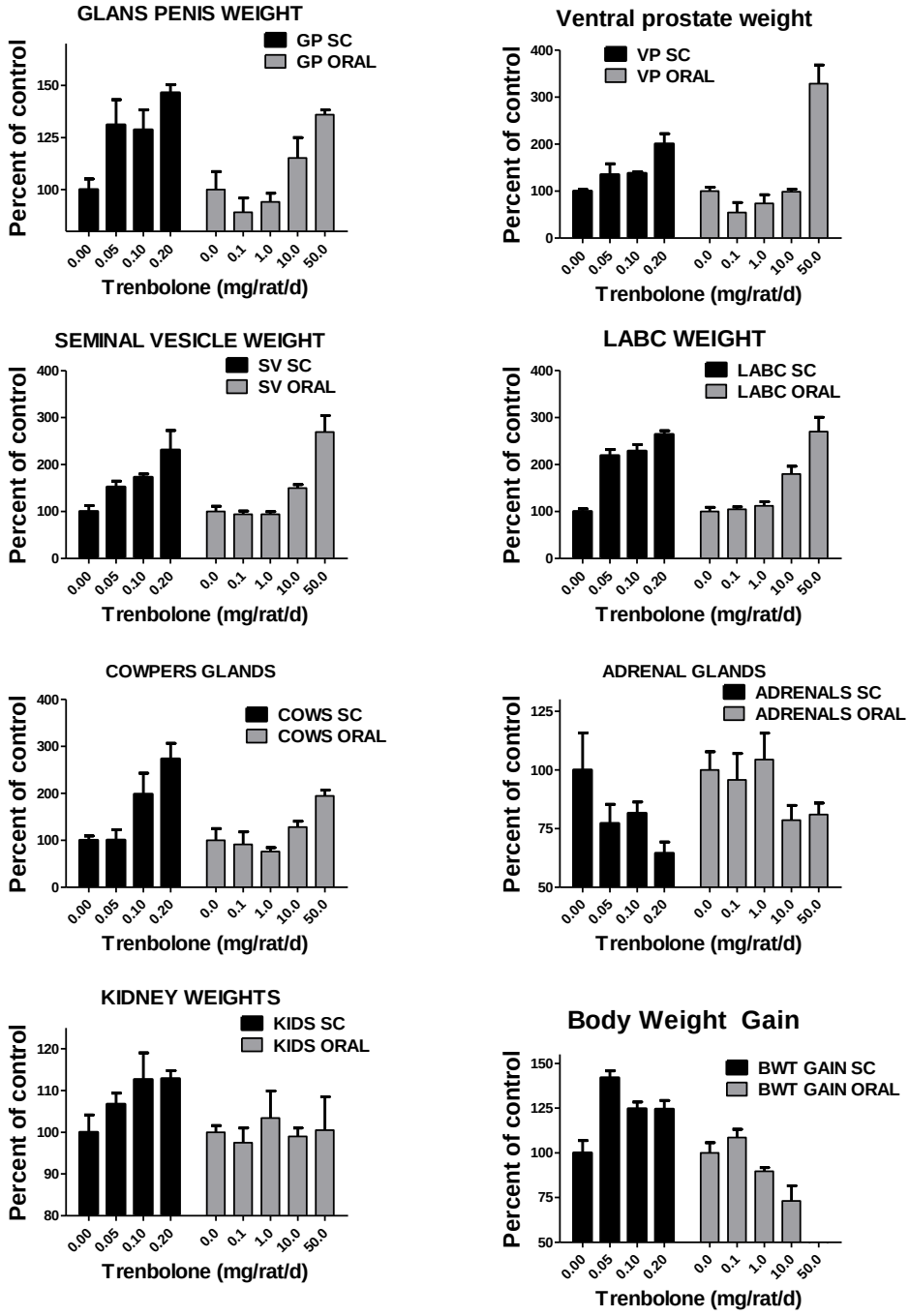

Figure B.29 reproduced from Wilson et al. (2002).

The androgenic effects on the reproductive tissues were all dose related, and none were NMDR.), Sc administration of TB, however, produced an NMDR effect at a high dose on body weight gain over the ten day dosing period in the Hershberger Assay (Fig. B.30 below).

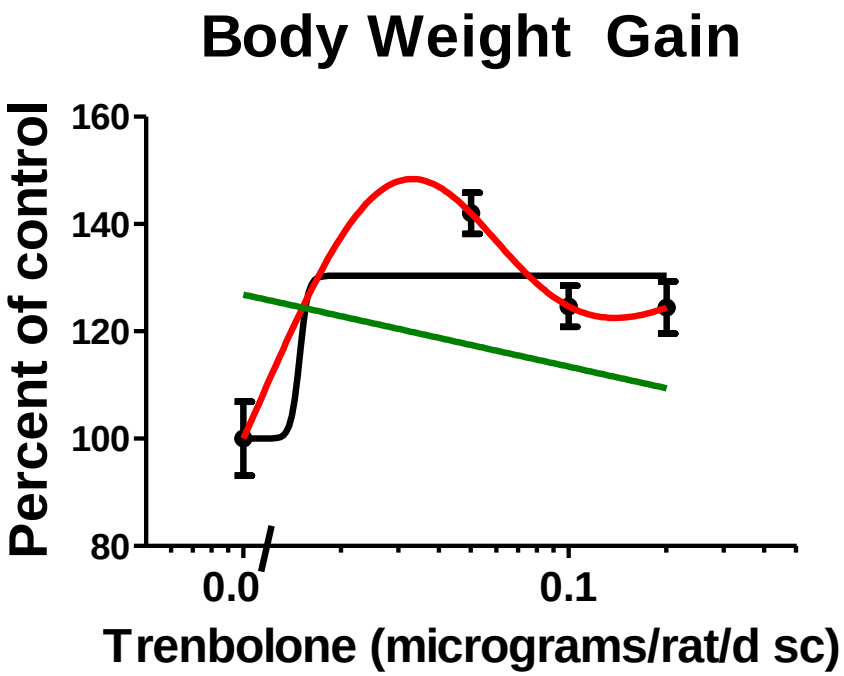

Figure B.30 from Wilson et al. (2002).

TB also was administered to pregnant rats (sc) from GD 14-19 at 0, 0.1, 0.5, 1 or 2 mg/kg/d and the F1 male offspring examined after birth. Wilson et al. (2002) found that TB increased F1 female rat AGD at birth and induced abnormal nipple/areolar development at 0.5, 1 and 2 mg/kg/d in a dose related manner (no NMDR). Pup weight at birth was reduced in the high dose group, but this effect was not seen at 13 days of age. In addition, male AGD and nipple development were not affected by TB at any dose.

The assessment of the subsequent postnatal development of the male and female offspring was reported by Hotchkiss et al. (2007).

In addition to the NMDR seen here, Ankley et al. (2003) noted NMDR for the effects of TB on female fathead minnow serum levels of estradiol, T and vitellogenin (see sections 3 and 4.1.3 of this NMDR state of the science document. Fecundity, however, was reduced in a monotonically dose-related manner at concentrations lower than those inducing these NMDRs.

B.6.a.2 Hotchkiss et al. (2007)

In the continuation of the above *in utero* study, F1 females puberty was delayed in the high dose group, and the increased AGD seen at birth persisted throughout the study in the high

dose group. Adverse effects were detected in F1 females all dose groups except 0.1 mg/kg/d (sc) with increased incidences of external genital malformations and the presence of male prostatic tissue noted in the 0.5 mg/day, 1.0 mg/day, or 2.0 mg/day groups. In addition, TB-treated females a displayed abnormal nipple development in the two highest dose groups with very few normal nipples in females in the 2.0 mg/kg/d dose group; this contributed to the high rate of neonatal F2 pup mortality in this dose group. Some F1 females in high dose group also displayed the following effects: vaginal agenesis; retained male seminal vesicle tissue; hydrometrocolpos; cleft phallus; and a permanent vaginal thread.

No reproductive effects displayed an NMDR in the Hotchkiss et al. (2007) study.

B.6.a.3 Owens et al. (2007); Owens et al. (2006)

TB was administered to immature castrate male rats by oral gavage for ten days using the OECD Hershberger Assay Protocol (3 labs, 6 rats/dose/lab). This assay is very sensitive to AR agonists. The weights of all five androgenic tissues was increased, as expected for this synthetic androgen; this effect was dose dependent at 8 and 40 mg/kg/d (glans penis and LABC muscles) or only at 40 mg/kg/d (ventral prostate, seminal vesicle, and Cowper's glands (Fig. B31 below). None of the effects displayed an NMDR.

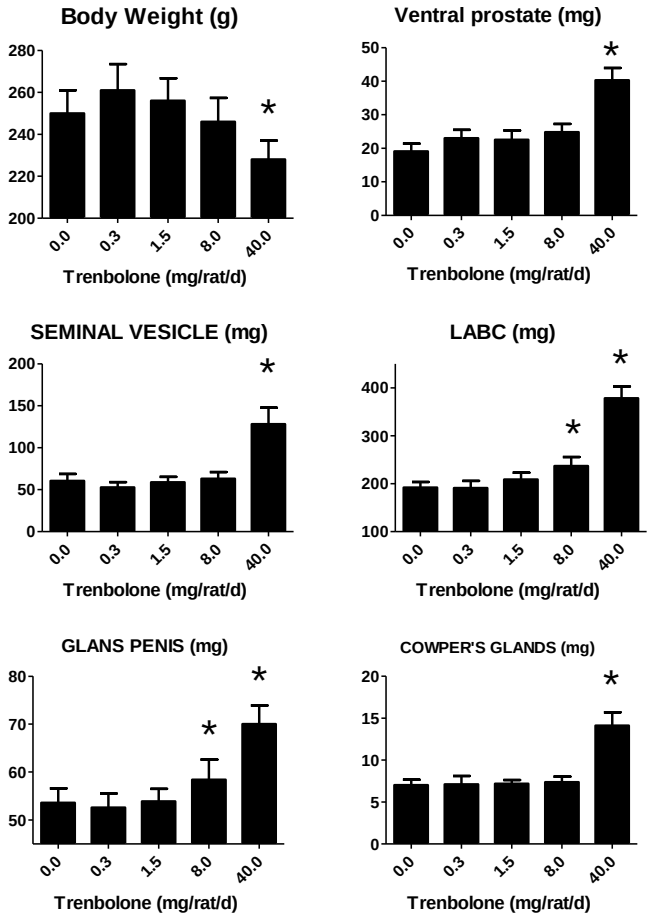

Figure B.31 from Owens et al. (2007).

B.6.b Testosterone

B.6.b.1 Robaire et al. (1979)

The Robaire et al. (1979) study is one of a series of papers by these authors and others demonstrating pronounced NMDR effects of sc or injected T on the testis. It has been repeatedly demonstrated in young adult male rats (Robaire et al., 1979; Ewing et al., 1977; Walsh and Swerdloff, 1973), rabbits (Ewing et al., 1973), and rhesus monkeys (Ewing et al., 1976) that increases doses of T cause a reduction in LH, followed by declines in testis androgen levels, sperm production and testis weight, without causing increases in serum T of androgen-dependent organ weights. However, as T dosage levels are increased above the nadir of the NMDR testis weight and sperm production levels are partially restored due to increasing levels of intratesticular T from the serum. Although LH levels decline within 2 days after the initiation of treatment, the effect on testis sperm production is not fully expressed for almost two months (Dykman, 1981). Thus the dose reponse relationships for the various responses are quite dynamic over this two month time period and do not stablize until then.

For example, Robaire et al. (1979)Robaire *et al*. (1979) administered implants containing T sc in young adult male rats (5-6/group); the T implants measured0, 1, 2.5, 4, 6 or 12 cm. T is released constantly over extended periods of time from these implants with the dose released increasing linearly with the surface area of the implant. Implants 2.5 cm long restored T in castrate animals to normal levels while shorter and longer implants released subphysiological and superphysiological levels of T, respectively. As seen below, these data demonstrate that the lowest dose of T (1 cm implant) reduces serum LH and sperm production followed by a reduction testis weight (2.5 cm) and increases in ventral prostate and seminal vesicle weights (4 cm). However, 6 and 12 cm implants cause a superphysiogical increase in serum T, along with increases testis weight and sperm production, albeit to less than normal levels.

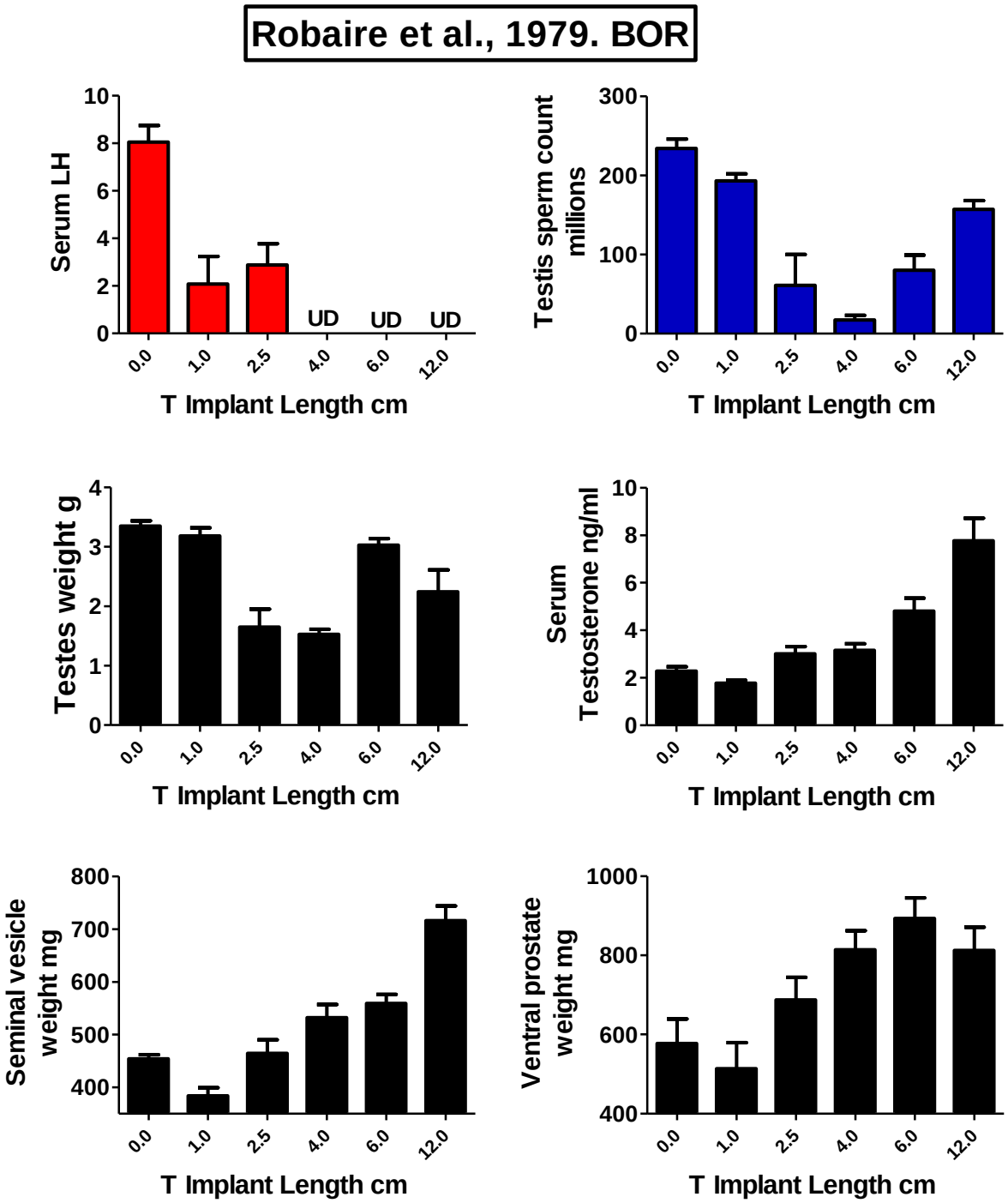

Figure B.32 adapted from Robaire et al. (1979).

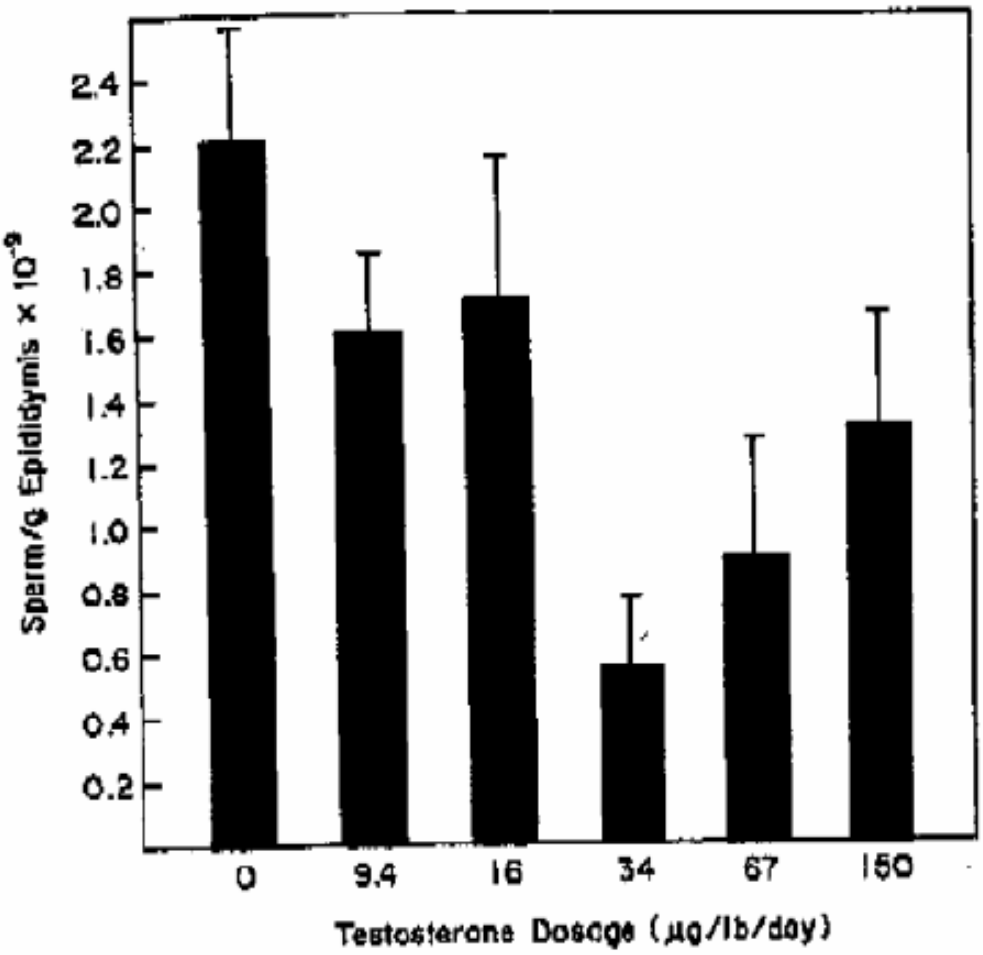

Figure 2

Concentration of spermatozoa in one cauda epididymis (sperm/g epididymis x10^{-9}) in adult male rhesus monkeys treated with increasing amounts of testosterone for 70 days. Each bar represents the mean ± standard error (vertical bar) of six monkeys.

Figure B.33 reproduced from Robaire et al. (1979).

B.6.b.2 Wolf et al. (2002)

Wolf et al. (2002) administered testosterone priopionate (TP) sc on GD 14-18 at doses of 0, 0.1, 0.5, 1, 2.5, 5 or 10 mg/rat/d. Although most effects, including the low dose effects displayed monotonic, dose-related changes, three effects displayed pronounced NMDRs ; these effects were uterine weight, a specific uterine malformation, and survial of female offspring after puberty.

Parturition was delayed at 2, 5 and 10 mg TP, litter size was reduced at 5 and 10 mg TP, and pup weight was reduced at 0.5 mg TP and higher doses. Androgenic effects were seen in F1 females at 0.5 mg TP inlcuding reduced numbers of permanent nipples, cleft phallus, and retained male-like prostatic tissue. At 1 mg TP and above, female AGD was increased, all nipples were eliminated, and females displayed male-like LABC muscles, Cowper's glands and seminal vesicles. The 1 mg dose of TP T levels 30 fold increased dams , but this dose increased female fetal levels by only 80%. Counterintuitively, the only reproductive effect seen in male offspring was reduced AGD.

The weights at weaning of F1 male and female offspring appeared to display NMDRs; however, when these data were fit to several different models (linear, sigmoidal, bell, and polynomial) none of the fits were statistically significantly better than the linear monotonic response (fig. B.34 below).

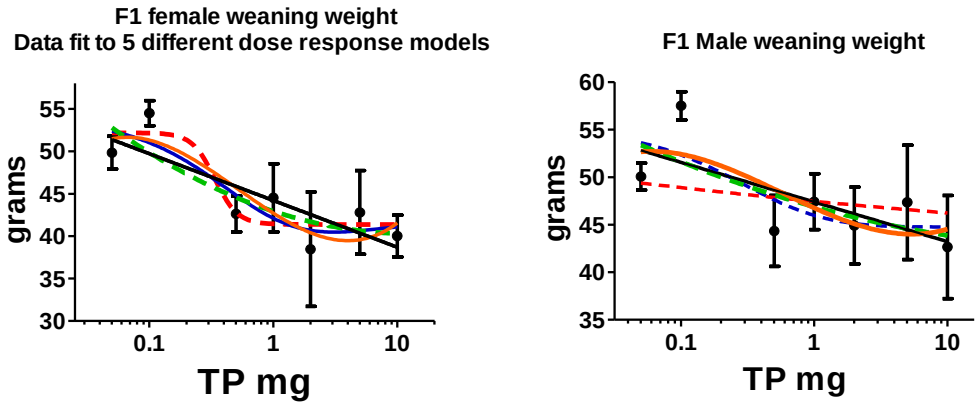

Figure B.34 from Wolf et al. (2002).

NMDRs were seen for the induction of uterine hydrometrocolpos and postweaning female mortality in the current study. Hydrometrocolpos with increased uterine weight was seen in 80%, 60% and 9% of the female offspring whose dams were treated with 1, 2 and 5 mg TP/d, respectively, but not at lower or higher dosage levels. This study replicates a similar observation by Greene *et al.* (1939) who provided a detailed anatomical description and explanation for the NMDR for hydrometrocolpos. Similar to that seen by Wolf et al. (2002), Greene *et al.* (1939) noted a sensitivity for retention of male tissues in androgen-treated

females (order of sensitivity: prostate> seminal vesicle and coagulating gland> vas deferens>epididymis (Table 6-9 Greene *et al.* (1939).

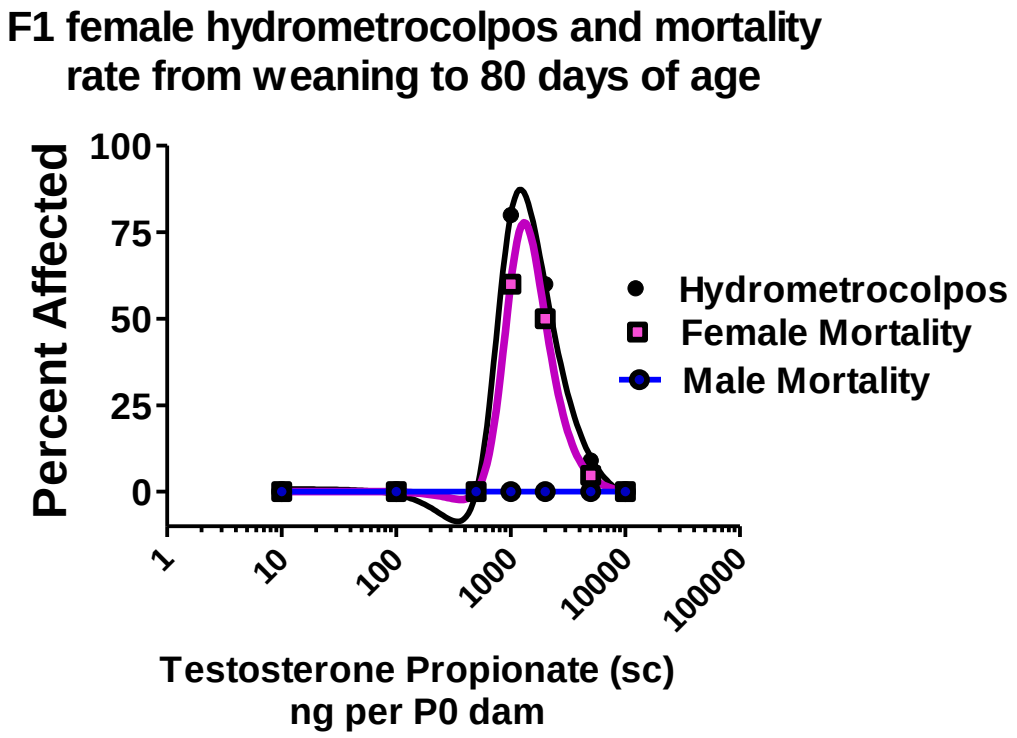

Figure B.35 fromWolf et al. (2002).

B.6.b.3 Hotchkiss et al. (2007)

Hotchkiss et al. (2007) – Reevaluated the lower dose effects of T on F1 female rat offspring reported by Wolf *et al*. (2002). These authors administered TP sc, as above, at 0, 1.5 and 2.5 mg/kg/d to pregnant rats on GDs 14-18 Their goals were These: to characterize the natural prenatal androgen environment of rats including the magnitude of the intrauterine position (IUP) effect; to characterize the permanent effects of prenatal androgen exposure on female rats; and to determine the ability of AGD and areolas to predict these permanent androgenic alterations in female rats.

IUP of male and female fetuses did not affect T concentrations or AGD in male or female rats at GD 22. TP reduced maternal weight gain, newborn pup weights, increased AGD in females (with no effect on male AGD) and decreased nipples in female offspring. High dose females displayed onset of puberty and irregular estrous cycles. Reproductive tract malformations were seen in both TP-treated groups but were more severe in high dose female offspring. Table 6 of Hotchkiss et al. (2007) presents a long list of alterations seen in androgen-treated female offspring, which could be included in a multigenerational study specifically tailored to detect the lose dose effects of environmental androgens.

B.6.b.4 Owens et al. (2007); Owens et al. (2006)

In the first phase of the OECD inter-laboratory validation study, TP was administered sc to castrate-immature-male rats at 0.1, 0.2, 0.4, 0.8, and 1.6 mg TP/kg/d (6 rats per dose group in each of the laboratories). After ten days of dosing, rats were euthanized, and the tissues weighed included five androgen-dependent tissues (ventral prostate, seminal vesicle, LABC, Cowper's glands and glans penis). These doses of TP provided serum T levels ranging from levels below those seen in early puberty to supraphysiological. The order of sensitivity of the androgen-dependent tissues to TP was glans penis> LABC, slightly> ventral prostate, slightly > Cowper's glands> seminal vesicle. None of the effects displayed an NMDR, whereas all responses appeared to be linear in the low dose.

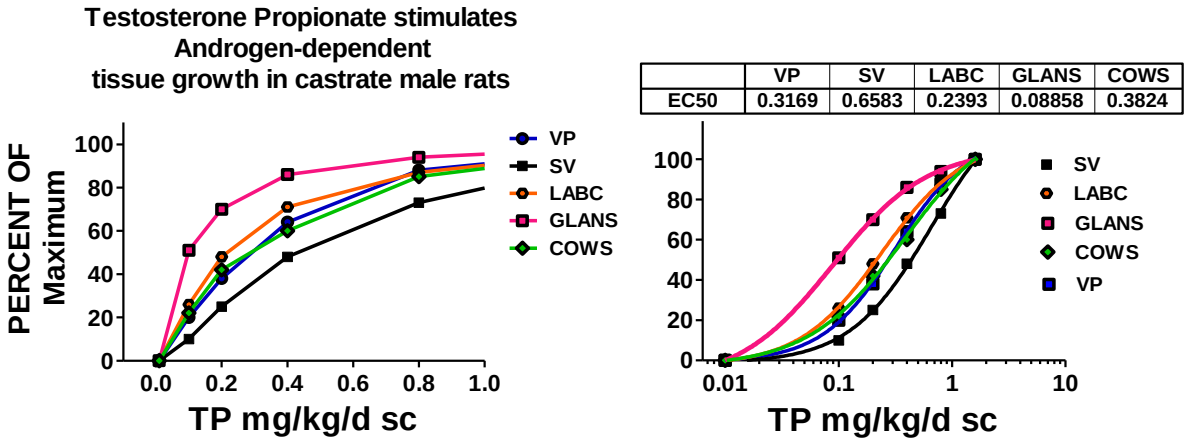

Figure B.36 Dose related effects of T on healthy young and old men

The next set of papers represents the effort of a group of scientists to examine the dose-related effects of T on a number of physiological processes. In these studies, healthy, eugonadal men, 18–35 years of age received monthly injections of a long-acting GnRH agonist, to suppress endogenous T secretion, as well as and weekly injections of 25, 50, 125, 300, or 600 mg of T enanthate for 20 wk. (Bhasin et al., 2005; Coviello et al., 2005; Storer et al., 2003; Singh et al., 2002; Sinha-Hikim et al., 2002; Bhasin et al., 2001). The serum levels of T attained with these injections ranged from sub-physiological (25 and 50 mg), to normal levels (125 mg) and supra-physiological levels (300 and 600 mg).

B.6.b.5 Bhasin et al. (2001)

Bhasin et al. (2001) examined the effects increasing doses of T on human body composition, muscle size, strength, power, sexual and cognitive functions, prostate-specific antigen, plasma

lipids, hemoglobin, and insulin-like growth factor I levels. The administration of the GnRH agonist plus T produced serum T concentrations of 253, 306, 542 (approximate level pretreatment), 1,345, and 2,370 ng/dl in the 25, 50, 125, 300, and 600 mg doses, respectively. The authors concluded that their "data demonstrate that different androgen dependent processes have different T dose-response relationships. Some aspects of sexual function and spatial cognition, and PSA levels, were maintained by relatively low doses of testosterone in GnRH agonist-treated men and did not increase further with administration of higher doses of testosterone. In contrast, graded doses of testosterone were associated with dose and testosterone concentration-dependent changes in fat-free mass, fat mass, muscle volume, leg press strength and power, hemoglobin, IGF-I, and plasma HDL cholesterol." No NMDR were noted.

B.6.b.6 Sinha-Hikim et al. (2002)

Testosterone increased quadriceps and vastus lateralis muscle volumes and cross sectional area of type I and II muscle fibers in dose related manner, increasing linearly with serum testosterone levels. No NMDRs were noted.

B.6.b.7 Singh et al. (2002)

Administration of testosterone reduced HDL-cholesterol, and apolipoprotein A-I, in the plasma in a dose-related manner but did not affect total cholesterol, LDL cholesterol, VLDL cholesterol, triglycerides, apolipoprotein B or C-III in the plasma. No NMDR were seen.

B.6.b.8 Storer et al. (2003)

Storer *et al*. (2003) found that testosterone administration produced dose-related increases in leg press strength and leg power but did not affect muscle fatigue. These effects did not display an NMDR.

B.6.b.9 Woodhouse et al. (2004)

Testosterone treatment was associated with a dose-related increase in body weight, as well asreductions in total body adipose tissue in several body regions. All effects were clearly dose related and did not display NMDRs.

B.6.b.10 Coviello et al. (2005)

Hemoglobin and hematocrit increased in linear, dose- and time- dependent manner in both young and older age groups, the increases being more robust in older men. No NMDR were noted.

B.6.b.11 Bhasin et al. (2005)

The participants received a long-acting GnRH agonist to suppress endogenous testosterone production and 25, 50, 125, 300, or 600 mg testosterone enanthate weekly for 20 wk .Prior to treatment serum total and free T levels were considerably lower in older (60 to 75 year old) men than in young men (mean 26 years of age). In older men, adverse reactions resulted in some participants (dose related-events) resulting in termination of their treatments. Free fat mass and leg press strength increased, and whole body fat mass decreased in older men with increasing doses of T. The 125 mg dose produced high-normal T levels in older men and induced significant improvements in fat remodeling and muscle strength without inducing the adverse effects (very high hemoglobin levels, leg edema and prostate events) seen in older men given higher doses of T. No NMDR were noted.

B.6.b.12 Gray et al. (2005b) –

In the Gray et al. (2005b) study, questionnaires were used to determine if sexual function in older men (libido, sexual activity, and erectile function) was affected by graded doses of T. All subjects received a long-acting GnRH agonist to suppress endogenous testosterone production; they randomly receive one of five doses (25, 50, 125, 300, and 600 mg) of testosterone enanthate weekly for 20 wk. Libido was increased by T dose, but only in men who reported being sexually active prior to treatment. In addition, free T levels during treatment were associated with increased sexual function, waking erections, spontaneous erections, and libido but not with the frequency of intercourse frequency or masturbation. The authors concluded that “Different aspects of male behavior respond differently to testosterone”, and these responses also differed from those seen in younger men. Such responses in older men were rather variable but some effects did display NMDRs including the overall sexual function score and waking erection frequency.

B.7 Selective Androgen Receptor Modulators (SARMS)

Over the last ten years SARMS have been developed for oral treatment of androgen-responsive tissues, which provide the beneficial effect with minimal side effects. None of the currently available SARMS are completely selective for the desirable anabolic effects on muscle and/or bone without producing undesirable androgenic side effects in sensitive tissues such as the prostate gland. SARMs have been developed that are orally active without causing liver damage; in contrast to testosterone, more potent and selective SARMs have been developed with enhanced tissue-selectivity. In elderly men with osteopenia or osteoporosis, it is desirable to have a SARM targeting bone and muscle tissue but with lesser or no activity on the prostate or testes. A SARM for women would ideally stimulate bone retention, or libido and other sexual function, without negative side effects such as masculinization, increased LDL/HDL ratios, or liver dysfunction. Several nonsteroidal androgens show favorable anabolic to androgenic ratios as compared to testosterone from 3:1 up to 10:1 (testosterone having a ratio of 1:1).

Since the typical target populations of SARMS are aging men and women, the literature contains few multigenerational test guideline-type studies. The animal models used by the pharmaceutical industry to evaluate the SARMS is often the adult castrate male or female rat. In addition, as is the case for SERMS, *in vitro* assays are useful to screen chemicals for tissue specific effects using cell lines derived from the different steroid-responsive tissues since some of the selective response of different tissues to SARMS arises from differences in tissue specific coactivators or corepressors. In addition, differential metabolism of the different steroidal androgens can also significantly alter the toxicity profile of the chemical (e.g. aromatizable or not or activated or inactivated by 5α reductase).

B.7.a Miner et al. (2007)

Miner et al. (2007) conducted an experiment in which young castrate male rats were dosed orally for two weeks with the SARM LGD2226 at 0, 1, 3, 10, 30 or 100 mg/kg/d. After the last dose, males were necropsied, and ventral prostate (considered to represent and androgenic response) and levator ani muscle (considered to represent and anabolic response) were weighed. The two androgen sensitive tissues both displayed monotonic dose-related increases with the muscles tissue being relatively more affected than the prostatic tissue.

B.7.b Chen et al. (2005)

This is a study of a novel SARM *S*)-3-(4-chloro-3-fluorophenoxy)-2-hydroxy-2-methyl-*N*-(4-nitro-3-trifluoromethylphenyl) propionamide (C-6). *In vitro*, C6 is about one tenth as potent an AR ligand as is DHT. C6 was administered at eight dosage levels to young castrate male rats by sc injection for 14 days at 0, 0.05, 0.1, 0.3, 0.5, 0.75, 1 and 3 mg/day. The rats were about 200 g at the start of the study. At necropsy ventral prostate, seminal vesicles and levator ani muscle were weighed. C6 induced monotonic dose related increases in the three tissues and inhibited serum LH and FSH in a dose-related manner. None of the endpoints displayed an NMDR.

B.7.c Basaria et al. (2013)

In this study, 76 healthy men (21 – 50 years) were exposed orally to daily doses 0, 0.1, 0.3, or 1.0 mg LGD-4033 for 21 days. LGD-4033 is novel nonsteroidal oral, selective androgen receptor modulator, blood counts, chemistries, lipids, prostate-specific antigen, electrocardiogram, hormones, lean and fat mass, and muscle strength were measured during and for 5 weeks after treatment was initiated. Hemoglobin, prostate-specific antigen, aspartate aminotransferase, alanine aminotransferase, or QT intervals were unaffected by this SARM. Whereas LGD-4033 administration caused dose-dependent suppressions of total T, sex hormone – binding globulin, high density lipoprotein cholesterol, and triglyceride levels; lean body mass increased in a dose dependent manner. In addition, FSH and free T were suppressed only in the high dose group (1.0-mg dose only). None of the endpoints in this study displayed an NMDR.

B.7.d Hanada et al. (2003)

In this study the SARM S-40503 (0, 1, 3, 10, 30 mg/kg/d sc) and DHT (0, 0.01, 0.1, 1, 10 mg/kg/d sc) were administered to young adult castrate male rats. After 4 weeks of treatments, ventral prostates and levator ani were weighed, and bone mineral density of the femurs was measured. Both androgens increased all of the above endpoints in a dose related manner, with DHT being about ten-fold more potent than S-40503. None of the responses were NMDRs.

B.7.e Furuya et al. (2012)

The SARM S-101479 displays agonist activity *in vitro* and is about ten-fold less potent than is DHT, the reference androgen. In the Furuya et al. (2012) study, S-101479 was administered orally once daily to ovariectomized female rats (12/group) at 0, 0.1, 0.3, 1, 3, and 10 mg/kg/d for 12 weeks, after which the females were necropsied, and bone mineral density (BMD), bone strength, and clitoral gland and uterine weights were measured. All effects were dose-relate, but none were NMDR. S-101479 increased bone mineral density and strength at 0.3 to 1 mg/kg/d and higher, whereas clitoral gland (10 mg/kg/d) and uterine weights (3 and 10 mg/kg/d) were affected at higher dosage levels.

B.7.f Allan et al. (2007)

The Allan et al. (2007) study includes three experiments that evaluated the effects of the SARM JNJ-28330835 on the prostate, levator ani and lean body mass in intact and castrated male rats. In the first experiment the SARM was administered orally to castrate male rats at 0, 1, 3, 10 and 30 mg/kg/d for two weeks, after which the ventral prostate and levator ani were weighed and serum FSH assayed. Both organs increased in weight and serum FSH decreased due to treatment in a monotonic fashion. In the second study, the SARM was administered orally for 6 weeks to intact male rats, after which the males were necropsied and the prostate and levator ani were weighed. In intact males, the SARM did not affect levator ani weight or serum FSH, whereas prostate weight was decreased in dose-related monotonic manner in the two higher dose groups. In the third experiment, 6 month old rats (castrated at 2 months of age) were

dose orally with the SARM for 2.5 months at 0, 2.5, 10 and 30 mg/kg (10 per group), and lean body mass was measured weekly by MRI. The SARM induced NMDRs on lean body mass and body weight in this experiment, increasing lean mass and body weight at 2.5 and 10 but not 30 mg/kg (see Figure below adapted from Fig 6 in Allan et al. (2007)).

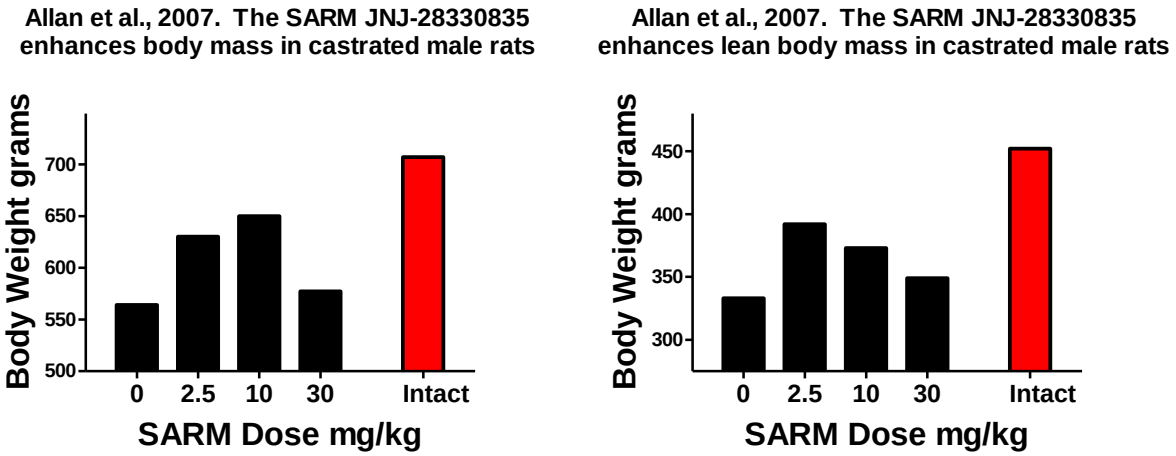

Figure B.37 adapted from Fig. 6 in Allan et al. (2007).

B.7.g Schmidt et al. (2009)

In this study, the SARM TFM-4AS-1 was administered to adult ovariectomized female rats (10-16/group) by sc injection for 24 days at 0, 1, 3, and 10 mg/kg. The authors measured bone formation rate by histological analysis of the periosteal surface of the distal femur as well as uterine weight. TFM-4AS-1 increased the periosteal surface, mineral apposition rate, and bone formation rate without affecting uterine weight. No NMDRs were detected.

B.8 Disruption of androgen-dependent tissues via the AhR

Chronic 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) in the rat produces adverse reproductive effects at remarkably low dosage levels (Bell et al., 2007c). Doses as low as 2.4 ng/kg/d cause delays in puberty in F1 male rat offspring, this being the low dose effect in these studies. None of the effects of TCDD in these studies (Bell et al., 2007a, b) were NMDRs. Bell et al. (2010) observed that fetal TCDD levels produced a linear delay in the onset of puberty in male rats. The acute and chronic studies each reported dose response data for approximately 50 endpoints including maternal, neonatal, and F1 male growth, organ weights, and andrology. The dose response studies by Gray et al. (1997a); Gray et al. (1997b) examined the effects of low doses of TCDD administered orally on gestational day 15 on these endpoints: maternal, neonatal and F1 male growth; epididymal histopathology; organ weights (30 endpoints); andrology; and F1 female, growth, ovarian histopathology and reproductive anatomy (20 endpoints). In summary, data from these three studies with developmental exposure to TCDD reveal that TCDD produces monotonic dose related alterations on affected endpoints, but NMDR were not detected for any of the 100 endpoints examined by these authors.

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