Appendix A:

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

Contents of the Appendix of studies describing the effects of chemicals that disrupt the Estrogen signaling pathways

***Numbers of studies reviewed (studies with 6 or more dose groups, total studies examined)**

A. ESTROGEN SIGNALING PATHWAY

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A.1 Estrogens

For several potent estrogens found in the environment due primarily to pharmaceutical usage or from animal/human effluent, there are robust, low- to high dose studies that included a number of estrogen-sensitive endpoints; among these were studies on ethinyl estradiol and estradiol. Studies on DES included a broad range of dosage levels, including low levels, but none of these were life-cycle multigenerational test guideline type studies. It should be noted that several multigenerational as well as mechanistic studies with estrogens were performed or are being executed by various groups specifically to examine the low dose issue and the shape of the dose response curve.

There also was a very robust data base for the phytoestrogen, genistein, including multigenerational studies and for the estrogenic fungal mycotoxin, zearalenone. However, the literature is predominantly from the 1970s and 1980s, when the reproductive problems in domestic animals were attributed to this chemical, and many of the studies including the critical study used in the 2011 European Food Safety Agency (EFSA) risk assessment were done with pigs.

Literature for several well-known estrogenic pesticides and toxic substances were reviewed including octylphenol, bisphenol A (BPA), nonylphenol (branched chain), methoxychlor (MET) and chlordecone (kepone).

There are several robust multigenerational studies on BPA. The BPA review here was not intended to revisit the issue of whether or not BPA produces low dose effects of concern, as this has been thoroughly reviewed by multiple independent regulatory and governmental agencies; rather the purpose was to examine the shape of the dose response curve over a broad range of dosage levels.

The literature reviewed on octylphenol included a multigenerational study with low- to high dosage levels and another study examining the effects of neonatal octylphenol administration of reproductive function.

Three robust multigenerational/transgenerational studies of nonylphenol and of MET were reviewed; however, none of the six studies used low doses (defined for assessment of E and A pathways as less than 1 mg/kg/d for a weakly estrogenic chemical).

One multigenerational study was found using rats exposed to the estrogenic and neurotoxic pesticide, Kepone. As this study was conducted in the late 1970s, it did not include low dosage levels, although the exposure levels were “environmentally relevant” for occupational exposures that occurred at the time. Neither did it include any of the endpoints considered most sensitive to estrogen treatment. A reproduction study in mice from 1965 also examined

the dose related effects of kepone at moderate to high dosage levels, and this study included measures of reproductive performance and estrous cyclicity in females.

A.1.a Ethinyl Estradiol (EE2)

There are several robust, well designed, comprehensive, low to high dose, multigenerational and transgenerational EE2 studies with rats. In addition, there are a number of shorter-term, mechanistic studies that examined the effects of EE2 over a broad dose range, which included upstream endpoints. The literature on the estrogen EE2 provides a rich data base to address questions about dose response, sensitivity of endpoints and critical life stages for induction of adverse effects of estrogenic chemicals.

A.1.a.1 NTP EE2 Report

Latendresse et al. (2009) – mammary gland abnormalities
Delclos et al. (2009)

The NTP executed a series of studies with EE2 and other well characterized estrogenic chemicals specifically to examine the shape of the dose response curve in the low dose and to identify endpoints sensitive to estrogens for potential inclusion in multigenerational studies of chemicals displaying estrogenicity in screening assays. These studies included the following measurements: fertility, fecundity, maternal and litter measures; repeated observations of growth and food consumption throughout the life cycle; AGD at birth; neonatal developmental landmarks; pubertal landmarks; estrous cyclicity organ weights (>15 organs); histopathology (> 18 tissues); ovarian oocyte counts and sperm counts.

Dose range finding one generation study – 7 dose levels

The first study was a dose range finding, one generation study with 7 dose levels. Exposure began on gestation day (GD) 7 and continued through gestation, lactation and directly to F1 in the diet until necropsy at 50 days of age. Doses were 0, 0.1, 1.5, 5, 25, 100 or 200 ppb, n>5 per group, and there were more than 50 individual endpoints per animal. These dietary exposure concentrations resulted in ingested doses of approximately 0.008, 0.08, 0.39, 1.77, 7.26, or 13.33 µg ethinyl estradiol/kg body weight per day to the dams. Dietary exposure of the dams continued through lactation, during which time ingested doses were approximately 0.03, 0.26, 1.37, 6.53, 29.68, or 51.93 µg/kg per day.

Pups from five litters were culled to eight per litter with an equal sex distribution on postnatal day (PND) 2; the animals were maintained on the same dosed feed as their mother after weaning until sacrifice at PND 50. Ingested doses were approximately 0.02, 0.22, 1.14, 5.48, 21.00, or 45.24 µg/kg per day for male pups and 0.02, 0.22, 1.18, 5.60, 22.92, or 45.87 µg/kg per day for female pups.

Maternal body weight gain was reduced during pregnancy by about 50% in the 200 ppb dose group and by 30% at 100 ppb, and mean pup weight at birth also was reduced in these two dose groups. The percentage of F1 males displaying preputial separation (PPS) at 50 days of age was reduced from 85% in controls to 20% in the 200 ppb dose group. However the age at PPS displayed an NMDR, being accelerated by about 1.6 days at 5 and 25 ppb.

In F1 males, terminal body, ventral prostate, testis sperm count and testes weights were reduced at 200 ppb, whereas the dorsolateral prostate weight was significantly increased only at 5 ppm (NMDR). Histopathological alterations were noted in reproductive and nonreproductive organs at 100 and 200 ppb (no NMDR) and mammary gland ductal hyperplasia was present at doses of 25 ppb and higher dose levels Latendresse et al. (2009).

In F1 females, vaginal opening (VO) was accelerated in the 25, 100, and 200 ppb groups, and body and ovarian weights were reduced at 200 ppb; ovarian, uterine and vaginal tissues displayed histological abnormalities at 200 ppb. No NMDRs were noted.

Five generation study – 4 dose levels

The data from the dose range finding study was used to select dosage levels for a 5 generation study. In this study (n>30 mated pairs per group were fed 0, 2, 10, 50 ppb in the diet (see protocol Figure below for details). Exposure concentrations of 0, 2, 10, or 50 ppb resulted in ingested doses of approximately 0, 0.1, 0.7, or 4 μg ethinyl estradiol/kg body weight per day. Higher dosage levels were not used because of the effects in the F1 females indicated that these levels would preclude successful mating (histological reproductive tract abnormalities and anestrus); EE2 exposure was terminated for all groups after weaning of the F3 generation.

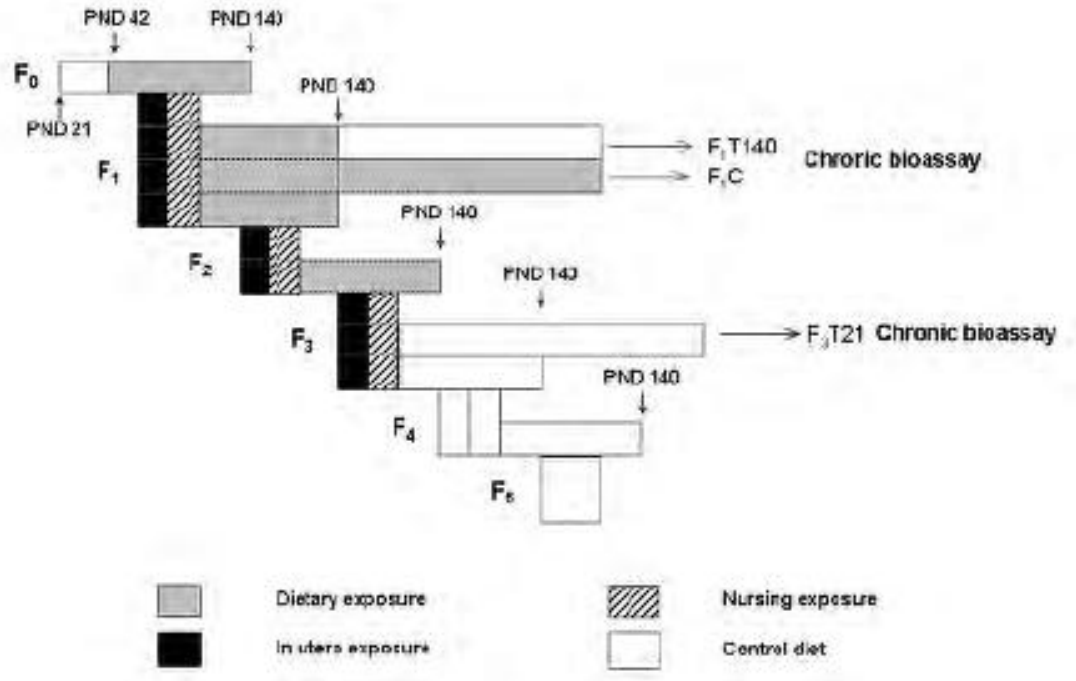


FIGURE 1
Dosing Schedule for the Multigenerational Reproductive Toxicology and Chronic Studies

Figure A.1 Reproduced from Latendresse et al. (2009).

In the multigenerational study, VO was accelerated at 50 ppb in the F1, F2 and F3 generations but not in the F4 generation (unexposed) (Delclos et al., 2009). Ethinyl estradiol administered at exposure concentrations of 2, 10, or 50 ppb in a low phytoestrogen diet to NCTR CD (Sprague-Dawley) rats showed clear biological activity including potentially adverse effects. Both preweaning and postweaning body weights of males and females were decreased during periods of direct exposure to dosed feed.

EE2 accelerated the attainment of puberty at 50 ppb in females under continuous exposure conditions (F_1 and F_2) and in the F3 where dosing was terminated at weaning. Perturbation of the estrous cycle (prolonged cycles, aberrant cycles, time in estrus) in young females after VO and prior to mating was observed in the F_1 and F_2 generations. In males, statistically significant inductions of male mammary gland hyperplasia (F_0 through F_3 generations) and mild mineralization of renal tubules (F_1 and F_2 generations) were observed. The majority of these effects in male and female rats were observed at 50 ppb, but statistically significant effects on body weight reduction and male mammary gland hyperplasia were observed at the lowest exposure concentration (2 ppb).

In summary, sporadic NMDRs that did not repeat in similarly exposed groups of rats from different generations were seen in the very large and comprehensive study of a model

estrogenic chemical. The lowest observed adverse effect level (LOAEL) for an adverse effect was induction of mammary gland hyperplasia in male rats, an endpoint not generally included in multigenerational or transgenerational studies for EDCs. No no observed adverse effect level (NOAEL) was detected for this effect.

As shown in the figure below, PPS displayed an NMDR in the dose range finding study; however, that dose range did not accelerate PPS in similarly exposed males from the F1 and F2 multigenerational study. In the multigenerational study, several endpoints (out of the hundreds measured over 4 generations) displayed NMDRs; that is, they were increased or decreased in a statistically significant fashion versus control and an intermediate dose but not at a higher dosage level. None of these effects were consistent across similarly exposed generations.

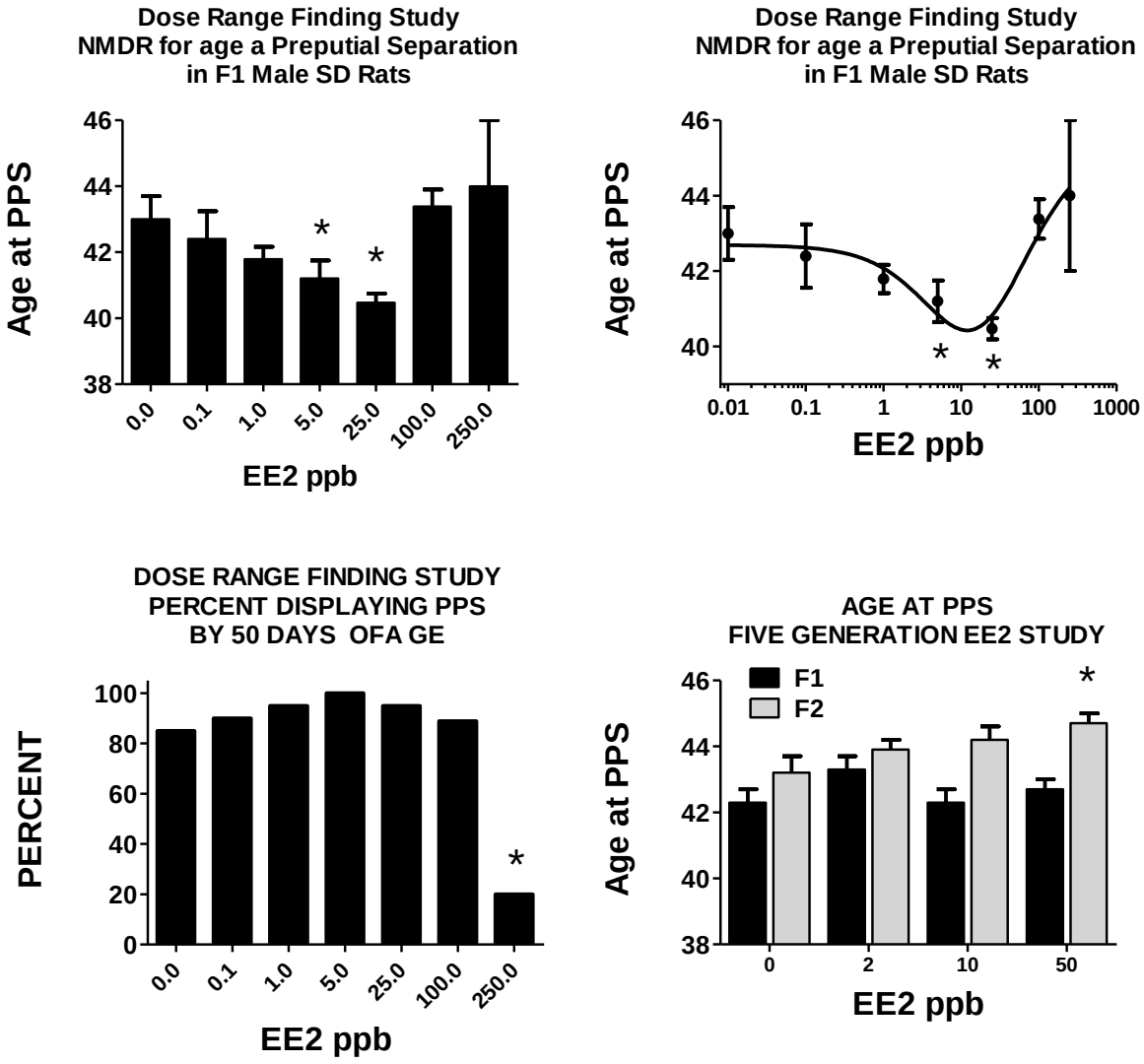


Figure A.2 Preputial Separation in Male F1 SD Rats

As shown in the figure below, although dorsolateral prostate weight displayed an NMDR in the dose range finding study, that dose range did not increase the weight of this tissue in similarly exposed males from the F1 and F2 multigenerational study. In addition, no histopathological lesions were detected in this tissue.

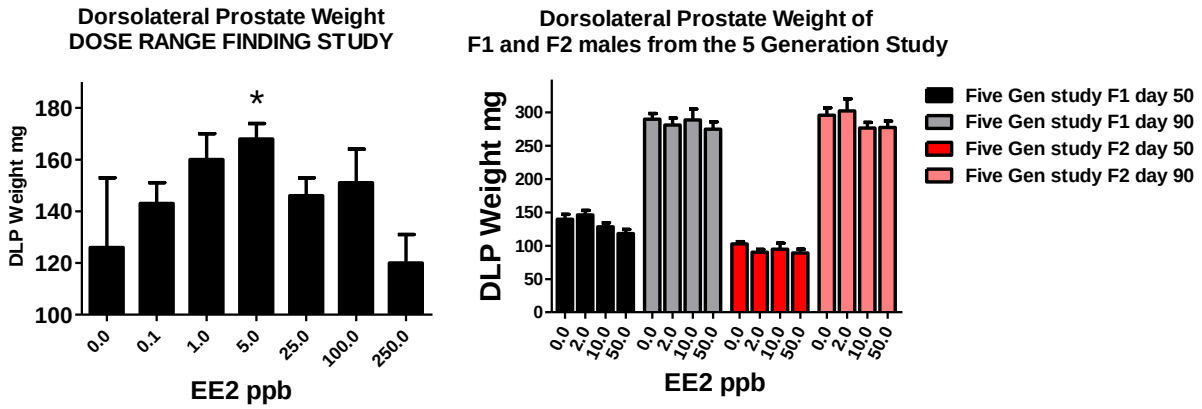


Figure A.3 Dorsolateral Weight

Another publication from this group (Ferguson et al., 2003) examined the effects of 0, 1, 5 or 200 ppb EE2 fed to rats from GD7 to necropsy, Growth and behavior (play, open-field activity, running wheel, and saccharin- and sodium flavored water intake) in male and female offspring were observed. Body weights and food consumption of F1 males and females were reduced in a monotonic manner, being statistically significant at 200 ppb; intake of sodium flavored solution was increased at 200 ppb in both sexes. No NMDRs were noted.

A.1.a.2 Sawaki et al. (2003)

EE2 was administered by oral gavage to female rats from GD 6 to PND 18 at 0, 0.5, 5 and 50 µg/kg/d (n=10/group), and the postnatal development of the F1 females was studied. One of the major differences between this study and the NTP EE2 study is that in this study EE2 was only administered to the dam with no direct exposure to the F1 generation after weaning, through puberty or in adulthood. Such studies are useful for identifying the permanent effects of early developmental exposure, as shown by Odum and Ashby (1999); however effects like pubertal development in the female, for example, may be less affected by exposure during early development than by direct exposure during pubertal life.

Endpoints measured in this study included these: maternal and neonatal litter observations; AGD (AGD) at 4 days of age; F1 growth through 25 weeks of age; fertility; estrous cyclicity in middle aged females; and morphology and morphometry of the external genitalia of the females (since it was malformed by EE2).

In this study, all dose related effects displayed normal monotonic dose responses. Body weight of F1 males and females was reduced at 50 µg/kg/d until 8 weeks of age, after which body weights did not differ among the groups. The only other statistically significant effects were on the female external genitalia and the delayed onset of abnormal estrous cycles at 6 months of age in high dose group F1 female rats. It is noteworthy that the effects seen in this study were all confirmed in a subsequent study by Ryan et al. (2010).

A.1.a.3 Ryan et al. (2010)

Ryan et al. (2010) studied the effects of gestational and lactational oral gavage exposure to EE2, on the postnatal development and reproductive function of the female offspring. EE2 was administered from GD 7 to PND 18 at 0, 0.05, 0.15, 0.5, 1.5, 5, 15 and 50µg/kg/d. Endpoints measured included AGD at 2 days of age, growth, VO, fertility and fecundity (over 4 month breeding period), sexually dimorphic behaviors (saccharin preference, locomotor activity, and female sexual behavior), and morphometry of the female external genitalia (>20 endpoints). The maternal and litter data and effects of EE2 on the male offspring were published by Howdeshell et al. (2008).

EE2 showed the following effects: increased female pup AGD (at 50 µg/kg/d and above); accelerated the age at VO (at 5 µg/kg/d); reduced t weight at VO (at 5 µg/kg/d); increased percentage of females with malformed external genitalia (at 5 µg/kg/d); reduced fecundity (at 5 µg/kg/d); reduced saccharin preference (to male-like levels at 5 µg/kg/d); reduced female sex behavior display (at 15 µg/kg/d); and reduced weaning weight (at 50 µg/kg/d). None of these data displayed an NMRDC.

A.1.a.4 Howdeshell et al. (2008)

Howdeshell et al. (2008) studied the effects of gestational and lactational oral gavage exposure to of EE2, on the postnatal development and reproductive function of the male offspring. EE2 was administered from GD 7 to PND 18 at 0, 0.05, 0.15, 0.5, 1.5, 5, 15 and 50µg/kg/d. The >30 endpoints measured included these: maternal and neonatal litter information; AGD at 2 days of age; growth; female-like nipple retention in infant F1 males; male hormone levels (estradiol, testosterone, LH, prolactin, T4, and corticosterone); histopathology of the reproductive organs (testes, epididymis, glans penis, lateral and ventral prostate, seminal vesicles, Cowper's glands, and levator ani bulbocavernosus muscle); organ weights; and sperm counts). EE2 treatment affected the following measures: reduced maternal body weight gain (at 1.5 µg/kg/d and above); the numbers of implantation sites (at 50 µg/kg/d); reduced numbers of live pups (at 15 µg/kg/d); reduced body weight at 2 days and at necropsy (at 50 µg/kg/d); increased fetal/neonatal mortality (at 50 µg/kg/d); decreased seminal vesicle and testis weight (at 5 µg/kg/d); decreased ventral prostate, levator ani bulbocavernosus muscle weight and glans penis weight (at 50 µg/kg/d); and reduced epididymal sperm counts (at50 µg/kg/d). All these effects displayed monotonic responses to EE2. The numbers of live pups at weaning was increased at 0.15 µg/kg/d and 1.5 µg/kg/d but not higher dosage levels, potentially indicative of

an NMDR. If indeed this NMDR were found to be reproducible, the prevalence in these two companion papers would be 1/>50 endpoints or less than 2%. Increased survival is not considered an adverse endpoint.

A.1.a.5 Thayer et al. (2001)

Thayer et al. (2001) fed pregnant mice EE2 in the diet at 0, 0.002, 0.02, 0.2, 2 or 200 µg/kg/d from GD 0 to 17 (n=10-12 dams/group). F1 males were necropsied at 50 days and 5 months of age; five reproductive tissues were weighed, testis sperm production was determined, and prostate androgen receptor (AR) levels were measured. A transient reduction was seen in sperm numbers in the testis, and a monotonic increase was seen in prostate weight. In addition, AR per prostate displayed an NMDR, and body weight at necropsy displayed an unusual dose response relationship (as seen in the figure below).

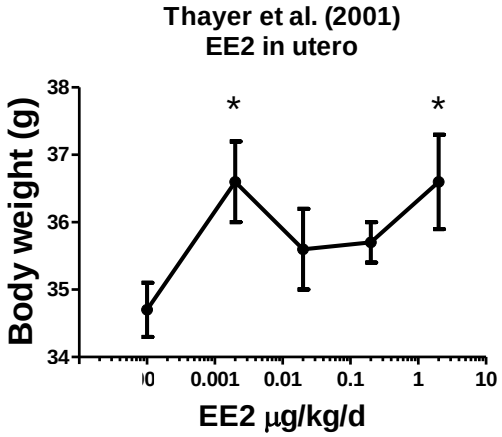


Figure A.4 Date from Thayer et al. (2001)

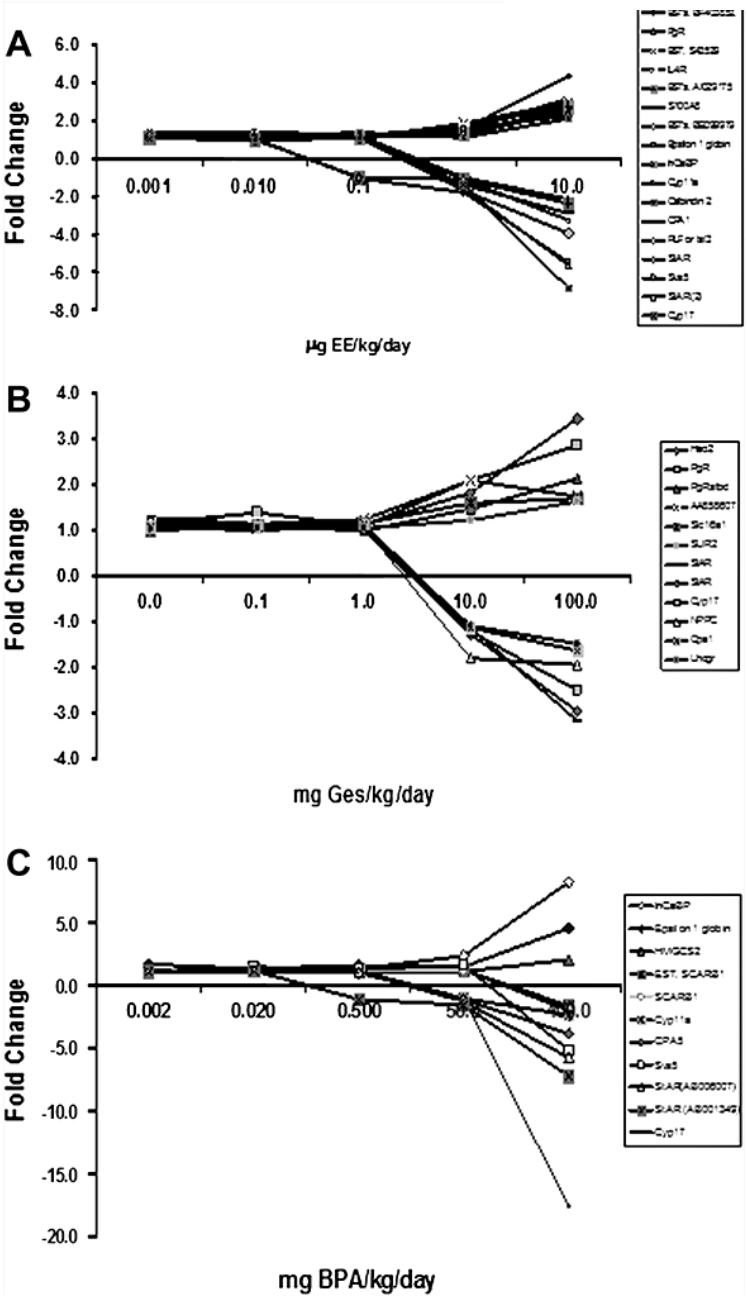
A.1.a.6 and A.1.a.7 Naciff et al. (2005); Naciff et al. (2002)

In addition to the above studies, which focused on downstream (or apical) adverse endpoints, three studies from one laboratory have examined the effects of EE2 (administered by sc injection) on global gene expression in developing male and reproductive rat tissues on GD 20. These studies were executed to determine if there were low dose NMDR alterations at this level of biological alteration, which in turn, might contribute to NMDR effects on downstream, adverse effects. In addition to EE2, the fetal studies also examined the low dose effects of genistein and BPA (discussed in section A.1.d and A.1.k respectively).

Two studies examined the effects of EE2 administered from GD11 to 20 on gene expression (microarray profiling of over 7000 genes and 1000 sequence tags followed by rtPRC). One study examined the effects of EE2 at 0, 0.5, 1, or 10 µg/kg/d on the developing uterus and ovaries of female rats (Naciff et al., 2002), and another examined the effects of EE2 at 0.001, 0.01, 0.1, 1, or 10 µg/kg/d on the developing testis/epididymis (Naciff et al., 2005). A third study (Daston

and Naciff, 2005)examined gene expression profiling in the immature female rat uterus and ovaries of Sprague Dawley (SD) rats at PND 24, 24 h after exposure to EE2, at 0.001, 0.01, 0.1, 1 and 10 µg/kg/d (sc), for four days (dosing from PND 20 to 23).

The following are extracted from the author's conclusions (Daston and Naciff, 2005). They evaluated the changes in gene expression in response to estrogens in the female reproductive tract of rats during embryo/fetal development and in the juvenile rat. "The results of these experiments indicate that a number of genes (dozens to hundreds) are changed in a reproducible, dose-related manner in response to estrogens. In regards to dose response relationships, these studies indicate that the dose–response for gene expression in fetal and juvenile rat reproductive tissues are monotonic suggesting that gene expression do not follow patterns that are unpredictable based on response at higher dosages."

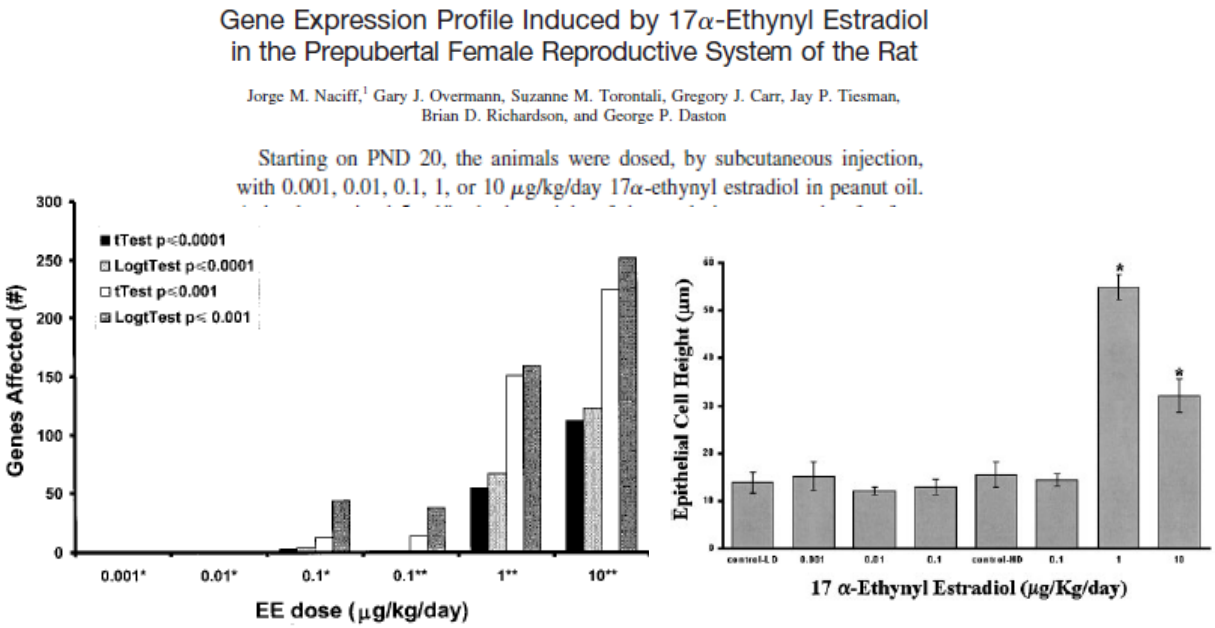


Example of the gene expression changes induced by transplacental exposure to (A) EE, (B) genistein, or (C) BPA, from GD 11 to GD 20.

Example of the gene expression changes induced by transplacental exposure to (A) EE, (B) genistein, or (C) BPA, from GD 11 to GD 20. The expression of the indicated genes changed in the fetal testis/epididymis in a statistically significant manner ($p \leq 0.001$) in each case. The fold change is the average fold change on the expression of each gene as compared to vehicle treated control (n = 5, in all dose groups,

for Ges and BPA and n = 6 for EE).

Figure A.5 Reproduced from (Naciff et al., 2005).



"The transcript profiles were compared between treatment groups and controls using oligonucleotide arrays to determine the expression level of approximately 7000 annotated rat genes and over 1740 expressed sequence tags ".

"Quantification of the number of genes whose expression was modified by the treatment, for each of the various doses of EE tested, showed clear evidence of a dose-dependent treatment effect that follows a *monotonic* response, concordant with the dose-response pattern of uterine wet-weight gain and luminal epithelial cell height.

The number of genes whose expression is affected by EE exposure increases according to dose."

TOXICOLOGICAL SCIENCES 72, 314–330 (2003)

Figure A.6 Reproduced from Naciff et al. (2002)

A.1.a.8 Kanno et al. (2001)

In the OECD interlaboratory validation of the rat uterotrophic assay, EE2 was administered to intact weanling female rats at 0, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0 and 10.0 µg/kg/d for three days, and uterine weight was measured in animals necropsied 24 hrs. after the last dose (16 laboratories, n=6 rats/dose/lab). Most laboratories detected an increase in uterine weight at 1 µg/kg/d and above, and all responses were monotonic in nature.

A.1.a.9 Andrews et al. (2002)

Administration of EE2 by gavage to adult female and male rats (following OECD Test Guideline no. 407) produced adverse effects at relatively high dosage levels as compared to dosage levels producing effects in developing rats. EE2 was administered at 0, 10, 50 or 200 µg/kg/d for 28 days, and the following were reported: clinical pathology; behavioral tests; estrous cyclicity; spermatology and organ weights (brain, heart, kidneys, liver, spleen, thymus, adrenals, testes,

epididymides, ovaries and uterus); organ weights after fixation (pituitary gland, thyroid, seminal vesicles, coagulation glands, and dorsolateral and ventral lobes of prostate. The study was conducted in two blocks (n=5/block/group), and many consistent monotonic alterations were seen at 200 µg/kg/d; these included reduced coagulating gland, seminal vesicle, dorsolateral and ventral prostate weights in males and increased liver weight in females. In females, thyroid stimulating hormone (TSH) levels displayed an NMDR in the first block, being significantly increased at 50 µg/kg/d, but not higher, but this effect was not seen in the second block.

A.1.b 17β Estradiol (E2)

E2 is a potent natural steroid produced primarily by gonadal tissues in most vertebrates. The normal physiological level of E2 varies greatly among different species, genders, life stages and reproductive status. Levels required for normal reproductive function at one stage of life can produce adverse effects at another stage of life. In adulthood, both higher and lower than normal E2 levels also can have adverse effects.

This hormone affects many, if not all, tissues in a tissue-specific manner via one of two nuclear receptors (ERα or ERβ), or cell membrane receptors. Tissue-specific responses arise from differences from differences in ERα or ERβ levels, levels of coactivators and corepressors, E2 metabolism, receptor stability, different estrogen response elements of E2 sensitive genes, gene silencing and other factors.

After a chemical binds to the ER, the hormone-receptor complex forms homodimers, and this complex recruits coactivators and corepressors that moderate the endocrine activity of the transcription complex. As the 3D shape of the homodimer complex varies from chemical to chemical, some toxicants or drugs that bind ER do not bind some or all of the cofactors necessary to activate all of the different E2-dependent genes.

Most xenoestrogens bind ER with affinities orders of magnitude lower than E2; thus very high levels, not low levels, of exposure are required to produce estrogenic effects *in vivo.* For this reason, the endocrine activity may be displayed only at doses equal to or above the induction of some other systemic toxicity. Taken together, these factors indicate that that fact that a chemical interacts with ER or induces some form of estrogenicity does not enable one to predict with certainty what other estrogenic responses will occur after exposure, the dose that will produce and effect, or the shape of the dose-response curve in each tissue. Tissues display tissue-specific responses, responding at different dosage levels and the dose response curve can be linear-no-threshold, threshold or NMDR for a single chemical.

A.1.b.1 Biegel et al. (1998b); Biegel et al. (1998a)

In the late 1990s the Dupont-Haskell laboratory conducted several long term studies using dietary E2 exposure as a model estrogen. This was done to examine the dose range over which

estrogenic effects are seen and to provide "benchmark data for a risk assessment for chemicals with estrogenic activity". The 90-day/one generation study (Biegel et al., 1998b) was conducted in male and female Crl:CD BR rats using dietary concentrations of 0, 0.05, 2.5, 10 and 50 ppm of E2. Endpoints included standard toxicological endpoints as well as several endpoints considered at the time to be mechanistic/biochemical effects. These dietary levels resulted in exposures of approximately 0, 3-5, 150, 600 and 3500 µg/kg/d. Viability, litter sizes, growth, food consumption, maternal parameters, pubertal landmarks, reproductive and non-reproductive organ weights and histopathology, clinical chemistry, hematology, and urinalysis were evaluated. There are more than 65 individual dose response curves in the current study, and the lowest dosage level was a lowest observed effect level (LOEL) for accelerated VO in F1 female rats. Several effects were noted at the next highest dosage level of E2 and above**.** No statistically significant NMDRs were noted in the current study with E2.

A companion paper (Biegel et al., 1998a) present the effects of the above dietary E2 exposures on P1 and F1 female rat serum hormone concentrations and estrous cycles. At the one week time point, the lowest dosage level that significantly accelerated VO in the F1 females did not elevate serum E2 levels or affect any other hormone level in P1 generation female rats; these included luteinizing hormone (LH), follicle stimulating hormone (FSH), progesterone (P4) and prolactin). The next highest E2 dosage level essentially doubled serum E2 levels and increased serum prolactin by about 50% but did not affect other serum hormone levels. Prolactin displayed an NMDR peaking at 10 ppm then declining nearly to control levels at 50 ppm, although the effect at 50 ppm was still statistically significantly higher than the control value. LH was reduced in the two highest dose groups, and P4 and FSH were unaffected by E2 in the diet.

At the 28 day time point, E2 was elevated at 2.5 ppm and above, and LH was reduced at 10 and 50 ppm; at this time point prolactin did not display an NMDR being increased at the highest dosage level and unaffected at lower exposure levels.

After 90 days of E2 exposure, E2 and LH in the serum were increased (at 2.5 ppm and above), P4 was reduced at 0.05 ppm (the lowest dose) and above, prolactin remained elevated in the highest dose group, and FSH was unaffected.

Estrous cycles were abnormal in all females monitored (10/group) in the P1 generation at 2.5 ppm and above. All indices of cyclicity displayed monotonic responses.

In summary, these two studies using E2 as a model estrogen detected effects at the lowest dosage level of 3-5 µg/kg/d and detected many adverse effects at higher dosage levels, all of which displayed monotonic dose responses. The only effect suggestive of an NMDR was found in the intermediate to high dose E2 groups with serum prolactin increasing and then declining (but still elevated versus control prolactin levels). However, this response was only observed at one of the three sampling times. While chronic exposure to E2 may induce NMDRs on some other endpoints that were not included in the current study, they are not common.

A.1.b.2 Tyl et al. (2008a)

In a one generation dose range finding study, the higher dose study of the two (Tyl et al., 2008a), adult CD-1® mice (10/sex/group) were administered E2 in the diet at nine dose levels (0, 0.005, 0.05, 0.5, 2.5, 5, 10, and 50 ppm; approximately 0, 1, 10, 100, 500, 1,000, 2,000 or 10,000 µg/kg/d) during a 2-week pre-breed period, mating, gestation and lactation. F1 weanlings (3/sex/litter) were necropsied; 2/sex/litter were retained, and exposed until VO or PPS at which time they were necropsied. The study examined the following: maternal and neonatal endpoints, pubertal development, body and organ weights (>70 dose response curves) in male and female P0, F1 (weaning and at puberty), growth, food consumption, F1 viability, and F1 male and female AGD.

E2 induced complete infertility at 2.5–50 ppm. At 0.5 ppm (and above), F0 adult female uterus plus cervix plus vagina weights were increased, gestational length was prolonged, and F1 stillbirths were increased along with reduced litter sizes. F1 males had decreased testes and epididymal weight, delayed PPS, undescended testes; however, prostate weights and AGD were unaffected. F1 females displayed earlier VO, enlarged vaginas, and fluid-filled uteri. At 0.05 ppm no reproductive effects were seen in the parent generation whereas subtle effects were seen in the F1 generation, and 0.005 ppm was a no observed effect level (NOEL). Of the 100 or so measures (with multiple contrasts for each versus control values) only one (seminal vesicle weight in the PO generation) displayed an apparent NMDR being increased versus control at 0.005 and decreased at 5 ppm and above.

A.1.b.3 Tyl et al. (2008c)

This two generation study followed the OECD Test Guideline 416 with enhancements. In this study, CD-1 mice (F0 generation, 25 mice/sex/group) were exposed to lower doses of E2 than in the previous study. E2 was administered in the diet at 0, 0.001, 0.005, 0.05, 0.15, or 0.5 ppm (~0, 0.2, 1, 10, 30, or 100 µg/kg/d); this was done for 8 weeks prebreeding, 2 weeks mating, ~3 weeks gestation, and 3 weeks lactation. At weaning, selected F1 offspring (F1 parents; 25/sex/group) and extra retained F1 males (one per litter) were exposed to the same dietary concentrations and durations as the F0 generation. Study termination occurred at F2 weaning; F1/F2 weanlings (up to three per sex per litter) were necropsied with organs weighed.

The NOEL was 0.005 ppm E2 (~1mg/kg/day). At 0.05 ppm and above, uterus, cervix and vaginal weights were increased in F1/F2 weanlings, and PPS was delayed in F1 males. In addition, at 0.15 ppm, testis and epididymal weights also were decreased in F1/F2 weanlings. Severe effects were seen on fertility and fecundity at 0.5 ppm. This dose increased F1/F2 perinatal loss, prolonged F0/F1 gestational length, and reduced F1/F2 litter sizes in addition to the previously cited effects seen at lower dosage levels.

There were no biologically significant or treatment-related effects at any dose on these measures; F0/F1 parental body weights, feed consumption, or clinical observations; or on F0/F1

estrous cyclicity; F0/F1 andrology; or F1/F2 AGD. The authors concluded that the CD-1 mouse model was sensitive to E2 by oral administration, with effects on reproductive development at doses of 10- 100 µg/kg/d. The NMDR observed for P0 seminal vesicle weight in the previous study at 0.005 ppm was not a statistically significant effect in the current study. In the current study, thyroid weight was the only endpoint among more than 60 endpoints to display an apparent NMDR being decreased by about 26% only at 0.005 ppm (in the F1 but not F0); however, this effect was not seen in the previous study.

In summary, 2 of 160 endpoints displayed apparent NMDRs in these two E2 studies with mice; however, since neither effect replicated it is likely that these are occurred by chance due to the large number of statistical contrasts between individual groups versus the control. When so many contrasts are made, the probability of detecting an effect at the $p<0.05$ levels that actually occurred by chance is far greater than one in twenty ($p=0.05$).

A.1.c Diethylstilbestrol (DES)

DES is a recognized developmental toxicant in humans. It was synthesized in 1938 for treatment of menopausal symptoms in women. Studies that followed shortly after this identified that *in utero* administration of DES produced uterine malformations in female rat offspring and long term treatment was carcinogenic. For this reason, the study authors recommended that it only be used in menopausal women. In 1941 DES was approved for use by the FDA for treatment of menopausal symptoms; however DES was used off-label by physicians in pregnant women globally with disastrous results. It was prescribed to women for preventing early pregnancy losses, other pregnancy problems and for "healthy babies" since 1940s; recommended dose was 5 mg daily (corresponding to approximately 80 µg/kg body weight in a 60-kg woman), increasing in 5-mg increments every 2 weeks throughout the pregnancy, until doses of 150 mg daily. In 1971, DES was banned due to its association with a rare vaginal carcinoma (clear-cell adenocarcinoma) in daughters exposed *in utero* during the first 18 weeks of pregnancy; subsequently, several defects in the morphogenesis and histogenesis of female as well as of male reproductive tract have been detected consequent to DES exposure. DES represented also a significant issue for food safety since it was one of the first anabolic hormone-like compounds used in intensive animal production until it was banned for this use in the US in 1979. DES is still considered an environmental estrogen of concern as it is currently used in aquaculture in some countries to induce sex reversals and control growth in some fish species.

A.1.c.1 vom Saal et al. (1997)

vom Saal et al. (1997)examined the effects of feeding DES to pregnant mice on GD 11 to 17 at 0.002, 0.02,0.2, 2, 20 and 200 µg/kg/d on F1 male prostate weight (no other endpoints were reported). They reported increasing prostate weights versus control at .02 to 2 µg/kg/d with a significant decline at 200 µg/kg/d. However, Ashby et al. (1999) and Cagen et al. (1999a) did not replicate the increase in prostate weight reported by vom Saal et al. (1997) at 0.2 µg/kg/d.

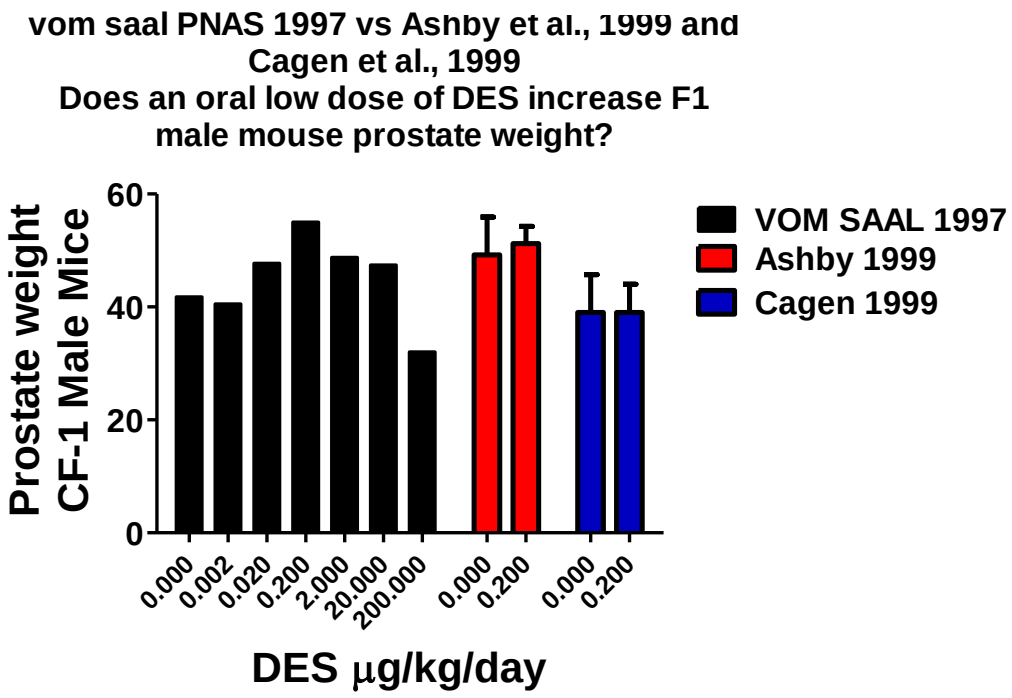


Figure A.7 Prostate weight data from vom Saal et al. (1997), Ashby et al. (1999) and Cagen et al. (1999a)

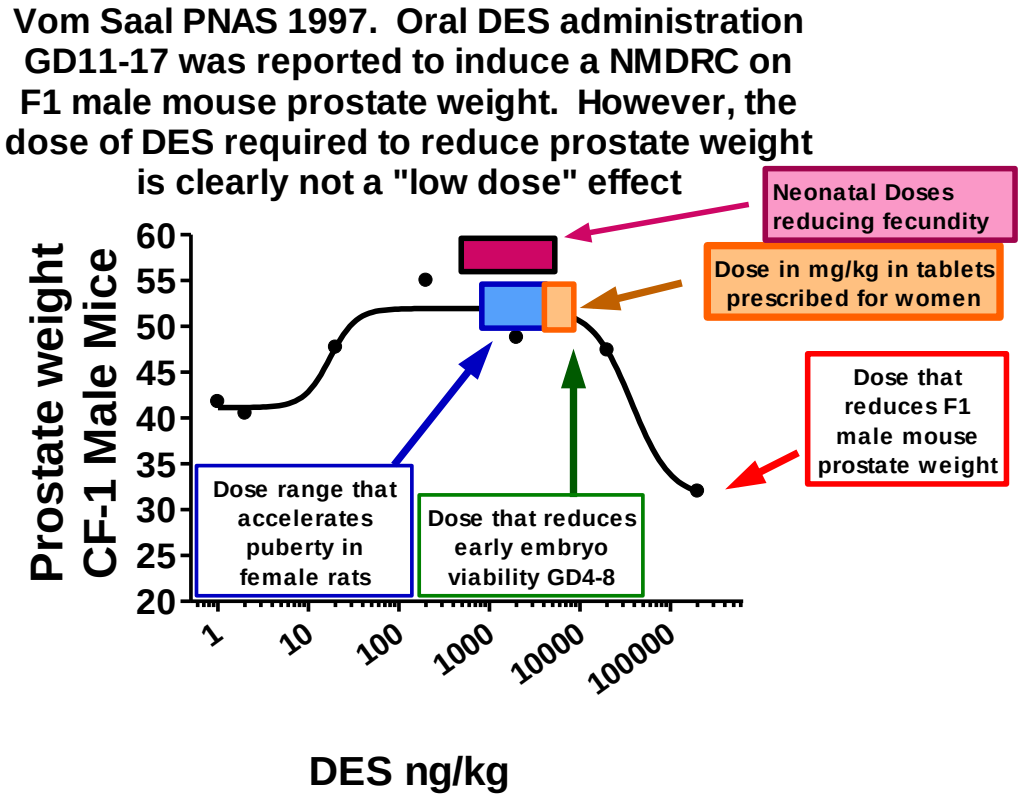


Figure A.8 Prostate Weight Data from vom Saal *et al.* (1997)

The significant reduction in prostate weight induced by gestational exposure to a very high dose of DES (at 200 µg/kg/d) is an effect that would not be expected in a long-term multigenerational study in rats or mice because they rodents display numerous adverse effects at doses 100 fold lower than this high dosage level. As documented in the discussion that follows, effects occurring at dosage levels below 200 µg/kg/d included infertility, reduced F1 body weight, altered pubertal development and histopathological lesions of reproductive and endocrine tissues. For example, DES reduces fertility when administered at 10 µg/kg/d from GD 4 to 8 during implantation and placental development (Nagao and Yoshimura, 2009).

A.1.c.2 Nagao et al. (2012); Nagao and Yoshimura (2009)

Nagao and Yoshimura (2009) found that oral DES treatment reduces fertility in mice when administered at 10 or 100 µg/kg/d from GD 4 to 8 during implantation and placental development. In a second study, Nagao *et al*. (2012) examined placental function and gene expression after oral treatment with DES (1, 5, 10, 15 µg/kg/d) on GD 4 to 8. Embryonic mortality was increased from 2.8% in controls to 68.5% and 88.2% after exposure to 10 and 15 µg DES/kg/d, respectively. Placental weight was reduced at 15 µg DES /kg/d. All effects displayed monotonic dose responses except for expression for ERα mRNA in the placentas of male (but not female) embryos. ERα mRNA in male embryos was increased only in the group exposed to 5 µg DES/kg/d (NMDR in male). ERβ, estrogen related receptor (ERR)β and ERRλ mRNA were not significantly affected at any dosage level.

A.1.c.3 Maranghi et al. (2008)

Maranghi et al. (2008) administered DES daily by oral gavage on GD 9-16 at a dose of 10 µg/kg/d and studied the development of F1 female mice. This treatment did not affect maternal weight gain or food consumption during pregnancy, but the body weight of the F1 female pups was significantly reduced, the age at VO was accelerated and ovarian histopathology was adversely affected in a majority of the females at PND 60. Since the study only used one dosage level of DES no conclusion can be made about the shape of the dose response.

A.1.c.4 Ohta et al. (2012)

DES was administered by oral gavage directly to neonatal rats at doses of 0.05, 0.5 and 5 µg/kg/d for 5 days after birth (Ohta et al., 2012). VO was accelerated at 5 µg/kg/d, and females displayed cleft phallus at this time. Abnormal estrous cycles were observed throughout the study in all females from the 5 µg/kg/d group and in 40% from the 0.5 µg/kg/d group from 24 weeks of age. PPS in males was unaffected.

The conception rate of 12-week-old females in the 5 µg/kg/d group was 0%, and that of the 23-week-old females in the 0.5 µg/kg/d group was 33.3%, but male fertility was not affected. No effect of DES was observed at the first parturition in any group, except for the 5 µg/kg/d group. However, litter size was significantly reduced in the 0.5 µg/kg/d group at the second parturition. In addition, dose-related changes were seen in several organs at 54 weeks; pituitary and adrenal weights were increased at 54 weeks and ovary weight was reduced at 0.5 and 5 µg/kg/d. However, there were no effects on male organ weights or sperm counts at 26 and 52 weeks of age. Altered antibody response to sheep red blood cells (SRBC) at all dose groups at 26 but not 52 weeks of age. No effect on shuttle-box avoidance test of males and females.

By contrast to report that neonatal DES is an obesogen when given 1 µg/kg/d sc to mice during neonatal life (Newbold et al., 2005)**,** there were no consistent effects on either male or female body weights in this study that administered DES during neonatal life by oral gavage to neonatal rat pups (measured approximately 30 times during the course of the study).

When terminated at about 100 weeks of age, neonatally treated females displayed dose-related increases in acinar dilation and galactoceles in the mammary glands (0/20, 2/20, 8/20 and 5/20 females of the control, 0.05, 0.5 and 5 µg/kg/d groups, respectively). By contrast, there were no statistically significant differences in the incidence of neoplastic lesions in the >30 tissues examined in male or female rats between the control and DES-treated groups. The median of the survival days of the females in the 5 µg/kg/d group was shortened by 17 weeks compared with that of the control group.

The authors concluded that these results suggest that a prolonged observation period, at least to 23–24 weeks of age, is necessary to determine the LOEL of EDCs reliably (referring to the OECD One-Gen protocol TG 443) because some adverse low dose effects were only detected in middle-aged female rats. In summary, these results demonstrate that neonatally exposed female rats are more sensitive to DES than are male rats, and many effects were more obvious, or only evident as the animals aged. Although a few subtle effects were detected at 0.05 µg/kg/d, the reproductive system of the female rat was more severely affected in a dose-related manner at 0.5 and 5 µg DES/kg/d.

Pubertal female rat exposure to DES

A.1.c.5 Odum et al. (2002)

Odum et al. (2002) studied the effects of several low doses DES administered in the drinking water at 10, 30 and 60 µg/L (equivalent to 1.3, 3.5 and 6.2 µg/kg/d in females and 1.2, 3.4 and 6.4 µg/kg in male rats) on pubertal development of immature male and female rats exposures. They also conducted an uterotrophic assay with DES in the drinking water with intact weanling female rats. DES delayed PPS in male rats (LOEL of 3.4 µg/kg/d) and accelerated the age at VO in female rats (LOEL of 1.3 µg/kg/d, with no NOEL). DES in the drinking water stimulated uterine weight with a LOEL of 1.6 µg/kg/d. The daily DES dosage levels producing adverse effects when administered during pubertal development did not induce NMDRs with effects occurring at levels below those reported by vom Saal et al. (1997) as inducing an NMDR response in the prostate weight of male offspring after *in utero* exposure in mice.

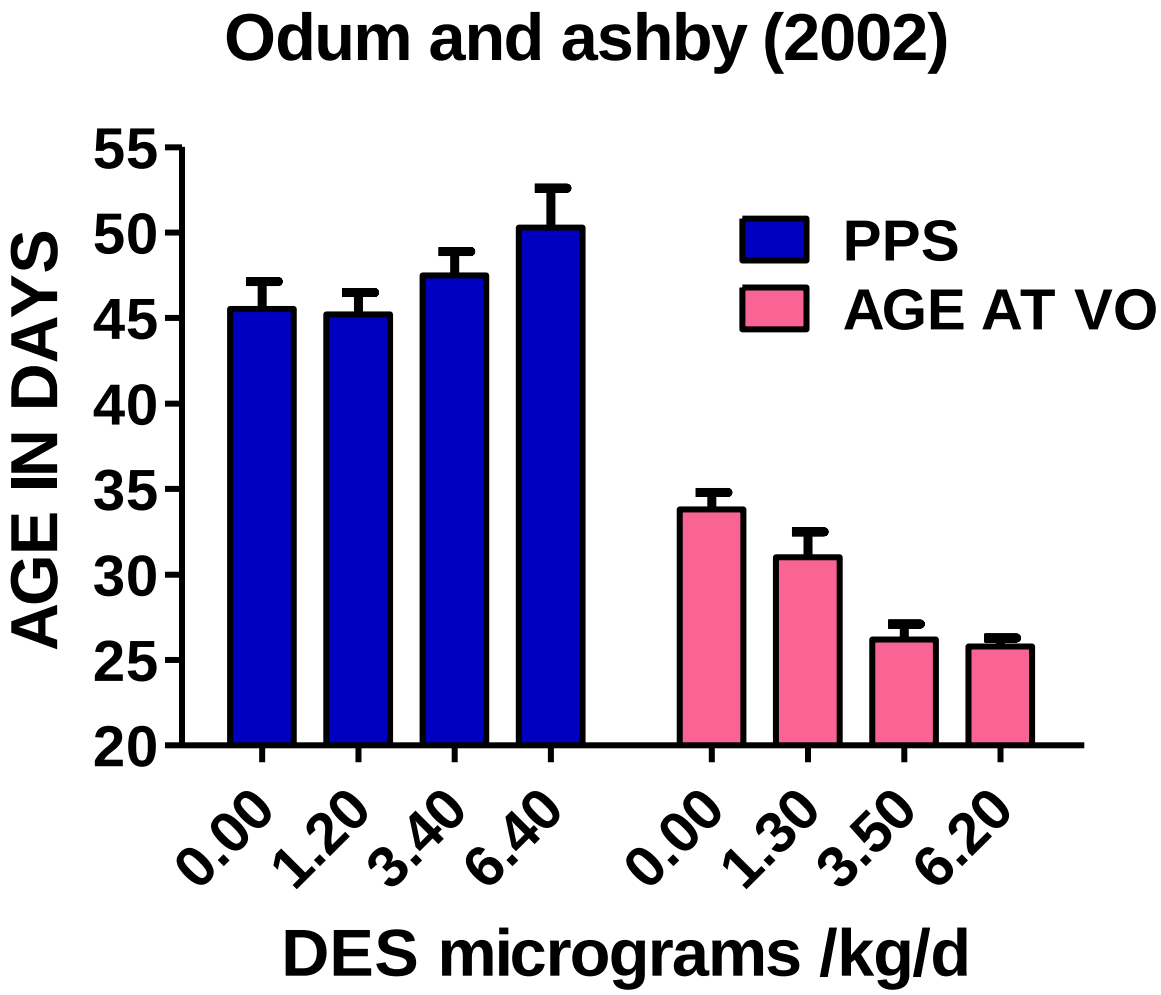


Figure A.9 Data from Odum *et al.* (2002)

A.1.c.6 Kim et al. (2002c) –Pubertal female rat assay

Kim et al. (2002c) exposed 21 to 41 day old female rats to low doses of DES by oral gavage at 0.2, 1 and 5 µg/kg/d and observed increased thyroid gland weight (5 µg/kg/d) and increased serum T3 (1 µg/kg/d, the LOEL, no NOEL) reduced body weight at VO and ovary weights (5 µg/kg/d), persistent vaginal estrous from 25 to 36 days of age and accelerated age at VO (5 µg/kg/d). NMDR were reported for liver, and kidney weights were reported being decreased only at 1 µg/kg/d.

A.1.c.7 Shin et al. (2009) - Pubertal male rat assay

Shin et al. (2009) studied the effects oral gavage administration of DES on pubertal development in male rats, exposed from PND 33 to 53 to 10, 20 or 30 µg/kg/d (10 per group). They were more than 20 endpoints: growth; hormone levels (testosterone [T], LH, thyroxine [T4]); age at PPS; reproductive and non-reproductive organ weights; and testicular and thyroid histopathology (>20 endpoints). Statistically significant effects were seen at the lowest dose on T, LH, and some reproductive tissue weights. Liver weight was the only endpoint showing an NMDR increasing at 10 and 20 but declining at 40 µg/kg/d concurrent with a 20% reduction in terminal body weight. This effect on body weight effect exceeds that acceptable in the US EPA Endocrine Disruptor Screening Program (EDSP) Pubertal Male Assay.

A.1.d Genistein

Genistein is one of several phytoestrogens found at high levels in many animal and human diets; concern has been expressed about the human health effects of phytoestrogens. Clover-disease is one of the few examples of a causal relationship between xenoestrogens and adverse mammalian health effects. Clover disease in sheep is caused by exposure to high levels of the isoflavone formononetin in some older subterranean clover varieties, especially red clover. The ingestion of clover pasture, containing high levels of this chemical, causes infertility in sheep (Bennetts et al., 1946). The main estrogenic compound in red- and subterranean clover is the isoflavone formononetin (7- hydroxy-4'-methoxyisoflavone), which is indirectly responsible for this reproductive dysfunction (Millington et al., 1964). Formononetin is metabolized by the rumen microorganisms in sheep and cattle, mainly to daidzein (7-hydroxyisoflavone) and further to the equol (7,4'-dihydroxyisoflavandiol), which is the major metabolite in serum; it is equol which causes the effect on estrus (Shutt and Braden, 1968). About 30% of human adults can be characterized as equol producers. White clover and other plants grown under stressedconditions can produce high levels of the phytoestrogen, coumestro, which also may impact ewe fertility, but it is generally reversible and less severe than the effects of formononetin. Genistein and other phytoestrogens are also present in most clovers and many other plants, but these phytoestrogens have much less impact on the fertility of sheep, and uncertain effects (beneficial versus adverse) on human health.

A.1.d.1 Genistein Studies with Rats: NTP (2008), NTP (2007) Dose Range finding study: Delclos et al. (2001)

The study protocols for this chemical follow the dose-range finding study described above under section 5.a.1 for the NTP EE2 report. The dose range finding study administered genistein in the diet (10 per group) at 5, 25, 100, 250, 625, or 1,250 ppm; this provided exposures of approximately 0.5, 2.5, 10, 30, 75 and 165 mg/kg/d, doses which span human exposure levels. F1 offspring (paraphrased from the report) in the 1,250 ppm groups had statistically significantly decreased body weights relative to controls at sacrifice (males, 9% decrease; females, 12% decrease), and statistically significantly decreased ventral prostate gland weight (28%) was observed in 1,250 ppm males.

Histopathologic examination of female pups revealed increased incidences of mammary gland ductal/alveolar hyperplasia at 250 ppm and greater, abnormal cellular maturation in the vagina (625 and 1250 ppm), and abnormal ovarian antral follicles (at 1,250 ppm). Control females had a high incidence of renal tubule mineralization, and the severities of this lesion were statistically significantly increased in groups exposed to 250 ppm or greater. In F1 males, there were increased incidences of mammary gland ductal/alveolar hyperplasia and hypertrophy (at 25 ppm and greater) and hyperplasia (at 250 ppm and above). Males also displayed increased incidences of aberrant or delayed spermatogenesis in the seminiferous tubules and a deficit of sperm in the epididymis at 625 and 1,250 ppm relative to controls. Males showed no renal

tubule mineralization below 250 ppm, but incidences and severities increased with exposures of 250 ppm and greater.

Of the >60 individual dose response curves one showed an apparent NMDR for the effects of genistein on the righting reflex in F1 female (but not male) pups. The NMDR effects of genistein on F1 male AGD, body weights at weaning and testis weights, reported by Akingbemi et al. (2007) were not replicated in this or other studies.

NTP (2008) – Multigenerational study of Genistein

A 1,250 ppm exposure concentration was clearly ruled out for further testing based on the effects on body weights, histopathological observations in males and females, and a reduction in the proportion of mated dams producing litters seen in the dose range finding study (above). Accordingly, the highest exposure concentration chosen for the multigenerational reproductive toxicology study (NTP, 2008) and the 2-year study was 500 ppm. A low exposure concentration of 5 ppm, where no statistically significant effects were observed in the reproductive dose range finding study (NTP, 2007), and an intermediate exposure concentration of 100 ppm were also selected. The 5, 100 and 500 ppm dietary levels resulted in exposures of approximately 0.3, 7, and 35 mg genistein/kg body weight per day for males and 0, 0.5, 10, and 51 mg/kg per day for females, respectively.

Dietary exposure to 500 ppm genistein decreased body weights, accelerated VO, decreased AGD, and altered estrous cyclicity in females continuously ingesting genistein. There were no consistent effects on male or female AGD, or growth, although there was some evidence for reduced litter size in the F1 and F2 generations that were continuously exposed to 500 ppm of genistein. No other impacts on fertility and no histopathologic lesions were observed in females. The male reproductive tract did not show significant alterations, but increased incidences of hyperplasia of the mammary gland and calcification of renal tubules were observed in continuously exposed 100 and 500 ppm males examined at 20 weeks of age.

This study included >200 individual dose response curves. Body weights were measured about 20 times in the F1, F2, F3 and F4 the generations and repeatedly in the F0 and F5 generations. There were 4-5 cases each where body weights, food consumption or water consumption displayed an apparent NMDR at one week, but these effects were not consistent with respect to age, sex, dose or the direction of change versus control. They likely reflect random variations around the control mean**.**

In males in the F0, (but not F1, F2 or F3) generation male adrenal weights were increased only at 5 ppm; spleen weights in males were increased only at 5 ppm in the F0 and F2 (but not F1 or F3) generations; and thymus weight was decreased at 100 ppm in the F2 (but not F0, F1 or F3) generation.

In females, the age at VO was accelerated in an NMDR fashion at 5 ppm in the F3 generation but not in any other generation. The percent time spent in different stages of the estrous cycle

also displayed NMDRs in a few cases, but this effect also was not consistent from generation to generation. Female adrenal weights were reduced at only in the 5 ppm in the F2 generation (but not F0, F1 or F3 generation), pituitary gland weight was increased only at 100 ppm in the F0 generation (but not F1, F2 or F3), spleen weight was increased at increased only at 5 ppm in the F1 generation (but not F0, F2 or F3), and thymus weight was decreased at 100 ppm in the F3 generation (but not F0, F1 or F2).

The NMDR effects of genistein on F1 male AGD, body weights at weaning and testis weights, reported by Akingbemi et al. (2007) were not replicated in this study in neonatal males from the F1, F2, F3 or F4 generations who were exposed to genistein *in utero* and during lactation.

The NTP (2008) study indicates that NMDRs may arise in a study where a large number of endpoints are measured. However, these appear to be random variations and result from the many hundreds of statistical comparisons with control values. None of these NMRDCs was observed consistently across generations, sexes, or doses; these changes were not of a magnitude considered to be adverse.

NTP (2007) - Toxicology and Cancer Studies of Genistein

NTP (2007) describes three separate studies in which rats were exposed to genistein from the time of conception until weaning through their mothers, who were given genistein in their feed. At the end of each study, tissues from more than 40 sites were examined for every animal. The studies also included assessment of body and organ weights, estrous cyclicity, and survival.

- In one study, feed contained 0, 5, 100, or 500 parts per million (ppm) of genistein (50 rats per sex per group) from conception through two years.
- In the second study, groups of 50 male and female rats were given the same feed concentrations up to 20 weeks following birth, followed by untreated feed for the remainder of the two years.
- In the third study, groups of 50 male and female rats were exposed to the same feed concentrations from conception through weaning, and then given untreated feed for the duration of the study.

There were no increased rates of cancer in male rats in any of the three studies. In female rats exposed to genistein from conception and throughout two years, the rates of adenoma or adenocarcinoma of the mammary gland and pituitary gland adenoma or carcinoma were statistically significantly increased. In female rats exposed to genistein for 20 weeks following birth, the rates of pituitary gland adenoma or carcinoma were slightly increased, and in female rats exposed to genistein just from conception through weaning, the rates of mammary gland adenoma or adenocarcinoma were slightly increased. In summary, exposure to genistein for two years caused tumors of the mammary gland and pituitary gland in female rats. Exposure to genistein for shorter durations following birth was also possibly associated with increased rates of pituitary gland and mammary gland tumors. Exposure to genistein also accelerated the

onset of abnormal estrous cycles in F1 females exposed to 500 ppm genistein in the diet, regardless of the exposure regime.

In these studies, there were more than 200 dose responses; 40 tissues were examined histologically, and many of them were weighed in the 3 studies. The following NMDRs responses were observed; however, in no case was the same NMDR seen in both sexes or in more than one of the 3 cohorts (studies). Furthermore, several of the NMDRs listed below could be deemed to be beneficial (noted below with *) rather than adverse effects:

- Reduced incidence of fibroadenomas of the mammary gland of female rats in the 2 yr. study in the 5 ppm group in one of 3 cohorts.
- Reduced incidence of pituitary adenomas in male rats at 5 ppm in one of 3 cohorts
- Increased incidence of preputial gland squamous cell carcinoma in male rats in one of 3 cohorts at 100 ppm
- Reduction in benign neoplasms in all organs at 100 ppm in one of 3 cohorts
- *Reduced incidence of uterine stromal polyp in1 of 3 cohorts at 5 ppm
- Occasional variations in body weight (repeatedly measured once every 4 weeks over 2 years) in different cohorts and sexes.
- Increased brain weight in females in 1 of 3 cohorts at 100 ppm
- Increased pituitary weight in females in 1 of 3 cohorts at 100 ppm
- Increased spleen weight in females in 1 of 3 cohorts at 5 ppm

A.1.d.2 Dalu et al. (2002) –

In this study, F0 rats were fed 0, 5, 100, or 500 ppm genistein prior to mating and through pregnancy and lactation over two generations. At weaning, male pups were selected in each of the F 1 and F 2 generations with half of the pups continued on the same diet as their dams (G/G, continuous exposure) while their litter mates were placed on control chow (G/C, gestational and lactational exposure) until necropsy at 140 days of age. Male reproductive organ weights, serum levels of T and dihydrotestosterone (DHT), and ERα and ERβ protein levels in the ventral and dorsolateral prostate were the endpoints measured. Prostate sections were also evaluated microscopically. Statistically significant elevations in T and DHT were observed in PND 140 animals from the F 1 generation, but they were not accompanied by organ weight changes. Body weight in the continuously dosed 500 ppm F1 PND 140 animals was depressed relative to control, but organ weights in animals of either generation showed few treatment-related effects. While estrogen receptor (ER) levels were quite variable, levels of ERβ in the dorsolateral prostate were significantly depressed in all dose groups in the G/C exposure and the high dose group of the G/G exposure in F 1 rats, but not in F 2 rats.
Of the >35 individual dose response curves, Dalu *et al*. (2001) reported an NMDR for ERα in the dorsolateral prostate at 100 ppm in the F1 but not F2 in only the G/G cohort. They also reported an NMDR for ERβ protein levels in the ventral prostate of the F1 but not the F2 at 100 ppm in only the G/C cohorts. However, they did not replicate the NMDR for serum T which Akingbemi *et al.* (2007) reported as increased at 5 ppm but not at higher dosage levels.

A.1.d.3 Flynn et al. (2000)

Flynn et al. (2000) fed F0 rats 0, 5, 100, or 500 ppm genistein in the feed from GD 7 through pregnancy and lactation; their pups were fed the same diets through to 77 days of age. The levels of open field activity, play behavior, running wheel activity and saccharin- and sodium chloride flavored drinking water solutions were evaluated. The authors reported "subtle" behavioral alterations at levels that also decreased maternal and offspring body weights. Of the >20 endpoints, there were no statistically significant NMDR response.

A.1.d.4 Nagao et al. (2001b)

Nagao et al. (2001b) conducted two dose response studies with genistein using doses (five treated groups and a control) ranging from 12.5 to 200 mg/kg/d. In the first study, 10 male pups were administered genistein orally by gavage at a dose of 0, 50, 100 or 200 mg/kg on PND 1 through 5. Body weight in pups was significantly reduced throughout lactation at 100 and 200 mg/kg, and pups' viability was markedly reduced at 200 mg/kg.

In the full study that followed, neonatal SD rats were dosed daily by gavage with 12.5, 25, 50, or 100 mg/kg genistein), or 2 mg/kg EE2 (as a positive control) from PND 1 through 5. The dose of EE2 used as a positive control was based upon a preliminary study with this chemical which exposed male rat pups orally to EE at 0.1, 0.5, 1, or 2 mg/kg/day from PND 1 through 5. Pups in the highest EE2 dose group showed growth retardation during the lactational period, and the development of the reproductive organs was disrupted. Pups in the other treated groups showed no evident alteration in their growth and development of gonads. In this study, the ages at VO, estrous cyclicity, PPS, reproductive performance, sperm measures and serum T were measured. Body weight was significantly reduced in a monotonic manner in female and male offspring at 12.5 mg/kg and above. The ages at VO in F1 females, PPS and epididymal sperm concentration in males were not affected at any dosage level. The incidences of normally cycling females prior to mating were 87.5% in the controls, 43.3±19.4% in the 12.5 mg/kg group, 43.8±15.7% in the 25 mg/kg group, 22.9±15.7% in the 50 mg/kg group, and 33.4±12.4% in the 100 mg/kg group, respectively. The fertility of F1 males was unaffected at any dosage level whereas the F1 female fertility index was significantly reduced in a monotonic fashion at all dosage levels, from 100% in controls to 44% in the high dose group. At necropsy, epididymal weights were reduced by 8-10% in each treated group whereas testis, ventral prostate and seminal vesicle weights were unaffected. A variety of histopathological lesions were detected in the ovaries and uteri of F1 females, none of which displayed an NMDR. F1 males did not show any histopathological alterations in the testes, epididymides, prostates or seminal vesicles.

In summary, neonatal exposure to genistein did not produce any NMDR in this study that included many estrogen-sensitive endpoints.

A.1.d.5 Masutomi et al. (2003)

Masutomi et al. (2003) fed SD rats genistein at 0, 20, 200, or 1000 ppm from GD15 to PND10. AGDs, prepubertal organ weights, onset of puberty, estrous cyclicity, and organ weights and histopathology of endocrine organs at adult stage (week 11) as well as the volumes of sexually dimorphic nucleus of preoptic area (SDN-POA) were assessed. Genistein reduced body weight at necropsy (11 weeks of age at all dosage levels) but did not affect endocrine-regulated parameters at any dose level; nor was the SDN-POA volume altered. There were no low dose NMDRs among the >35 endpoints measured in this genistein study.

A.1.d.6 Akingbemi et al. (2007)

In the study by Akingbemi et al. (2007) several of the effects reported were NMDRs. This study also, reported effects that were in the opposite direction (body weight) of dose related effects from other studies, or effects reported were not seen in other studies with genistein at similar exposure levels (AGD and serum T). Akingbemi et al. (2007) did not administer genistein, but rather they fed pregnant LE rats casein-based diets containing whole soybean as sources of protein with isoflavone concentrations of 0, 5, 50, 500, or 1000 ppm from GD 12 to PND21. They measured serum concentrations of free and conjugated insoflavones in male rats and dams at 21 d postpartum. Akingbemi et al. (2007) reported NMDR for increased body weights and longer AGD in male rats at PND 5 at 5 and 50 ppm but not at higher exposures. In addition, serum T levels and *in vitro* Leydig cell T production were increased only at 5 ppm in prepubertal males, and serum LH was increased only at 50 ppm in adult male offspring (fed the same diet as the dam until PND 90). The authors also reported that testis weight was reduced at all dose levels; this has not been seen in other studies with genistein (e.g. see NTP studies above).

A.1.d.7 Naciff et al. (2005); Naciff et al. (2002)

As described above in the discussion of EE2, two studies from one laboratory have examined the effects of genistein (administered by sc injection) on global gene expression in developing male and reproductive rat tissues on GD 20. These studies were executed to determine if there were low dose NMDR at this level of biological alteration, which in turn, might contribute to NMDR effects on downstream adverse effects. Genistein was administered from GD11 to 20, and gene expression (microarray profiling of over 7000 genes and 1000 sequence tags followed by rtPRC) was measured in the tissues. One study examined the effects of genistein at 0, 0.1, 10, or 100 mg genistein/kg/day on the developing uterus and ovaries of female rats at GD 20 (Naciff et al., 2002). Another examined the effects of genistein at 0, 0.001, 0.01, 0.1, 10, and 100 mg genistein/kg/day on the developing testis and epididymis of male rats (Naciff et al., 2005). These studies indicate that the dose responses for gene expression in fetal rat reproductive tissues after gestational genistein exposure are monotonic; this further suggests that gene expression follow patterns that are predictable based on response at higher dosages of genistein.

A.1.d.8 Fielden et al. (2002)

In this study, mice were dosed orally by gavage with genistein at 0, 0.1, 0.5, 2.5 and 10 mg/kg/day or DES at 0, 0.1, 1, 10 µg/kg/d (a positive control) throughout pregnancy and lactation (GD12 to PND 20). Female offspring were weaned on PND 21; mammary gland whole mounts were examined for growth (length and area of the epithelial tree), proliferation (number of terminal end buds [TEBs]), and differentiation (density of alveolar buds [Abs]) on PND49. They reported that DES induced statistically significant monotonic increases in mammary gland development at 1 and 10 µg/kg/d (P<0.05) and decreased the number of TEBs in the highest dose group (P<0.06).

A.1.d.9 Fielden et al. (2003)

This study examined the long-term reproductive effects in F1 male mice of gestational and lactational exposure to 0, 0.1, 0.5, 2.5 and 10 mg genistein /kg/day by gavage; the authors (describe these treatment levels as comparable to or greater than human dietary exposures). Testicular growth, sperm counts and motility, and sperm fertilizing ability *in vitro* were assessed at PND 105 and 315, and selected genes were examined by real-time PCR. The authors did not find any statistically significant treatment-related effects on male offspring including these measures: AGD; body, seminal vesicle or testis weights; sperm count; the percent of motile sperm; or the number of motile sperm at any age.

A.1.d.10 Montani et al. (2008)

These authors found that genistein, modulates gene expression in the whole body of male mice in a dose- and time-dependent manner, at all ages through an ER–mediated action These mice were engineered to express a reporter (luciferease) of ER transcriptional activity (ERE-tK-LUC mouse) providing a functional picture of the map of ER activation. They exposed these ERE-tK-LUC male mice to a single oral gavage dose of 0, 5, 50, 500, 5000 µg/kg genistein and measured luciferase activity in tissue extracts 12 hours after dosing. Results showed that genistein induced ER activation in reproductive and non-reproductive organs of these mice. Luciferase activity was statistically significantly increased in the liver and the thymus at 50 µg/kg genistein and above; in the lung, heart, spleen, testis, hippocampus, and cortex at 500 and 5000 µg/kg; and in the pancreas, kidney, eye, and cerebellum at 5000 µg/kg. In addition prolonged exposure over a 15 day period to genistein also reduced prostate weight at 5000 µg/kg but had no effect on seminal vesicle, body or testis weights. All of the effects of genistein in the current study displayed monotonic dose response curves.

A.1.e Zearalenone (ZEA) and Zeranol (ZER)

Zearalenone

Zearalenone (ZEA) is a nonsteroidal estrogenic mycotoxin that is found on cereal crops and their derived products. It is considerably more potent than the estrogenic pesticides and toxic substances, and it also is one of the few environmental estrogens known to have an adverse effect on domestic animal reproduction. Estrogenism due to ZEA was first clinically recognized in prepubertal gilts fed moldy corn in the late 1950s, but ZEA is still occasionally reported as causing sporadic outbreaks in dairy cattle, sheep, chickens, and turkeys (WHO, 2000; Kuiper-Goodman et al., 1987).

Pigs are the most sensitive species, whereas high dietary concentrations are required to produce disease in cattle and sheep, and extremely high levels are required to affect poultry. This sensitivity of the different species cannot be predicted from a reported *in vitro* assay (citation) of estrogenicity, and the ability of ZEA and ZER to induce tissue-specific estrogenicity also varies greatly from species to species. The first report implicating the consumption of moldy feed with estrogenism in pigs was in 1928 (McNutt et al., 1928), but it was not attributed to a specific chemical in mold until the late 1950s, and the specific chemicals produced by mold displaying estrogenic activity were not identified until the late 1970s. Outbreaks of this syndrome were reported in North America, Europe, Africa, Asia and Australia at that time.

Since ZEA produces adverse effects at low, environmentally relevant concentrations in domestic animals, toxicity studies have been conducted in a wide range of domestic animal species (e.g. pigs, cattle, mink, sheep, turkeys, and chickens) and laboratory animal species (e.g. rats, mice, rabbits, guinea pigs and hamsters).

In 1987 and 2000 there were two extensive reviews of the risk assessment for ZEA (WHO, 2000; Kuiper-Goodman et al., 1987). They reported that ZEA caused alterations in the reproductive tract of females, both in laboratory animals and in domestic animals including persistent estrus, decreased fertility, increased embryo-fetal resorptions, reduced litter size, alterations in the weight of adrenal, thyroid and pituitary glands, and changes in serum progesterone and estradiol levels. However, no teratogenic effects were reported in mice, rats, guinea pigs or rabbits. Pigs and sheep were more sensitive than rodents to reproductive effects of ZEA. In 2011, the European Food Safety Agency (EFSA, 2011) based their tolerable daily intake (TDI) determination on studies in the pig since it is the most sensitive species, with immature female pigs being more sensitive than female mature pigs or male pigs.

The following discussion will focus primarily on rodent studies that provide information about the shape of the dose response curve in the low dose region to determine the prevalence of NMDRs for this chemical since this is the species of choice for multigenerational and one generation test guideline studies. However, since the critical studies used by the EFSA to establish the TDI were done using data from the most sensitive species, the pig (Edwards et al., 1987a) some of these studies also are discussed herein.

A.1.e.1 Heneweer et al. (2007)

Heneweer et al. (2007) exposed immature female rats to ZEA at 0, 0.03, 0.1, 0.3, 1 or 10 mg/kg/d and EE2 (as a positive control at 0, 0.03, 0.1, 0.3, 1 or 10 µg/kg/d) in the diet for 3 days, after which they assessed uterine weight and epithelial cell height and gene expression by microarray (44K whole genome 60-mer oligo, G4130A, Agilent arrays) and rtPCR. All effects displayed a monotonic response to both chemicals. Uterine weight (at 1 and 10 mg/kg/d) and epithelial cell height (at 10 mg/kg/d) were increased dose-dependently after a 3-day oral exposure of rats by ZEA. At 0.1 mg/kg/d ZEA, uterine edema, and vacuolization were observed in some animals. Exposure to 1 mg/kg ZEA resulted in severe damage of the uterine epithelial layer. Microarray and rtPCR gene expression were altered only in the highest ZEA dose group. In summary, this study provides no evidence of an NMDR for measure of ZEA-induced low dose alterations of uterine morphology, histology or gene expression, in a total of about 50 individual responses (weight, histology, 40 genes altered as assessed by microarray and 5 using rtPCR).

A.1.e.2 Becci et al. (1982)

Becci et al. (1982) administered ZEA in the diet to Wistar rats through two generations at dosage levels of 0, 0.1, 1.0 and 10.0 mg/kg body weight/d in all generations.

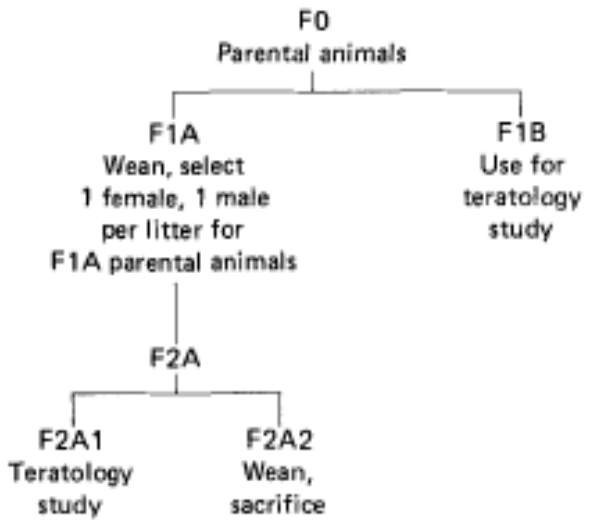


Figure A.10 Reproduced from Becci et al. (1982)

ZEA treatment significantly reduced body weight gain and the numbers of live born pups, ovarian corpora lutea/dam and uterine implantations/dam and increased the resorptions/dam in the high dose group (no NMDR) in both generations. In the teratologic assessment, ZEA induced several skeletal and soft tissue abnormalities, but none of these displayed clear NMDRs. Adrenal, thyroid and pituitary gland weights were increased in a monotonic, dose-

related fashion in F1 and F0 male rats. The organ weights in female rats were less affected, and no effects were noted in ovary, testis, uterus, seminal vesicle or prostate gland weights.

A.1.e.3 Collins et al. (2006)

Collins et al. (2006) administered ZEA by oral gavage at 0, 1, 2, 4 or 8 mg/kg/d on GD 6-19. Dams were euthanized on GD20, and fetuses were examined for skeletal and soft tissue abnormalities. In addition maternal serum was taken for hormone analysis.

Monotonic, dose-related decreases were seen in maternal feed consumption and body weight gain in all treated groups; gravid uterine weight, percent pregnant, and fetal size and weight were reduced in the two high dose groups. The percent of early fetal deaths and full litter resorption were increased in the high dose group. Delayed fetal development (reduced skeletal ossification in the two high dose groups) was concurrent with reduced fetal weight and increased maternal toxicity. Uterine endometrial gland necrosis was observed in the 4, and 8 mg/kg females and appeared to increase with dose; liver effects included treatment-related increases in the incidence and/or severity of vacuolated hepatocytes in females from all treated groups. In addition, in the high dose group maternal serum LH and prolactin were increased, and E2 was reduced as compared to control values. Progesterone tended to be reduced at 2 mg/kg/d and above, being statistically significant at 4 mg/kg/d. The authors concluded that based upon the dose-related maternal and fetal toxicity in all treated groups, the NOEL for a reproductive and teratogenic effect was less than 1 mg/kg.

A.1.e.4 Belli et al. (2010)

Belli et al. (2010) exposed rats to ZEA by sc injection at 0, 0.2 µg/kg/d, 20 µg/kg/d, 1 mg/kg/d or 5 mg/kg/d during the last 14 days of gestation to PND 5. Mammary gland development was assessed at 30 and 180 days of age. Monotonic, dose related increases in the number of terminal end buds were noted in all dose groups at 30 days of age at in cell proliferation at 180 days of age. By contrast, irregular hyperplasia (in 4/18 females) was only seen in the highest dose groups at 6 months of age.

A.1.e.5 NTP ZEA Cancer Bioassay Report (1982) (NTP, 1982)

The NTP conducted a series of dose response studies in rats and mice with ZEA including high dose acute and 14 day studies, a 13 week subchronic study (0, 30, 100, 300, 1,000 and 3000 ppm) and a chronic study (0, 25, and 50 ppm). The 13 week studies in the rat and mouse with six groups are reviewed here. At the end of the 13 week dosing period animals were necropsied, and histopathological examinations were conducted on the pituitary, adrenals, testes or ovaries, prostate or uterus and seminal vesicles. Other organs were examined but only in the control and high dose groups.

In rats, ZEA did not induce any mortality, but weight gain was depressed by more than 17% in

rats of either sex receiving 100 ppm and higher. ZEA also caused dose related (monotonic) atrophy of the seminal vesicles (11% at 30 ppm) and testes (70% at 300 ppm) and fibromuscular hyperplasia of the prostate in 30%, 90% and 100% of the male rats fed 300, 1,000 or 3,000 ppm, respectively. Ductular hyperplasia of the mammary gland occurred in 60% of males and 100% of females fed 3,000 ppm ZEA. Chromophobe hyperplasia of the pituitary gland occurred in 60% of males and 70% of females fed 3,000 ppm; 22% of males and 20% of females fed 1,000 ppm; and 10% of females fed 100 ppm. This lesion was not seen in controls. Osteopetrosis (hardening of the bone) was seen female rats in all the ZEA groups; it was observed in 50% of females at 30 ppm and 90% to 100% of the females fed 100 ppm or more and in 90%-100% of the males fed 1,000 or 3,000 ppm (Table 4 or the NTP report). None of these effects displayed an NMDR.

In mice, weight gain was reduced but only in male mice at 1000 and 3000 ppm. Histopathological effects in male mice included cytoplasmic vacuolization of the adrenal gland, squamous metaplasia of the prostate, atrophy of the seminal vesicles and osteopetrosis and myelofibrosis of bone (disorder of the bone marrow). All the histopathological effects in male mice were monotonic responses; most were present at 1000 and 3000 ppm, whereas osteopetrosis was also displayed in the 100 and 300 ppm groups. In female mice, all the effects were monotonic including histopathological lesions of the adrenal (300 ppm and above), uterus (1000 and 3000 ppm) and bone (osteopetrosis at 100 ppm and above and myelofibrosis in the two highest dose groups).

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A.1.e.7 Edwards et al. (1987a)

The critical study used by the EFSA to establish the tolerable daily intake (TDI) was a feeding study published by Edwards et al. (1987a). In this study, sexually mature non-pregnant gilts, were given 2 kg per day of feed containing 0, 1, 5 or 10 ppm purified ZEA/kg of feed between day 5 and day 20 of estrus (equivalent to 0, 40, 200 or 400 µg/kg/d; n = 24-25 gilts per dose group). ZEA treatment at 5 and 10 ppm increased the inter-estrous interval significantly from 21.0 ± 0.3 days in the control group to 29.2 ± 2.9 and 32.7 ± 3.3 days in gilts, respectively; this effect was not seen in gilts fed 1 ppm ZEA in the diet, and increased levels of progesterone and prolonged maintenance of corpora lutea were observed in the gilts with prolonged estrous cycles. A NOEL of 40 µg/kg/d was taken from this study by the EFSA (2001) to determine the TDI. In summary, this study reported NMDR at environmentally relevant concentrations of the chemicals tested.

A.1.e.8 Young and King (1986)

Young and King (1986) fed gilts diets containing 0, 3, 6 or 9 ppm (n=16/group) ZEA starting the day after pubertal estrus and they were artificially inseminated twice at subsequent heat periods. No effects were noted at 3 ppm. Eighty-eight percent of the gilts fed 6

or 9 ppm ZEA ZEA became pseudo-pregnant as confirmed by elevated plasma progesterone levels and examination of their reproductive tracts. In addition, only three animals fed the two higher levels of ZEA conceived and farrowed. Histological examination of the ovaries of higher dose gilts revealed the presence of abnormal corpora luteal function (corpora hemorrhagica and corpora albicans). All effects seen in the current study were monotonic dose responses.

A.1.e.9 Gajęcka et al. (2011)

Gajęcka et al. (2011) fed ZEA at 20 or 40 µg/kg/d (n=12/group) for 48 days *per os* in gelatin capsules to sexually immature gilts. ZEA-induced estrogen-like ovarian alterations including lowered proliferation of granulosa cells of the follicle walls and connective tissue of the ovarian stroma. In addition, in the ovary there were fewer proliferating follicles and reduced connective tissues (both dosed groups were similarly affected). By contrast, serum estradiol and T levels were unaffected in either dose group by comparison to controls.

Taken together, the studies with swine, including the low dose studies by Edwards et al. (1987a) (Edwards et al., 1987b) and Young and King (1986) do not indicate that administration ZEA, a relatively potent environmental estrogen, produces any low-dose NMDR effects in the most sensitive species or in the endpoints used in the recent EFSA risk assessment.

A.1.f Zeranol (ZER)

α-Zearalanol (Zeranol® or ZER), a metabolite of ZEA, is used in implants to promote growth in beef cattle and lambs in the United States and Canada. However, the use of α-zearalanol for growth promotion in food animals was banned in the European Union in 1985 along with five other hormonally active anabolic agents including estradiol, progesterone, T, melengesterol acetate, and trenbolone acetate.

ZER also was evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) as a veterinary drug for use as a growth promoter (FAO/WHO, 1988). ZER is estrogenic in mice, rats, dogs, and monkeys, and it induces tumors of the pituitary gland, presumably from the estrogenic properties. The safety assessment by JECFA was based on determination of a no-hormonal effect level. The JEFCA determined that the no-hormonal-effect level was 0.05 mg/kg/d from studies in ovariectomized female cynomolgus In addition to its use in domestic animal species, ZER has been administered to women in a number of clinical trials to determine the effectiveness of this chemical as a drug for the treatment of menopausal symptoms (e.g., Utian, 1973), and Dai et al. (2004) proposed the use of ZER for the prevention of atherosclerosis in middle-aged women.

ZER is more potent *in vitro* and *in vivo* than is ZEA (Katzenellenbogen et al., 1979), however, the *in vivo* potency relative to estradiol is not a great as one would expect from the *in vitro* results. The reviews of ZER include several low dose multigenerational studies with rats (primarily done

in the 1970s and 1980s) and reproductive studies in other mammalian species but, as is often the case, these studies were not published. As a result, while robust summaries of these studies are available, we are unable to examine the dose response curves. However, published summaries of the early risk assessments and summaries of the toxicity studies are provided in reviews by Baldwin et al. (1983) and Lindsay (1985).

Fertility

In a fertility and reproduction study, 0.3 mg zeranol/kg/day administered orally to male and female rats for 60 days prior to their mating with untreated rats had no effect on the fertility or reproductive capacity of the animals (Baldwin, Williams & Terry, 1983). Dosage levels of 1.25 and 5.0 mg/kg/day in males and females increased the number of days of cohabitation before first insemination. The higher doses decreased litter size at birth, increased the number of stillbirths and reduced neonatal survival. The reproductive no-effect level for repeated oral exposure of rats to zeranol was 0.3 mg/kg/day.

In vivo a fivefold increase in uterine weight was obtained in female rats by subcutaneous administration of either 2 μg oestradiol/rat/day or 550 μg zeranol/rat/day (Katzenellenbogen *et al.* 1979). Similar results have been obtained in studies of the effects on vaginal cornification in the mouse and rat (Parekh & Coulston, 1983). In castrated female rhesus monkeys, 10-μg/kg dosages of oestradiol were required to depress serum gonadotrophin levels. In comparison, a 1000 times greater dose of zeranol was required for the same effect (Fuller, Burnett, Graham & Hobson, 1982). The hormonal no-effect level for zeranol has been shown to be at least 0.05 mg/kg/day, as revealed by the absence of effects on vaginal cornification in castrated female monkeys (Griffin, Parekh, Singh & Coulston, 1983).

A.1.g Octylphenol

A.1.g.1 Tyl et al. (1999)

Tyl et al. (1999) conducted a two generation reproduction study in which they administered octylphenol over a broad range of dosage levels (five groups of rats (30/sex) at dietary concentrations of 0, 0.2, 20, 200, or 2000 ppm in the diet, equivalent to doses ranging from 0, 0.034–0.011, 3.3–1.05, 32.6–10.9, 369–111 mg/kg/day, respectively, depending on the age and sex of the animals and the phase of the study). Body weights were consistently reduced in the high group in each generation. In addition, the age of puberty was delayed in a monotonic fashion in F1 and F2 males (high dose only), and in females (VO was delayed in F1 and F2 females (statistically significant at 2000 ppm in the F2 and 200 and 2000 ppm in the F1). Epididymal and uterine weights were reduced in the high dose group but only in P0 generation. In addition, AGD was lengthened in F2 females in a monotonic manner being statistically significant at 20, 200 and 2000 ppm.

Tyl et al. (1999) did not detect any other effects on reproductive parameters, prostate, or ovary weights or morphology, on sperm counts, motility, morphology, production, or on estrous cyclicity. The authors concluded that "No estrogen-like effects were evident." In summary, this study examined a large number of effects across several orders of magnitude of exposure and did not observe any NMDR.

A.1.g.2 Gray and Ostby (1998) and Laws et al. (2000)

By contrast to the lack of effect of octylphenol on the age at VO noted above, Gray and Ostby (1998) and Laws et al. (2000) observed a significant estrogen-like acceleration in VO (6.9 days and 3.2 days, respectively) when octylphenol was administered by oral gavage from weaning at 200 mg/kg/d. The shorter-term dosing used by Gray and Ostby (1998) and Laws et al. (2000) did not retard growth as compared to the effect seen in the high dose group by Tyl et al. (1999) with multigenerational dosing.

A.1.g.3 Nagao et al. (2001a)

In the study of octylphenol by Nagao et al. (2001a), neonatal rats were dosed with octylphenol by oral gavage at doses of 0, 12.5, 25, 50, or 100 mg/kg once daily on postnatal days 1 through 5 to examine its effects on male and female reproductive function after puberty.

Observations included these:

- reduced body weights in male and female rats throughout the study, statistically significant at 25, 50 and 100 mg/kg/d
- delayed puberty in males and females at 50 and 100 mg/kg/d
- no effect on estrous cyclicity

- no effect on any measure of male and female reproductive performance
- no effect on epididymal sperm concentration
- no effect on serum T
- no effect on testis or seminal vesicle weights
- reduced epididymal weight at 100 mg/kg/d
- reduced ventral prostate weight at 25, 50 and 100 mg/kg/d

In summary, oral neonatal administration of octylphenol did not disrupt reproductive development in an estrogen-like manner in male or female rats, and all the effects that were seen displayed monotonic responses.

A.1.h Nonylphenol (p-NP)

Nonylphenol (*p*-NP) is used for the production of plastics, nonylphenol ethoxylates in detergents and pesticide formulations and is ubiquitous in the environment. The nine alkyl carbon side chain may be branched or straight (4n NP), with the branched forms (CAS 25154–52-3 and CAS 84852-15-3) being the most common. p-NP is a mixture of approximately 20 para-substituted isomers with differently branched alkyl chains, which has greater estrogenic potency that does the 4n NP molecule; the individual isomers in the p-NP mixture vary significantly in the estrogenic potencies (Pruess *et al*., 2006). Several studies have shown that oral administration of 4-NP stimulates uterine weight and accelerates puberty in the female rat (Kim et al., 2002b; Laws et al., 2000); Gray and Ostby (1998) with an apparent no- and low-hormonal-effect levels of 25 and 50 mg/kg/d, respectively.

The following discussion includes two multigenerational studies with p-NP - CAS 84852-15-3 (Tyl et al., 2006; Chapin et al., 1999) and one with p-NP-CAS 25154–52-3 (Nagao et al., 2001a). However, none of these used low doses defined as less than 1 mg/kg/d for a weakly estrogenic chemical.

A.1.h.1 Tyl et al. (2006)

Tyl et al. (2006) exposed rats (25 per sex per group) toof 4-NP (CAS 84852-15-3) at 0, 20, 200, 650, and 2000 ppm (equivalent to 0, approximately 1.5, 15, 45, and 150 mg/kg/day) or to the positive control E2 (2.5 ppm) in the diet for three generations. In the male rat, body weight was significantly reduced at 2000 ppm in all generations, and there was a tendency towards reduced testis and epididymal weights and increased kidney weights at this dose. Males also displayed histopathological alterations in the kidney at 650 and 2000 ppm, which was statistically significant at some treatments. In addition, andrologic parameters all displayed subtle effects in the high dose group, but only one of the measures was statistically significant in one generation. In the female rat, body weight at necropsy was not reduced whereas ovarian weight was reduced at 650 and 2000 ppm. All responses were monotonic. There were no effects on reproductive or lactational indices in any generation. Accelerated VO and delayed

PPSwere seen in the E2 positive control group, effects expected from exposure to , an estrogen; however, these endpoints were not measured in the 4-NP groups.

A.1.h.2 Chapin et al. (1999)

Chapin et al. (1999) administered 4-NP (CAS 84852-15-3) in the diet over 3.5 generations at 200, 650, and 2000 ppm (reported as equivalent to 9-35, 30-100, 100-350 mg/kg/d, respectively). In the 650 and 2000 ppm treatment groups, body weight gain was reduced and VO was accelerated by 2 and 6 days; uterine weights at 21 days were increased 14% and 50%, respectively; ovarian weights were reduced; epididymal sperm density was reduced by 8% and 13% respectively. Kidney weights were increased, and histological alterations of the kidneys were noted in all the treated groups. In addition, in the high dose group, testicular sperm count was reduced by 13%. The authors concluded that NP had limited estrogenicity and other reproductive effects in the presence of measureable nephrotoxicity. The data from this study (Tables 1-3) contain >40 endpoints in each of 3 generations, none of which displayed an NMDR.

A.1.h.3 Nagao et al. (2001a)

Nagao et al. (2001a) administered a different mixture of NP (CAS 25154–52-3) than used by the studies cited above by oral gavage. There were two studies: a dose-range finding study (0, 10, 50 and 250 mg/kg/d) and the two generation study (0, 2, 10 and 50 mg/kg/d).

In the dose range finding study, NP induced a marked reduction in body weight gain, increases in liver and kidney weights in both sexes and a slight decrease in fecundity in the 250 mg/kg/d group. No other effects were noted in either male or female rats, and no effects were seen in the 10 or 50 mg/kg/day treatment groups.

The main study included these measurements; growth; reproductive and non-reproductive organ weights in the P0 generation and at weaning of the F1 and adulthood: histopathology (about 14/ generation/sex); fertility; litter size; three behavioral tests; eight serum hormone levels (at weaning of the F1 and adulthood); male and female pubertal landmarks; and estrous cyclicity (>60 endpoints in the P0 and F1 generations). In the main study, the high dose group induced salivation in males, increased kidney weight and reduced thymus weights across the generations. F1 females displayed estrogen-like acceleration of VO at 50 mg/kg/d, but estrous cyclicity and fecundity were unaffected at any dose level of NP Fourteen endpoints displayed NMDRs (of which 8 were hormonal measurements), but these were not consistent from generation to generation. The authors concluded that 50 mg/kg/d was a NOAEL.

In summary, this study did find NMDR responses. These were not reproducible from generation to generation, they were restricted to organ weights and hormonal measurements, and they did not include assessments of organ histopathology or reproductive performance.

A.1.h.4 Fourie et al. (2001)

The study design was in compliance with the OECD 415 guideline for the testing of chemicals (OECD, 1983). Adult male SD rats were exposed by oral gavage to p-NP at 0, 5, 20, 50, 100, 250 and 400 mg/kg/d from 12 until 22 weeks of age. The epididymis was separated from the testis, and one epididymis was used for histopathological evaluation while d the other epididymis was divided into the caput-corpus and cauda regions for examination of biochemical markers of epididymal function. Biochemical markers included L-carnitine, a-glucosidase activity, acid phosphatase (ACP) activity, and tartrate-resistant acid phosphatase (TRACP) activity. The aim of this study was to compare epididymal markers in control and exposed groups and to integrate these markers with histological findings.

Exposure to p-NP increased mortality in a dose-related, monotonic fashion from 100 to 400 mg/kg/d. p-NP treatment did not induce any major histopathological abnormalities in the epididymis; levels of p-NP below the 100 mg/kg/d did not affect biochemical marker values. At levels above the 100 mg/kg/d, biochemical markers of epididymal function were affected by the exposure of adults. L-carnitine was unchanged at all levels of exposure. Exposure of adult males to levels above the 100 mg/kg/d increased α-glucosidase in a dose related manner, suggesting increased epididymal secretory activity.

In summary, this study did not find any NMDR responses in the endpoints they measured.

A.1.i Methoxychlor (MET)

Methoxychlor (MET) is a formerly used estrogenic pesticide. Studies using high oral dosing regimens have demonstrated that MET exposure can produce the following effects: dramatic decreases in testis weight;, increased kidney weight; cystic tubular nephropathy (Tullner and Edgcomb, 1962) accelerated VO in female rats,; impaired reproductive performance of male and female rodents exposed *in utero* and during lactation (Swartz and Corkern, 1992; Harris et al., 1974) and induction of estrogen-dependent sexually dimorphic behavior in ovariectomized female rats (Gray et al., 1988).

There are several developmental and reproductive dose response studies with four or more dose groups using rats that administered MET by oral gavage or in the diet, the relevant route of exposure. However, none of these used low doses defined earlier as less than 1 mg/kg/d for a weakly estrogenic chemical. The dosage levels used in these studies range from about 1 to 400 mg/kg/d.

Several studies have been executed with MET using dose levels in the µg/kg/d range but these administered MET by injection and had only 1 or 2 MET treated groups; these are insufficient to define the shape of the dose response curve. One study that injected (sc) MET during neonatal life (PND 3 to 10) is discussed below because it included a control and five levels of MET (from 1 to 500 mg/kg/d) (Uzumcu et al., 2006).

A.1.i.1 Masutomi et al. (2003)

Masutomi et al. (2003) fed SD rats MET at 0, 24, 240, or 1200 ppm (calculated as 0, 1.9 to 3.4, 18.7 to 35.9 and 81.4 to 167.6 mg/kg/d, respectively) from GD15 to PND10. AGD, prepubertal organ weights, onset of puberty, estrous cyclicity and organ weights and histopathology of endocrine organs at adult stage (week 11) as well as the volumes of the brain sexually dimorphic nucleus of preoptic area (SDN-POA) were measured.

AGD at birth was unaffected by MET at any dosage level. In the high dose group neonatal body weight gain was reduced in F1 male and female rats; VO was accelerated; females displayed abnormal estrous cycles; PPS was delayed in males; and testis weight at puberty and in adulthood and ovarian weight in adulthood were reduced. However, no other organ weights were affected in any dose group. In females, histological alterations were seen in the ovary, uterus, vagina and pituitary gland, and there were few corpora lutea in the ovaries of high dose group females. In males, prostate weight and histology were unaffected at any dosage level. There were no low dose NMDRs among the >35 endpoints measured in the MET study.

A.1.i.2 Chapin et al. (1997)

Chapin et al. (1997) administered MET to pregnant rats by oral gavage in a dose range finding study (0, 20, 50, 100, 130, 170 or 200 mg/kg/d). In a multigenerational study rats were treated (0, 5, 50, or 150 mg/kg/day) for the week before through the week after birth; , after this time the pups were directly dosed from PNDs 7 to 21 in one cohort or from 7 to 42 days of age in the other cohort.

In the main study, MET reduced litter sizes at birth (at 150 mg/kg), reduced pup body weight during lactation (at 150), accelerated VO at all dosage levels (no NOAEL), and delayed PPS in male rats in the two highest dose groups. AGD was unchanged in either sex. However, the age at VO was accelerated in females in all treated groups, and male PPS preputial separation was delayed at the middle and high doses by 8 and 34 days, respectively.

In pubertal females, body, thymus and uterine weights were reduced in the highest dose groups. Ovarian weights were reduced in a monotonic manner in all dose groups. In animals exposed to MET until 42 days, all indices of reproductive performance and the numbers of ovarian corpora lutea in F1 females, were altered in the two high dose groups. Adult estrous cyclicity was disrupted at 50 and 150 mg MET/kg/d, doses which also showed reduced rates of pregnancy and delivery. At necropsy, uterine weights were reduced after pregnancy in all MET treated groups (no NOAEL), and thymus weight was reduced in the highest dose group. Serum estradiol/progesterone ratios were elevated in the two highest dose groups, and FSH was decreased at all dosage levels examined (5 and 50). All groups of treated females showed uterine dysplasias and less mammary alveolar development; estrous levels of FSH were lower in all treated groups; and estrus progesterone levels were lower at 50 and 150 mg/kg/d, attributed to fewer corpora lutea secondary to ovulation defects.

At the pubertal necropsy at 46 days of age, F1 male rats in the 50 and 150 mg/kg/d dose groups displayed monotonic dose-related reductions in body, thymus, testis, epididymis, seminal vesicle and prostate weights, whereas adrenal weights were increased in the high dose group alone. In adult F1 males, exposed to MET until 42 days of age, mating performance was reduced in the high dose group alone. At necropsy, testis and epididymal weights and testis sperm counts were reduced at 50 and 150 mg/kg/d; by contrast prostate and seminal vesicle weights were reduced only in the high dose group. High-dose males impregnated fewer untreated females; epididymal sperm count and testis weight were reduced at the high, or top two, doses, respectively.

These data indicate that 5 mg MET/kg/day is not a NOAEL based on changes in day of VO, pubertal ovary weights, adult uterine and seminal vesicle weights, and female hormone data. None of the endpoints affected by MET displayed an NMDR including those known to be sensitive to exogenous administration of estrogenic chemicals.

A.1.i.3 Johnson et al. (2002)

The dosing protocol for MET used in this study is similar to that used above by Chapin et al. (1997). Pregnant rats were dosed with MET at 0, 5, 50 or 150 mg/kg/d for the week before and the week after they gave birth, after which F1 male pups were dosed directly from PND 7 to 42 and necropsied at about 152 days of age. Testes were weighed, fixed, sectioned and examined histologically. Testis weight, the volume of the Sertoli cell nuclei, and the number of Sertoli cells were reduced in a monotonic, dose related manner, being statistically significant at 50 and 150 mg/kg/d.

A.1.i.4 Gray et al. (1988)

In this study, rats were dosed by oral gavage from weaning, through puberty, mating and gestation, to day 15 of lactation with MET at 25, 50, 100, or 200 mg/kg/day.

In P0 females, MET accelerated the age at VO and first estrus at all dose levels, and the vaginal smears were cornified at 50 mg/kg/d and above. Growth was retarded, and fertility was reduced at 100 and 200 mg/kg/day when the females were bred with untreated or similarly treated males. F1 litter sizes were reduced at 100 mg/kg/d and above. In the highest dose group, the mated females went from constant estrus into pseudopregnancy following mating, but they had no implants. All effects in the P0 females were monotonic.

In P0 males, MET treatment reduced growth at lower dosage levels than in the female, being statistically significant at all dosage levels (25 mg/kg and above). In addition, seminal vesicle, cauda epididymal, and pituitary weights and caudal sperm content were reduced in a monotonic dose related manner. Puberty was delayed in the two highest-dosage groups. Testicular sperm measures were much less affected than caudal sperm measures, which were reduced at 50 mg/kg/d and above. Testis weight and histology were slightly affected, and testicular sperm production, sperm morphology, and motility were unaffected at all doses.

In P0 males, endocrine function of the testes and pituitary was altered by MET administration. Leydig cell T production, in response to human chorionic gonadotropin challenge, was reduced and pituitary levels of prolactin, thyroid-stimulating hormone (TSH), and follicle-stimulating hormone (FSH) were altered. In contrast, serum levels of prolactin, FSH, and luteinizing hormone were unaffected. Serum TSH was reduced by 50% of control at 100 and 200 mg/kg/day, while pituitary levels were increased. Gonadotropin-releasing hormone concentration in the mediobasal hypothalamus was also elevated. In spite of the many reproductive alterations, the fertility of treated males was not reduced when they were mated with untreated females at doses up to 200 mg/kg/d. In P0 males, none of the dose response curves were NMDR.

In a parallel study, Goldman et al. (1986) examined the effects of eight weeks of oral exposure to MET at 25 and 50 mg/kg/d on hypothalamic-pituitary endocrine function in vitro. Of the

multiple measures taken in the study, only pituitary prolactin concentration (increased in both dose groups) and GnRH in the mediobasal hypothalamus (increased only in the high dose group) were affected.

These studies provide no evidence for the NMDR 'low dose" hypothesis, as all responses were monotonic.

A.1.i.5 Gray et al. (1999)

Due to a lack of effect of MET in the study by Gray et al. (1988) on many aspects of male rat reproductive function and fertility a follow-up study was conducted in which male rats were exposed to higher dosage levels of MET (0, 200, 300 and 400 mg/kg/d) for a longer time period. Combined with the earlier study by Gray et al. (1988) this extends the dosing range to 0, 25, 50, 100, 200, 300 and 400 mg/kg/d.

MET treatment delayed puberty at 200, 300 and 400 mg/kg/d by an average of 8, 16 and 34 days, respectively and reduced fertility and copulatory plug formation in a monotonic dose-related manner at all dosage levels. During mating, MET-treated males exhibited shorter latencies to mount and ejaculate versus control males, but the number of intromissions prior to ejaculation was unaffected; this indicates that MET enhanced the arousal level in the males in an estrogen-dependent manner. Most treated males eventually mated but time-to-pregnancy was lengthened. Body, liver, kidney, pituitary, epididymal and seminal vesicle weights were reduced in a monotonic manner. Very low cauda epididymal sperm counts were associated with infertility, whereas testis sperm production was much less affected. These data demonstrate that MET affects the CNS, epididymal sperm numbers, and the accessory sex glands and delays mating without significantly affecting the secretion of LH, prolactin, or testosterone. Furthermore, MET, unlike the estradiol positive control, did not alter pituitary endocrine function in either an estrogenic or antiandrogenic manner, indicating that MET acts as an estrogen in a tissues-specific manner. None of the effects in this study displayed an NMDR.

A.1.i.6 Martinez and Swartz (1991)

In this study, sexually mature female mice were treated with MET by oral gavage at 1.25, 2.5 or 5 mg/mouse (estimated to be about 41, 83 and 167 mg/kg/d) for 5 days a week, or the positive control, estradiol (250 µg/mouse/d), for 2 or 4 weeks. Vaginal smears were taken daily. The females were necropsied after the last dose, and the ovaries and reproductive tracts were removed, weighed and examined histologically. MET induced persistent vaginal estrus and reduced ovarian weights (at 4 weeks) in monotonic, dose-related manner in every treated group (no NOAELs). After 4 weeks exposure, the numbers of healthy large follicles was reduced (statistically significant only at 1.25 and 2.5 mg/kg), and the percent of the follicles that were atretric was increased by MET treatment in all dose groups. None of the effects in this study displayed an NMDR.

A.1.i.7 Staub et al. (2002)

In this study, rat dams were gavaged with MET at 0, 5, 50, or 150 mg/kg/day for the week before and through the week after they gave birth, and male pups were dosed directly from PND7 to 42. Testes were evaluated stereologically. Across dose groups, body weight was not affected, but testicular weight, daily sperm production were significantly reduced in a monotonic, dose-dependent fashion. Spermatogenic potential, based on number of spermatogonia and number of spermatids per testis, was significantly reduced by treatment in a monotonic manner in all dose groups; the ratio of spermatid number per spermatogonia was higher in all the MET-treated groups (no NOEL). None of the effects in this study displayed an NMDR.

A.1.j Kepone (Chlordecone)

Kepone is a well-known human neuro- and reproductive- toxicant. In rodent studies, kepone produces adverse effects on the reproductive system and neurotoxicity, with the reproductive effects occurring at dosage levels slightly below those that cause tremors. The potency of kepone as a reproductive toxicant is greater *in vivo* than for many other estrogenic toxicants and is greater than one might expect from its effects in *in vitro* ER binding and transcriptional activation assays.

A.1.j.1 Huber (1965)

Huber (1965) conducted a series of dose response studies in which he administered kepone in the diet to mice. In a subacute mortality study (0, 10, 30, 40, 60, 70, 80 and 100 ppm in the diet, >12 mice per group), he found that doses of kepone at 40 ppm and below did not produce mortality. However, a constant tremor appeared at 4 weeks, and liver weights were increased in mice fed 30 ppm and higher; livers doubled in size in 60-90 days at 40 ppm.

In a reproduction test, mice (8 breeding pairs per dose) were fed kepone at 0, 10, 30, 37.5 ppm in the diet for one month before mating and throughout the 100 day mating trial. Fecundity was reduced by about 24%, 79% and 87% at 10, 30, and 37.5 ppm, respectively. Taken together, these studies demonstrate that kepone produces monotonic responses with long-term dietary administration in the mouse with effects on reproduction occurring at dosage levels below those that induce tremors, increase liver weight or induce mortality. None of the effects in this study displayed an NMDR.

A.1.j.2 Good et al. (1965)

Kepone was administered in a series of studies in the diet to pairs of adult mice at 0, 5, 10, 17.5, 25, 30 or 37.5 ppm for one month before and 5 months after mating to determine the effect on reproductive performance (Good et al., 1965). In the first study (which did not include the 5 ppm dose group), there was “some effect on reproduction at all dosage levels. A progressive reduction in litter size and a decreased frequency of litter production as the dosage level increased. ...”. In a follow-up study, wherein adult mice were administered kepone for 128 days the percentage of pairs producing litters was significantly reduced. In addition, the fertility of the F1 mice exposed to 5 ppm *in utero*, in the milk and for a brief period prior to mating, was also was reduced by about 55% as compared to controls. None of the effects in this study displayed an NMDR.

A.1.j.3 (Larson et al., 1979)

Male and female **r**ats were fed kepone at 0, 1, 5, 10, 25, 50 and 80 ppm for up to 2 years (two studies). All rats exposed to 50 and 80 ppm died during the first six months of treatment. Growth was depressed in females at 10 ppm and above and in males at 25 ppm and higher.

Testis-to-body weight ratios were dramatically decreased as compared to controls at 50 (down 53%) and 80 ppm (down 70%) after three months of treatment and at 25 ppm (down 20%) at one year. Liver-to-body weight ratios also were increased in a monotonic dose-related manner. Histological changes in the testes and liver also were dose-related. In summary, long-term dietary administration of the weakly estrogenic pesticide kepone produced adverse effects on the male rat reproductive system, the liver, and viability; none of these effects were NMDRs.

A.1.j.4 Linder et al. (1983)

Linder et al. (1983) fed adult male rats kepone at 0, 5, 15 or 30 ppm (equivalent to 0.26, 0.83, and 1.67 mg/kg/d) for 90 days, after which the males were mated to untreated controls and necropsied. Sperm motility, sperm viability and the numbers of sperm in the cauda epididymis were reduced by treatment with kepone at 15 and 30 ppm. Body weight gain and seminal vesicle and prostate weights were reduced in the high dose group; and mild tremors were noted at 15 and 30 ppm. However, reproductive performance, testis and epididymal weights were not affected. In summary, all effects in the current study displayed monotonic responses, increasing in severity as the dose level increased.

A.1.j.5 Swartz et al. (1988)

Swartz et al. (1988) exposed sexually mature female mice by oral gavage to kepone at 62, 125 or 250 µg/mouse (estimated to be about 2, 4 and 8 mg/kg/d) (5 days a week) or the positive control estradiol (100 µg/mouse/d) for 2, 4 or 8 weeks and examined their ovulatory response to exogenously administered gonadotropins. Kepone induced persistent vaginal estrus (an estrogenic effect) in the majority of females at all ages examined and in all the treated groups. No NOAEL was determined for this estrogenic effect. This effect also was displayed by females in the positive control group exposed to estradiol. In the high dose group, kepone also reduced the number of ovulated oocytes after exogenous administration of gonadotropins at 4 and 6 weeks; this effect was present, but not statistically significant, after 2 weeks of treatment. This effect was not seen in the estradiol positive control group at any time point. None of the effects in this study displayed an NMDR.

A.1.k Bisphenol A

The selection criteria for studies reviewed herein on bisphenol A (BPA) differ from other sections in the review. Since the data and studies on this chemical have been subject to scores of scientific reviews by governmental and regulatory agencies, the studies included here were selected from those identified by these groups as being "adequate for evaluation" and "high utility" in the respective assessments. These groups based these determinations upon route of exposure, sufficiency of the methods and sample sizes and statistical and experimental design criteria, among others. All of these evaluations have noted limitations of the "low dose" literature that report effects at very low BPA doses, and these assessments all have concluded that current exposure levels do not pose a risk to human health. The current evaluation and this appendix does not revisit this controversy, but rather focuses solely on the shapes of the dose response curves from "adequate", "high utility" studies that used oral dosing, with a broad range of dosage levels (at least 3 treated levels and a negative control).

Throughout this section we have included notes on strengths and weaknesses of studies as described by Chapin et al. (2008) as well as their comments on utility and adequacy of the study for the CERHR evaluation process. In some instances we have included comments by an FDA review panel, a panel of the Bundesinstitut für Risikobewertung(Bfr)or one convened by the European Food Safety Agency (EFSA)(EFSA, 2010).

BPA Oral studies with *in utero* and/ or lactational exposures

A.1.k.1 Tinwell et al. (2002) – some text extracted from Chapin et al. (2008)

Tinwell et al. (2002) examined the effects of *in utero* exposure to low and high doses of BPA on sexual development of male rats. SD and Wistar-derived Alderley Park rats (6-7/group/strain) were gavaged on GD 6–21 with BPA at 0, 0.020, 0.100, or 50 mg/kg /d. A positive control group initially received 200 µg/kg/d ethinyl estradiol, but the dose was reduced to 100 µg/kg/d between GD 11 and 14 due to maternal toxicity. At birth, pups were counted, sexed, weighed, and AGD was measured 24 hours following birth. Pups were weighed throughout the post-lactation period. Ages at PPS preputial separation, VO, and first estrus were assessed. Males were killed on PND 90–91 and females on PND 98. Liver and reproductive organs were weighed. Daily sperm production was determined. Data were analyzed using the litter and grouped individuals as the statistical unit.

The only statistically significant effect observed in female rats exposed to BPA was a 1.6-day delay in VO in Alderley-Park rats of the high-dose group. In Alderley Park males of the high-dose group, statistically significant reductions were observed for total sperm count/testis [12% lower than controls**]**, sperm count/g testis [10% reduction], daily sperm count/testis [12% reduction], and daily sperm count/g testis [10% reduction]. In both rat strains, BPA treatment had no effect on litter size, sex ratio, birth weight, AGD, first day of estrus, or age of PPS. There were no significant effects on weights of liver, ovary, cervix, uterus, vagina, testis, epididymis,

seminal vesicle, or prostate. Rats treated with ethinyl estradiol also experienced decreased sperm counts, in addition to decreased weights of male reproductive organs and advanced age of VO. Several findings observed by Chahoud and colleagues (Schönfelder et al., 2002) were not duplicated in this study.

NTP CERHR evaluation: Strengths of this study are the range and appropriateness of selected measures, the use of 2 strains of rat, the verification of dosing solutions, and the use of ethinyl estradiol, which produced expected responses. An unfortunate weakness is the small sample size of 6-7 dams/strain/group. Nevertheless, data were appropriately analyzed with the litter as the experimental unit, and significance judgments were apparently based on 7/group. Modest effects were noted in male and female offspring in the 50 mg/kg exposure group, whereas effects on the lowest doses in this study were not observed. This study is adequate and of high utility for the evaluation process.

There were no NMDR noted among a >50 dose response curves among two rat strains and two sexes.

A.1.k.2 Cagen et al. (1999b) – some text extracted from Chapin et al. (2008)

Cagen et al. (1999b) conducted a study to examine the effects of prenatal and lactational BPA exposure on reproductive development of rats. Female Han-Wistar rats were dosed for 2 weeks prior to mating, during a 2-week mating period, and during the gestation and lactation periods (28 rats/group); dosing was through drinking water containing BPA at 0.01, 0.1, 1.0, or 10 ppm (0.001–0.004, 0.008–0.038, 0.100–0.391, or 0.775–4.022 mg/kg/d). Two negative control groups of 28 rats each were given un-dosed drinking water. A positive control group of 28 rats was given drinking water with DES at 0.1 ppm (0.006–0.036 mg/kg/day). Dosing solutions were prepared weekly, and concentrations were verified. Dams were evaluated for food and water intake, weight gain, and fertility endpoints. Pups were sexed, weighed, and counted at birth. During the postnatal period, pups were evaluated for growth and survival. At weaning on PND 22, up to 4 males/litter (86–109 pups/group) were randomly selected to continue in the study until 90 days of age. At necropsy, brain, liver, kidneys, and reproductive organs were weighed, daily sperm production was determined, and testes were examined histologically. The litter was considered the experimental unit in statistical analyses.

In the BPA groups, there were no statistically significant effects on dam body weight gain or food or water, fertility, mating, gestation index and duration, live litter size, or pup survival and body weight gain during the postnatal period. Male sex ratio was affected in an NMDR manner being increased in the 0.1 ppm BPA group (56.7% males versus 48.4% in control but not at any other dose level**.** Dams in the DES group experienced these effects: decreased body weight gain and food intake; increased duration of gestation; smaller litter size at birth; and decreased pup survival in the postnatal period. In adult F1 offspring from the BPA groups, there were no statistically significant effects on terminal body weight or organ weights including prostate, epididymis, preputial gland, seminal vesicle, or testis. There were also no statistically significant effects on epididymal sperm concentration, efficiency of sperm production, or daily sperm

production. No histopathological alterations were observed in the testis. Reproductive development in male offspring was also unaffected by prenatal exposure to DES.

NTP CERH evaluation: Significant strengths of this study include the large number of dose levels and animals per dose level and the technical care with which the study was performed, as well as the inclusion of a positive control group and two negative controls. The lack of much effect with DES treatment is a weakness. Although only weak effects were observed for the DES positive control the panel considered this study adequate and of high utility.

In summary one of the effects in this study displayed an NMDR, but the same effect was not seen in any other study of BPA in rats in this dose range.

A.1.k.3 Tyl et al. (2002)

Tyl et al. (2002) executed a multigenerational study of BPA in SD rats in which P0, F_1, F_2, and F_3 rats were exposed to BPA during gestation and lactation and directly through feed after weaning at dietary doses of 0, 0.015, 0.3, 4.5, 75, 750, or 7500 ppm (equivalent to about 0, 0.001, 0.02, 0.30, 5, 50, and 500 mg/kg/d, with 30 P0 pairs per sex per group). At the 7500 ppm dose there were fewer pups and live pups/litter, and body weight gain of pups was lower during the lactation period. Delayed puberty in both males and females of the 7500 ppm group was attributed to reduced body weights. BPA exposure during development did not increase the weight of the prostate in adult rats, although some decreases in epididymal sperm concentration and daily sperm endpoints were each observed in 1 generation of males from the high-dose group. The study authors identified an offspring and reproductive NOAEL of 750 ppm (~50 mg/kg/d). A systemic NOAEL for adult rats was identified at 75 ppm (~5 mg/kg/d) by the study authors; therefore, BPA was not considered a selective developmental or reproductive toxicant.

NMDRs were identified for some the endpoints below; -each curve had 6 dose groups compared to controls:

- Body and organ weights at necropsy (Table 2 of Tyl et al. (2002). Of 52 dose response curves (6 x 52=312 individual contrasts)
    - Six NMDRs in the male data that did not repeat from generation to generation
    - Three NMDRs in the female data that did not repeat from generation to generation
    - Histopathology of 11 organs in 4 generations of male rats (Table 3 of Tyl et al. (2002) No NMDRs
    - Histopathology of 12 organs in 4 generations of male rats (Table 4 of Tyl et al. (2002)No NMDRs
- Eleven reproductive parameters (Table 5 of Tyl et al. (2002) in male and female rats over 4 generations
    - Two NMDRs seen in only one of the 4 generations

- Seventeen reproductive development patterns (Table 6 of Tyl et al. (2002)
    - Three NMDRs seen in only one of three generations

In the evaluation of the authors of this state of the science evaluation, there were no reproducible NMDRs in the Tyl *et al.* (2002). Given the large number of statistical comparisons the number of NMDRs seen in the study does not appear to exceed chance variation.

NTP CERH evaluation: This study has numerous strengths, including the quality and number of the endpoints evaluated, the number of dose groups and generations examined, and the confirmation of dosing solutions. This study incorporated screening-level endpoints within the context of a multigenerational study. As such, it addresses gross issues but does provide helpful data regarding the NOAEL. This study is adequate and of high utility for the CERH evaluation process.

In summary the authors of the current state of the science document note that several NMDRs were seen in this study; however, none of these were considered to be reproducible since the same effects were not seen in a similarly exposed male or female rat from a different generation.

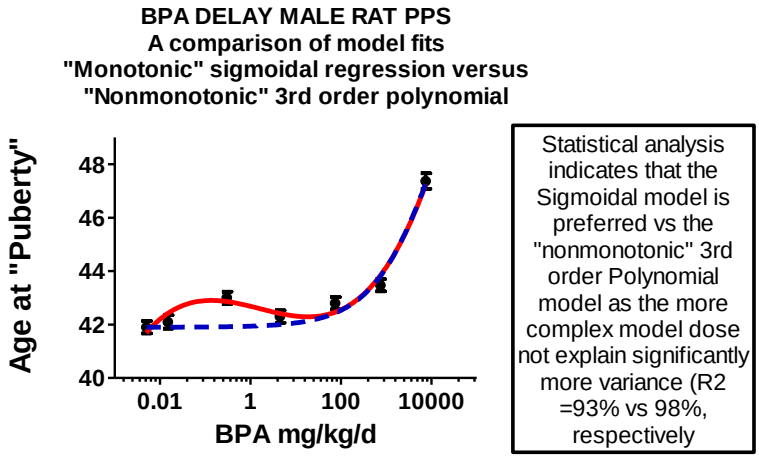

Figure A.11 Male Rat Preputial Separation

A.1.k.4 Tyl et al. (2008b)Tyl *et al.* (2008) (including 159 pages of supplementary data files)

BPA was administered to mice in the diet using a two generation study protocol at 0, 0.018, 0.18, 1.8, 30, 300, or 3500 ppm (0, 0.003, 0.03, 0.3, 5, 50, or 600 mg BPA/kg/d, 28 per sex per group) and an estradiol positive control (0.5 ppm in the diet; 28 per sex). There were no BPA-related effects on the following: adult mating; fertility or gestational indices; ovarian primordial follicle counts; estrous cyclicity; precoital interval; offspring sex ratios or postnatal survival; sperm parameters or reproductive organ weights or histopathology (including the mammary glands of F1 females or male testes and prostate). In the highest dose groups, systemic effects included centrilobular hepatocyte hypertrophy, reduced body weight, increased kidney and liver weights, centrilobular hepatocyte hypertrophy, and renal nephropathy in males. BPA also reduced F1/F2 weanling body weight, reduced weanling spleen and testes weights (with seminiferous tubule hypoplasia), and delayed PPS. At lower doses (1.8 and 30 ppm), male mice displayed consistent monotonic increases in kidney weight, being statistically significant at 30 ppm in both generations (P0, F1 and F1-retained). The study authors concluded that BPA was not a selective reproductive or developmental toxicant in mice.

NTP CERH evaluation: Strengths include the large number and range of doses examined, the rigor with which the study was performed (including evaluation of phytoestrogen content of feed), the large sample size in each group, the number of additional animals per litter that were retained and examined, the use of a concurrent estrogenic positive control group, and the thoroughness of the histologic evaluation.

This study is adequate and of high utility for the CERH evaluation process.

The following NMDRs were seen in the data.

- Seminal vesicle weight in F1 males was increased at 0.018 ppm and 300 ppm but not in any other dose groups or in P0 or extra retained F1 males.
- F2 but not F1 offspring survival indices were reduced at 300 ppm but not at any other dose.
- F1 male (but not F2 male and not F1 female) weanling thymus weight was increased at 300 ppm.
- F1 but not F2 weanling epididymal weight was increased at 0.18 ppm.
- Seminal vesicle weights in weanling males was increased at 300 ppm in F1 males, but seminal weight was reduced in F2 males.

In the opinion of the authors of this state of the science evaluation none of these were consistent among the generations/cohorts out of several hundred individual dose response curves

A.1.k.5 Ema et al. (2001)

Ema et al. (2001) conducted a multigenerational reproductive toxicity study of BPA in CD rats. Five-week-old males and 10-week-old females were gavaged with 0, 0.0002, 0.002, 0.020, or 0.200mg/kg/d BPA. Males were dosed for 10 weeks before mating and during the mating period; females were dosed from 2 weeks before mating, and during the mating, gestation, and lactation periods. At weaning on PND 22, 1 or 2 F1 weanlings/litter/sex (25 /sex group) were selected to continue in the study. Dosing of F1 began on PND 23 and continued for 10 weeks before mating and through the mating period, gestation and lactation periods. Twenty-five F2 weanlings/sex/group were selected on PND 22. Beginning on PND 22, male F2 rats were dosed for 4 weeks, and females were dosed for 11 weeks before being necropsied. Endpoints examined in adult rats included clinical signs, body weight, and food intake. Fertility, copulation, and gestational indices were examined in mating rats. Vaginal smears were evaluated for 2 weeks before mating in F0 and F1 females and at 9–11 weeks of age in F2 females. Sperm endpoints were measured in F0 and F1 adult males. Serum hormone levels were measured in 6 adult F0 and F1 males and proestrous females. Offspring were examined for the attainment of VO or PPS. AGD in pups was examined at several time points during the lactation period and through adulthood. Behavioral testing was conducted at 5–7 weeks of age. The litter was considered the experimental unit in data obtained before weaning. In F0 and F1 adult animals, there were no treatment related effects on clinical signs, body weight gain, or death.

Of the numerous measurements in 3 generations the following effects displayed an NMDR.

- AGD in males and/ or females was measured over 50 times (different ages, two generations, two sexes) and occasional small NMDR responses were displayed, whereas in most cases no effect was noted. Effects seen in the F1were not replicated in the F2.
- F2 males (but not F2 females or F1 males or females) displayed delayed negative geotaxis reflex (by less than one day)
- F1 males (but not F1 females or F2 males or females) displayed accelerated mid-air righting reflex (by 1.2 days)
- Of about 60 organ weights taken at necropsy of F1 and F2 weanlings, two showed an NMDR in males but not females. These effects were not present in F1 females or at the adult necropsy. One of the two NMDR was also seen in F2 weanling males but not F1 females or F2 adults of either sex.
- F1 adult males displayed an NMDR reduction in testis weight.
- Of about 24 sperm measures taken in F1 and F0 males, one of 24 was NMDR, but the effect is rather trivial with a decrease in abnormal sperm from 1.6% in controls to 0.6% in a mid-dose group in F1 but not F0 adult males.

NTP CERH evaluation: This well-designed comprehensive low-dose assessment of potential bisphenol A-related effects on multiple generations of rats examined a wide variety of hormonally sensitive endpoints. The study had appropriate power with an appropriate number of rats per group. Route of administration (oral) was appropriate. The concentrations of the

dosing solutions were verified (both prior and after). This study is adequate and of high utility for the CERHR evaluation process.

In summary, no consistent low dose effects were seen with BPA treatment in the current study in any generation or in either sex. No reproducible, consistent NMDRs were seen.

A.1.k.6 Kobayashi et al. (2010)

This study was conducted to examine the effects of low-dose exposure to BPA on reproduction and development in two generations of mice. Pregnant female C57BL/6J mice (P0) were fed a diet containing low doses of BPA(0, 0.33, 3.3, or 33 ppm) from GD6 through PND22, and the F1 and F2 weanlings from each P0 and F1 dam group, respectively, were also fed these same concentrations of BPA *ad libitum* until sacrifice. There were no treatment-related changes in body weight, body weight gain, food consumption, gestation length, or the number of live births on PND1 in P0 dams. Sex ratio and viability were similar in all F1 pups. No treatment-related changes were observed in body weight, food consumption, developmental parameters, AGD, or weight of any of the organs (liver, kidney, heart, spleen, thymus, testis, ovary, or uterus) in F1 and F2 adults in either sex. There were no treatment-related effects of BPA on cauda epididymal sperm count or sperm motility in F1 or F2 males. In addition BPA did not induce any gross or histopathological lesions in the testes or epididymides.

NTP CERH evaluation: This multigenerational study is adequate and very useful for the evaluation since it administered three low to moderate dosage levels of BPA via a relevant route of administration, and it was properly designed and analyzed, using the litter as the unit of analysis. These findings indicate that dietary exposure to BPA between 0.33 and 33 ppm does not adversely affect any measure of reproduction or development as assessed in two generations of mice. The lack of low dose effects of BPA in the mouse is consistent with the results of other multigenerational studies in this species.

A.1.k.7 Kobayashi et al. (2012)

The current study exposed pregnant P0 SD rats to low doses of BPA (0, 0.33, 3.3, or 33 ppm, n=10/group) in the diet from GD6 to PND21. F1 pups were not exposed to BPA directly after weaning and were necropsied at 5 weeks or 3 months of age. There was no effect on P0 reproductive performance or F1 litter sizes or pup weights.

A few monotonic low dose effects were seen at the 5 week necropsy, but none of these were NMDRs, and these effects were not seen at 3 months of age in F1 males. The only NMDR noted in the current study was seen for increased body weights of F1 males but not females at 0.33 ppm at 12 and 13 weeks of age (2 of 11 measurements of body weight) and in F1 females at 6 and 11 weeks of age at 33 and 0.33 ppm, respectively.

A.1.k.8 Stump et al. (2010)

This study was conducted to determine the potential of BPA to induce functional and/or morphological effects to the nervous system of F1 offspring from dietary exposure during gestation and lactation according to the OECD and USEPA guidelines for the study of developmental neurotoxicity (DNT). BPA was offered to female SD) rats (24 per dose group) and their litters at dietary concentrations of 0 0.15, 1.5, 75, 750, and 2250 ppm daily (target

doses of 0, 0.01, 0.1, 5, 50, and 150 mg/kg/d) from GD 0 through lactation day 21. F1 offspring were evaluated using the DNT test guideline.
Maternal body weight gains at 750 and 2250 ppm during pregnancy were reduced by 9.5% and 22.4%, respectively (GD 0-20). BPA did not affect the length of gestation or induce any additional overt maternal toxicity, nor did it or affect litter sizes at birth, sex ratio or pup survival. Maternal kidney and liver weights were not reduced at weaning of the F1.

Some transient NMDR were reported for F1 pup weights during lactation. F1 pup weights were reduced from PND 7 to 14. Statistically significantly higher mean male body weight gains were noted in 0.15 and 75 ppm groups during PND 14-21 compared to the control group. In addition, mean female pup body weights in the 75 ppm group were statistically significantly higher on PND 4 (pre-culling), mean male body weights in the 75 ppm group were statistically significantly higher during PND 4 (pre- and post-culling) through 11 and on PND 21, and the mean male body weight in the 0.15 ppm group was statistically significantly higher on PND 21 as compared to the control group values. They reported that differences from the control group were generally slight (≤ 8.5%). Mean post-weaning offspring body weights and body weight gains in the 0.15, 1.5, 75, 750, and 2250 ppm groups were unaffected by test substance exposure, with no statistically significant difference for any test substance-treated group.

Mean ages of attainment of PPS were unaffected by test substance exposure. Mean ages of attainment of VO were unaffected by test substance exposure with no statistically significant difference for any test substance-treated group.
F1 offspring survival was not affected by BPA treatment, and no gross abnormalities were noted in either sex.

Based on maternal and offspring body weight reductions, the NOAEL for systemic toxicity was 75 ppm (5.85 and 13.1 mg/kg/day during gestation and lactation, respectively), with no treatment-related effects at lower doses and no NMDR observed for any parameter.

Summary of the study evaluation by FDA: This multigenerational study is adequate and very useful for the evaluation since it administered several low, moderate and high dosage levels of BPA in the diet, a relevant route of administration. In addition, it used large sample sizes per group and it was properly designed and analyzed, using the litter as the unit of analysis. These findings indicate that dietary exposure to BPA between 0.01 and 150 ppm does not adversely affect gestation, fertility, fecundity or the ability to rear F1 pups throughout lactation. There were no effects of BPA on F1 pubertal landmarks in either male or female offspring, F1 viability or F1 morphology. The lack of reproductive effect of BPA in the current study is consistent with other multigenerational studies of BPA in rodents.
NMDR-

> Body weights were taken >15 times in the current study in P0 males and females. Comparing each of the five BPA-treated groups to controls indicated NMDRs at 5 preweaning ages at 75 ppm in males but in P0 females, only body weight was affected and only at one of these ages. These effects did not persist after weaning

In one of eight measures of F1 weight gain during lactation (two sexes, weight gain reported for four time periods) male, but not female, weight gain was increased at 0.15 and 75 pp.
None of the >40 morphometric measures of brain structure or weight affected in F1 males or females at any age and no NMDRs were detected.
While the authors concluded that there we no behavioral alterations in F1 males or females other groups have interpreted the Biel Water Maze results to show an NMDR low-dose effect in males, but not female F1 rats. The effects or lack of effects in the Biel Water Maze have been a controversial NMDR issue as described in the EFSA BPA review of 2010.

EFSA review: Overall, the Panel concluded that the study by Stump *et al*. (2009) cannot be used for the assessment of the effects of BPA on learning and memory due to methodological limitations (see part I). A number of studies addressing other neurobehavioural endpoints (e.g. learning and memory behaviour, anxiety-related behaviour and gender-specific behaviour) were considered invalid or inadequate for risk assessment purposes by the Panel. The Panel does not consider the currently available data as convincing evidence of neurobehavioural toxicity of BPA."

A.1.k.9 Howdeshell et al. (2008)

The following is quoted from the FDA draft review of Howdeshell et al. (2008):

"Howdeshell *et al*. (Howdeshell et al., 2008) addressed BPA exposure on male reproductive tract development. This study adhered to many of the criteria. Multiple doses of BPA were administered orally to pregnant Long Evans rats from gestation day 7 through postnatal day 18. As a positive control for hormone disruption, multiple doses of EE were similarly administered to pregnant rats. The authors chose the Long Evans rat because of its common use for reproductive behavior studies and reported reproductive tract susceptibility to endocrine-disrupting and antiandrogenic chemicals. This study addressed repeatability by modeling the dosing protocol and doses after published studies from other laboratories.
Briefly, the authors used adult Long Evan rats (Charles River) that were approximately 90 days of age, mated by supplier, and shipped on GD2. Animals were housed in polycarbonate cages that were 'without significant wear' (stated to control for BPA contamination). Animals received Purina 5008 diet during gestation and lactation and 5001 until study termination. On GD7 animals were treated with EE doses were 0.05, 0.5, 1.5, 5 15, or 50 µg/kg/d and BPA at 2, 20, or 200 µg/kg/d. The study was conducted in 2 blocks with 13-29 dams per treatment group in the first block and 6-14 dams per treatment group for the second block. Weanlings were housed two per cage with the exception of animals used for lordosis behavior analysis. Maternal endpoints included body weight gain during gestation (GD7-GD20) and lactation (PND2 – PND18). Pups were examined for sex, body weight, and anogenital distance on PND 2; pup weight, sex, and number of female-like areolae on PND 14; fetal/neonatal mortality. Necropsies on male offspring began on PND 150 for block 1 and PND 229 for block 2. Animals were examined for serum hormone levels (estradiol, testosterone, corticosterone, total T4,

luteinizing hormone, prolactin), number/location of retained nipples, hypospadias, cleft phallus, cleft prepuce, vaginal pouch, exposed os, abnormal glans penis, epididymal agenesis, testicular malformations, agenesis of vas deferens/prostate/seminal vesicles, hydroureter, hydronephrosis, bladder stones, blood in the bladder, organ weights (ventral and lateral prostate, seminal vesicles, testes, epididymis, glans penis, levator ani-bulbocavernous muscle, Cowper's gland, liver), epididymal sperm counts, kidney weight, adrenal weight, spleen weight, heart weight, lung weight, brain weight, and pituitary weight. Neonatal measurements were made by blind observers, pathology evaluation conducted by a contracted pathology laboratory, and statistics (ANOVA, ANCOVA) based on the litter not the individual pup.

A number of positive findings for maternal and pup endpoints were reported with EE treatment. No significant findings were reported for maternal endpoints with BPA exposure. Ventral prostate weight was unaffected by BPA treatment whereas EE treatment at 50 µg/kg/d resulted in a significant decrease as compared to controls; terminal body weight was also only affected in this group (decreased). Ventral prostate hyperplasia was reported to appear as dose related for EE (2/29 at 5 µg/kg/d (NS), 11/24 at 50 µg/kg/d ($p< 0.001$)) while only noted in one 20 µg/kg/d BPA treated male (1/18) as compared to zero incidence in control animals (0/31). Seminal vesicle and paired testis weights weight were unaffected by BPA treatment but decreased in animals treated with EE at ≥ 5 µg/kg/d. The study does report in the first block a significant increase in incidences of testicular degeneration in 35% of the 200 µg/kg/d BPA group and a non-significant increase in 16.7% of the 20 µg/kg/d BPA group. This effect was reported in the absence of effects on testes weight or epididymal sperm counts. Additionally, no significant findings were reported in the second block with regard to testis degeneration. When the blocks were pooled, the overall findings were non-significant.

This appears to be a well conducted study addressing multiple endpoints and many of the criteria. Although the authors used polycarbonate cages, they indicated that the cages were 'clear and without evidence of significant wear'. This and the municipal drinking water are possible sources of environmental contamination. One criticism of the study was the lack of range in the dose response assessment; utilization of additional BPA doses, including a high dose, would have aided in the interpretation of the results. However, this criticism is mitigated by the extensive dose response analysis of the positive control compound which characterized the sensitivity of the animal model. Otherwise, the remaining criteria were adequately addressed. This study's endpoints included a developmental assessment of the male reproductive system, and as such relates to the overall effects of BPA on the reproductive tract. No significant findings were reported for the prostate due to BPA exposure. The significance of the findings with regard to testicular degeneration of the first block of animals is diminished due the extensive negative related findings in concert with the lack of effect of the second block and pulled samples. Due to the adherence to a number of criteria, this study is useful for risk assessment. The overall findings suggest a severely limited or minimal overall effect of BPA exposure on male reproductive development and a NOAEL of 200 µg/kg/d (the highest dose tested)."

In summary as none of the measures taken in this study were affected by BPA it does not provide support for the hypothesis that BPA induces low-dose NMDRs on endocrine-sensitive endpoints.

A.1.k.10 Ryan et al. (2010)

The following is quoted from the FDA draft review of this study:

"*In utero* and lactational exposure to bisphenol A, in contrast to ethinyl estradiol, does not alter sexually dimorphic behavior, puberty, fertility and anatomy of female LE rats.

Ryan et al. (2010) is the second part of an original study published by Howdeshell et al. (2008) and was to determine the effects of BPA at low doses and a concomitant positive control ethinyl estradiol (EE2) on characterized sexually dimorphic behaviors and reproductive development and function in female rat offspring following maternal exposure during gestation and lactation. Female Long Evan rats (Charles River Laboratories, Raleigh NC) were individually caged in clear polycarbonate cages. Animals were fed (*ad libitum)* with Purina Rat Chow 5008 while pregnant or lactating and 5001 was fed to weanlings and adults. Pregnant females (two stocks; 13-29 dams/dose in first stock, 6-14 dams/dose in second stock) were gavaged with vehicle (corn oil), EE2 at 0.05, 0.5, 1.5, 5, 15, or 50 µg/kg/day, or BPA at 2, 20, and 200 µg/kg bw/day from GD 7 to PND 18 (GD 1 = sperm positive). For sexually dimorphic behaviors, lordosis, saccharin preference, and Figure-8 maze tests were conducted. For reproductive development and function, anogenital distance (Alonso-Magdalena et al., 2010) on PND 2 and corresponding body weights, age at vaginal opening (VO) and corresponding body weight, cleft phallus at weaning and body weights, genitalia urethra-vaginal distance (UVD) at necropsy, fecundity, and fertility were examined in the female offspring. The neurodevelopment component of this study consisted of three neurobehavioral assays on a F1 generation of females to assess potential effects of BPA gestational and lactational exposure on sensory, locomotor, and sexual behavior endpoints. Data were analyzed by PROC GLM for one-way analysis of variance (Casanova et al., 1999) followed by a t-test if the F value was significant ($p < 0.05$) or by Dunnett's test if the F value was not significant. For neurobehavioral endpoints, ANOVA was also used for statistical analysis with EE2 and BPA being analyzed individually if an initial significance was noted. Analyses were based on the litter mean values.

According to the study authors, EE2 at dosage of ≥ 1.5 µg/kg bw/day significantly reduced F0 dam body weight and weight gain during the gestation. In addition, reduced number of implantation site/scars, and decrease in number of live pups and body weight of pups on PND 2 were also observed in 50 µg/kg bw/day. Significant positive results including dose-dependent trends were reported in the EE2 exposed groups for AGD, VO, fecundity and fertility, and cleft phallus. Treatment of EE2 also reportedly significantly increased urethral slit depth and length and reduced urethral-vaginal distance at PND 300. For the neurobehavioral assessments, significant positive results were reported in EE2 exposed groups for saccharin preference and lordosis behavior, but not in the figure-8 maze. No significant results were reported for the

reproductive/morphological measurements and neurobehavioral assays at any level of BPA exposure tested.

The study appears to be well designed and executed. A sufficient amount of animals were tested for the targeted endpoints to provide sufficient statistical power to make relatively solid conclusion(s). The statistical methods used in the study were appropriate and generally accepted for the data generated from these kinds of studies. Oral exposure was used and the positive control EE2 demonstrated the estrogen sensitivity of each test by producing results on each reproductive-developmental or neurobehavior endpoint tested (except Figure-8 maze locomotor activity test) over a sufficient dose range, thereby validating the model. The dose levels of BPA in this study have covered the generally recognized "low-dose" range for BPA.

Limitations included: the use of polycarbonate cages as sources of potential environmental contamination in the study, although the study authors do note that only clear and without evidence of wear; the use of phytoestrogen containing diets (5001 and 5008); vaginal cytology of F1 females was likely not performed as an endpoint in EE2 or BPA treated groups; and the lack of use of high as well as low doses of BPA to fully characterize the dose response assessment. Utilization of additional BPA doses, including a high dose, would have aided in the interpretation of the results. However, this criticism is mitigated by the extensive dose response analysis of the positive control compound which characterized the sensitivity of the animal model. The use of 50 µg EE2/kg/day in this study may have produce excessive toxicity for certain reproductive and developmental parameters, such as number of live pups observed. Reported in the previous publication, available number of pups or F1 offspring was significantly reduced (6.5 pups/litter vs. 12.1 pup/litter for 50 µg EE2/kg bw/day vs. control, Table 1 in Howdeshell et al. (2008).

Based on clear responses to EE2 on selected endpoints in LE rats (thereby demonstrating efficacy of the model to characterize an estrogenic response), the dose-dependent responses of reproductive and/or developmental endpoints to EE2, and the available comparison data between different animal species, this study is considered relevant to human health and useful for safety evaluation of BPA. The findings in F1 female LE offspring suggest that BPA, at the doses tested and within the conditions of the study, has no effect on female offspring age at VO and the body weight, AGD on PND 2, external genitalia, fecundity, fertility, and the neurobehavioral endpoints tested. Based on the findings of this study, a NOAEL can be established at ≥ 200 µg/kg bw/day (the highest dose tested)."

Since none of the measures taken in this study were affected by BPA it does not provide support for the hypothesis that BPA induces low-dose NMDRs on endocrine-sensitive endpoints.

The following is quoted from BfR (2010):

"…. The test design of the study of Ryan *et al*. had a particular focus on the investigation of estrogen-sensitive endpoints, a pivotal issue in the current scientific debate. The results

revealed no adverse effects in the low-dose range on behaviour and the development of female rat offspring whose dams were treated with bisphenol A during gestation and lactation. In contrast, female offspring from dams treated under the same conditions with ethinyl estradiol showed irreversible abnormal behaviour, impaired fertility and malformations of the external genitalia.

According to BfR (2010), the results of the two studies do not substantiate the concerns for a specific toxic potential of bisphenol A adverse to neurological and behavioural development."

In summary for the Howdeshell et al. (2008) and Ryan et al. (2010) studies the authors of the current state of the science document concluded that no NMDRC were noted for any endpoint for either BPA or ethinyl estradiol out of more than 40 dose response curves for each chemical.

Below (*A.1.k.*11 and *A.1.k.*11) are discusssed two Hershberger Assay publications assessing potential androgenic and antiandrogenic effects of Oral BPA with nine dose groups ranging from 1 to 1000 mg/kg/d

A.1.k.11 Kim et al. (2002a)

A.1.K.12 Yamasaki et al. (2003)

These studies investigated the potential androgenic and anti-androgenic effects of BPA in the OECD/USEPA EDSP Test Guideline for the rat Hershberger assay; they were carried out using castrate immature male rats. In these studies immature castrated male rats were dosed orally by gavage for 7 or 10 days with BPA (6 males per dose group). The BPA dosage levels used in Study 1 were 1, 5, 10, 25, 100 and 1000 mg/kg/d whereas Study 2 used 50, 200, 600 mg/kg/d BPA. In this protocol, BPA was administered to castrate immature males to detect androgenic effects and concurrently with T (TP sc) to screen for antiandrogenicity. In these studies no androgenic or antiandrogenic effects were detected.

NTP CERHR evaluation:
Strengths: Specific *in vivo* assessments of the androgenicity and antiandrogenicity of BPA in an internationally validated protocol.
Limitations: Study is a mechanistic study in castrate male rats and effects in such studies are not generally considered as adverse, as per WHO definition
Conclusions: BPA does not display androgenic or antiandrogenic activities *in vivo* when administered orally to castrate immature male rats at a doses ranging from 1 to 1000 mg BPA/kg/d.

No NMDRC were noted in these studies.

BPA Oral studies with exposures to adult male rats

A.1.k.13 Ashby et al. (2003)

Ashby et al. (2003) examined the effects of BPA exposure on sperm production in rats. The study attempted to replicate earlier findings from **Sakaue et al. (2001)**. **Sakaue et al. (2001)** reported that oral exposure of sexually mature male rats to BPA at 13 weeks of age led reduced testis sperm production at 14 and 18 weeks of age with dose-related (monotonic) effects occurring over the dose range 20 microgram/kg-200 mg/kg BPA, with an absence of activity over the dose range 2 ng/kg-2 microgram/kg BPA. There was no evidence of a dose response relationship over the active dose range (five orders of magnitude range).

Ashby et al. (2003) conducted four independent studies following the protocol used by Sakaue *et al*. (2001), Doses of 20 microgram/kg, 2 mg/kg, or 200 mg/kg BPA were administered to adult SD rats over PND 91-97, and the studies were terminated when the rats reached the age of 18 weeks. Three different rodent diets were employed (RM3, Purina 5002, and CE2), the last of which had been used by Sakaue et al. (2001). BPA failed to give any evidence of endocrine disrupting activities, including the changes in daily sperm production (DSP) reported by Sakaue et al. (2001). There were no statistically significant effects of BPA exposure on sperm count, daily sperm production, or weights of body, liver, kidney, testis, prostate, epididymis, or seminal vesicle. The study authors concluded that there was no evidence in their study that BPA affected reproductive organ weights or daily sperm production, in contrast to observations of the Sakaue et al. (2001) study.

NTP CERHR evaluation: This study reports a well conducted, comprehensive assessment of the potential effects of BPA delivered by 6 daily doses on daily sperm production. The 6-day treatment period is a (understandable) weakness.
This study is adequate and useful for the evaluation process.

No NMDRC were noted under any of the conditions used in either of the two studies.

A.1.k.14 Chitra et al. (2003)

Chitra et al. (2003), examined the effects of BPA on the reproductive system of male rats. Animals were given ‘‘standard commercial laboratory chow.’’ [Bedding and caging materials were not reported.] Six 45-day-old male Wistar rats/group were orally dosed [gavage assumed] with BPA (97% purity) in olive oil at 0, 0.0002, 0.002, and 0.020 mg/kg/day for 45 days (six rats per group). Testes, epididymides, seminal vesicles, and ventral prostate were weighed. Epididymal sperm counts and motility were assessed. Antioxidant enzyme activities were measured in sperm.

BPA treatment did not affect body weight. Absolute and relative (to body weight) weights of testis and epididymis and were reduced, and absolute and relative ventral prostate weights

were increased at all dose levels. Sperm motility was decreased at all dose levels, and sperm counts were reduced at the mid and high-dose. There were dose-related decreases in activity of superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase in sperm at all dose levels. Hydrogen peroxide generation and lipid peroxidation in sperm increased dose-dependently at all dose levels.

NTP CERHR evaluation: Strengths include the use of oral and low multiple doses and appropriate measures. A weakness includes the marginal sample size. This study is adequate for inclusion but of *limited utility* based on small group size.
All effects in the Chitra et al. (2003) study displayed monotonic responses.

A.2 Selective ER Modulators - SERMS

SERMS are synthetic drugs used as options for the treatment of osteoporosis in women, breast cancer and other conditions arising from adverse effects of estrogens. In this regard, SERMS have been developed and studied *in vitro* using cell lines from different estrogen-responsive tissues. They have also been studied in rat models (orchidectomized males and ovariectomized female rats) for their ability to have selective, beneficial effects on tissues like bone, without inducing potentially adverse effects on other tissues (for example, breast and prostate in females and males, respectively). These chemicals can act as estrogens in some tissues and as antiestrogens in other tissues.

One of the target tissues beneficially affected by estrogens in postmenopausal women and aging males is bone. SERMs typically have a positive effect on bones, the serum lipid profile and the cardio-vascular system and some may protect against the development of some estrogen-dependent neoplasms. The first SERM used as a pharmaceutical was tamoxifen, but due to its negative, stimulatory effect on the endometrium, it is currently not indicated in osteoporosis. Similarly, raloxifene, also reduces the reduction of risk of bone fractures in an estrogen-like manner, but it does not stimulate endometrial or breast tissues. However, raloxifene aggravates vasomotor symptoms, and its bone-protecting effect is not as great as estradiol or some other SERMS. At present, third generation SERMs (ospemifene, lasofoxifene, bazedoxifene) are being introduced on the market or researched in clinical trials. Some other SERMs, such as arzoxifene or levormeloxifene have reportedly been withdrawn from the trials because they did not perform as well in phase III trials as expected. Many other compounds possessing estrogen activity have been identified, patented and studied *in vitro* and *in vivo,* and they display a wide array of potencies and target tissue specificities.

A.2.a Idoxifene (IDO)

A.2.a.1 Treinen et al. (1998)

A series of reproductive and developmental toxicity studies were conducted with idoxifene to determine how it affected male and female rat fertility, implantation and fetal development In the male rat fertility study, young adult rats were treated by oral gavage with 0.003, 0.3 or 3 mg/kg/d for about 65 days. After 28 days of treatment males were mated and then necropsied at end of dosing the dosing period. IDO did not affect the fertility of treated male rats, but doses of 0.3 and 3.0 mg/kg/d reduced seminal vesicle, prostate and epididymal weights and epididymal sperm counts in a monotonic, dose-related manner.

In the female fertility study, rats were treated by oral gavage for 2 weeks prior to mating until insemination with 0.003, 0.03 or 3 mg/kg/d. IDO treatment at doses of 0.03 and 3 mg/kg/d disrupted estrous cycles, impaired fertility and increased preimplantation loss. When female rats were treated with IDO at the same doses from days 0 to 6 after mating, preimplantation

loss was increased at 0.03 and 3 mg/kg/d; treatment from days 6 to 17 after mating increased maternal and fetal death, induced fetal edema and developmental delays at 3.0 mg/kg/d. All IDO effects were monotonic responses (>50 individual dose response curves).

A.2.b Tamoxifen (TMX)

In the analysis of the following papers on TMX, one NMDR was evident in the OECD 407 assay, displaying an increase in relative uterine weight at the low dose with reduced weights in the higher dose groups. Therefore, this study had no LOEL and the low dose effect for this study was 5 µg/kg/day, the NOEL. However, in the one generation study by Yamasaki et al. (2005) TMX induced adverse effects at doses as low as 0.12 µg/kg/day (also no NOEL in this study). F1 female rats exposed to doses of 0.6 and 3 µg/kg/day displayed malformations of the external genitalia, and P0 fecundity was reduced at 3 µg/kg/day. Taken together, these results indicate that even though an NMDR seen at 5 µg/kg/day in the OECD 407 study at the lowest dose tested, this NMDR would not alter the determination of a NOAEL for TMX as adverse effects were seen at a lower dose by Yamasaki et al. (2005) in the one generation study at 0.12 µg/kg/day.

A.2.b.1 Kim et al. (2002c) - Twenty-day pubertal female rat assay

Kim et al. (2002c) executed the EDSP pubertal female assay with several EDCs including tamoxifen. Weanling female rats were dosed orally for 20 days by gavage with TMX at 10, 50 and 200 µg/kg/day. TMX accelerated the age at VO (puberty) and reduced pituitary weights in the two highest dose groups. Ovarian, uterine, heart, liver, kidney, adrenal and necropsy body weights were reduced, and irregular estrous cycles were detected in the high dose group. These effects all displayed monotonic dose-responses. Thyroid gland weights, serum TSH, and serum T_3 displayed NMDR response, being increased at 10 and 50 µg/kg/day but not at 200 µg/kg/day. In addition, serum estradiol was increased only in the 10 µg/kg/day TMX group.

In this DSP pubertal female assay, four NMDRs were detected. Three of these were related to pituitary-thyroid function (thyroid weight, serum TSH, and serum $T_{3)}$ and one was related to ovarian estradiol production.

A.2.b.2 Kennel et al. (2003) - Twenty-eight day adult male and female OECD 407 Test

This study was executed using OECD 407 Test Guideline. In this guideline test, TMX was administered by oral gavage for 28 days at 0, 5, 30, or 200 µg/kg/day to young adult male and female rats. The study was conducted in two blocks. The study included an assessment of multiple measures: cauda epididymal sperm counts and morphology; serum TSH, T3 and T4 levels; estrous cyclicity (females were all necropsied on diestrus); body and organ weights at necropsy (ovary, uterus, thyroid gland, prostate gland [ventral and dorsolateral parts]), seminal vesicles with coagulation glands and pituitary gland); and histopathological examination of the pituitary gland, vagina, mammary gland, seminal vesicles with coagulating glands, epididymis, and prostate gland [ventral and dorsolateral parts]. Organ weights were analyzed relative to brain weights.

In males, analysis of the data from the two blocks combined revealed that body, liver, seminal vesicles, prostate, epididymis and adrenal glands were all reduced at 200 µg/kg/day.

Liver weight displayed an NMDR in one block being increased at 5 µg/kg/day, but this did not repeat in the other block and was not significant in the overall analysis**.** There were no effects on epididymal sperm numbers or morphology, testes or thyroid gland weights or serum T4, T3 or TSH levels.

In the female rat, analysis revealed that serum T4, liver, adrenal and pituitary glands and ovaries were reduced at 200 µg/kg/day, and terminal body weight was reduced at 30 and 200 µg/kg/day. Uterine weight (with cervix) displayed an NMDR being heavier at 5 µg/kg/day and lighter at 30 and 200 µg/kg/day (all groups differ significantly from control). Given what is known about the biological activity of this chemical, which acts as a SERM (being a partial estrogen agonist and antagonist), this NMDR seems biologically plausible (Figure below). There were no effects on thyroid gland weight, or serum T3 and TSH levels.

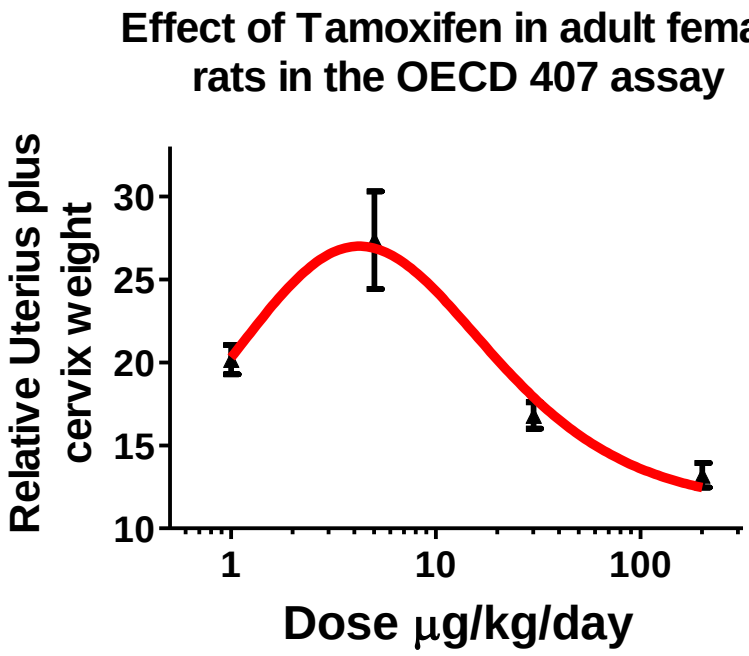

Figure A.12 Effect of Tamoxifen on Relative Uterus Weight

A.2.b.3 Yamasaki et al. (2005) - One generation reproduction test

Yamasaki et al. (2005) exposed orally pregnant CD (SD) IGS rats (TMX) at doses of 0.12, 0.6, or 3 µg/kg/day from GD6 to PND21, and the effects of TMX exposure on all offspring were examined at70 days of age; the reproductive performance of the offspring was also evaluated. Although the body weights of the dams treated with TMX remained normal from GD6 until PND 21, some of the dams in the 3 µg/kg/day group died during the pregnancy or partum periods. No

changes were detected in the reproductive measures in any of the TMX groups except for a decrease in the number of newborns/live newborns in the 3 µg/kg/day group. No abnormal clinical signs, body weight change, AGD, VO, or organ weight changes were detected in any of TMX groups; however, the day of PPS was delayed in the male offspring of all TMX groups in a monotonic manner, and cleft phallus was detected in the female offspring of the 0.6 and 3 µg/kg/day groups. No abnormalities were detected in the reproductive performance of the F1 male or F1 female offspring.

In summary no NMRD were seen in any of the more than 50 individual dose response curves.

A.2.c Lasofoxifene – LAS

Lasofoxifene is a nonsteroidal selective ER modulator (SERM) developed for the treatment of postmenopausal osteoporosis.

A.2.c.1 Weisenburger et al. (2004)

In the above investigation, a series of studies were executed to evaluate the effects of LAS on the postnatal development, behavior, and reproductive performance of offspring of female rats when the chemical was administered during pregnancy and lactation. Two range-finding studies examined the effects of LAS at doses 0.01, 0.1, 1, 5 and 10 mg/kg administered from GD 6 to PND 14 on parturition and lactation in pregnant rats and on the early postnatal development of the offspring. Note that some dams were not dosed from GD 18 until after delivery due to the induction of dystocia. In the two range-finding studies, the length of gestation was increased, parturition was prolonged, and dystocia was noted in the dams in the 0.1, 1, 5 and 10 mg/kg dose groups; the total numbers of live pups was reduced at 1, 5 and 10 mg/kg/d. All the effects of LAS were dose-related, monotonic responses.

In the pre- and postnatal development study, LAS was administered by oral gavage at dose levels of 0.01, 0.03, and 0.1 mg/kg on GD6-17 and lactation days 1-20 (not dosed GD18 to delivery to avoid dystocia). Maternal body weight gain and pups weights during lactation were reduced in all dose groups.

There was increased pup mortality in the F1 litters in the 0.1 mg/kg group, and all treated groups had decreased offspring body weights beginning at 1 week of age, continuing into the postweaning period and, for the F1 males, into adulthood. The study authors reported that F1 female offspring in the 0.03 and 0.1 mg/kg groups had increased body weights as adults. There were delays in the age of appearance of PPS in the males in the 0.1 mg/kg group and VO in the females in all treated groups. The sensory, behavioral, and functional measures, including the tests of learning and memory, were unaffected by treatment. Fertility was lower for the F1 animals in the 0.1 mg/kg group, but there were no effects on F2 litter size or pup viability.

In summary, a NOAEL for LAS was not identified in this study with adverse effects occurring in the lowest dose tested. LAS induced dystocia in all dose groups in a monotonic dose manner and reduced the total numbers of live pups/litter at 1, 5 and 10 mg/kg/d.

A.2.c.2 Ozolinš and Gupta (2004)

The purpose of this study was to evaluate the effects of LAS on fetal rat and rabbit development. Pregnant rats were dosed orally with LAS at 1, 10, or 100 mg/kg/d from GD 6-17 and necropsied on GD 21 to evaluate fetal viability, weight and morphology. Maternal weight gain (all treated groups) viable fetuses (high dose only) and fetal weight (top two dose groups) all were reduced in monotonic manner. In addition, malformations and variations were

increased in fetal rats in the top two dose groups, and placental weight was increased in all dose groups.

In the rabbit, as in the rat, all effects displayed dose-dependent monotonic responses. LAS treatment on GD 6-18 at 0.1, 1 or 3 mg/kg/d (necropsied on GD28) induced some toxicity in the rabbit in a monotonic dose-dependent manner, increased postimplantation loss (top two dosage levels), and reduced the number of viable fetuses (all dose groups).

In summary, LAS altered rat and rabbit development *in utero*,and all effects in the Ozolinš and Gupta (2004) study were monotonic in both test species.

A.2.c.3 Ke et al. (1998) –

Ke et al. (1998) examined the effects of LAS on of 5-month-old ovariectomized rats treated with vehicle, EE2 at 30 µg/kg/d or LAS at 1, 10, 100, or 1000 µg/kg/d for 4 weeks. The authors measured the following: body weight gain, uterine weight, serum cholesterol, serum osteocalcin, bone mineral density of the lumbar vertebrae and of femur. LAS completely prevented ovariectomy- induced increases in body weight gain, total serum cholesterol, and serum osteocalcin at doses between 10 and 1000 µg/kg/d after 4 weeks. In addition, LAS prevented ovariectomy- induced bone loss and inhibited the increased bone turnover.
All responses were monotonically dose-related; of >20 measured no NMDRs were detected.

A.2.c.4 Ke et al. (2000)

In this study, LAS was administered orally at 1, 10 or 100 µg/kg/d for 60 days to orchidectomized ten month old male rats, and prostate weight and histology serum cholesterol and bone structure, strength and mass (femur and fifth lumbar vertebra) were determined. LAS decreased body weight in all dose groups; improved bone structure and strength; and reduced serum cholesterol in the top two dose groups. Prostate weight and histology were unaffected. The authors concluded that LAS might be a useful therapeutic agent for preventing bone loss in elderly men. All responses were dose-related and monotonic; no NMDRs were detected.

A.2.c.5 Ke et al. (2004)

Ke et al. (2004) examined the long-term effects of lasofoxifene, on bone mass, bone strength, and reproductive tissues in ovariectomized rats. 3.5 month old female rats were ovariectomized and treated orally with LAS at 0, 60, 150, or 300 µg/kg/d for 52 wk. Body weight gain, induced by ovariectomy, and ovariectomy-induced bone loss were prevented by all doses of LAS. The effects on bone were due to decreasing bone resorption and bone turnover. In addition, the strength, energy, and toughness of the fourth lumbar vertebral body were increased by LAS treatment by comparison to sham control levels. In contrast, uterine weight was only slightly increased as compared to ovariectomized females, being much lighter than in

sham controls. The uteri of the intact females displayed histopathological uterine alterations consistent with aging; these included dilated gland, squamous metaplasia, cystic hyperplasia, inflammation and uterine dilatation. LAS treatment at 300 µg/kg/d also induced dilated glands but did not induce any of the other effects seen in the intact, sham control females. In summary,f or greater than >30 individual measurements, LAS did not induce any NMDR in the Ke et al. (2004) study.

A.2.c.6 Cappon et al. (2004)

In this study, LAS was administered orally to adult male rats at doses of 0.1, 1, 10 and 100 mg/kg/d for 66-70 days. Males were mated to untreated female rats after 28 days of treatment and then necropsied at the end of the dosing period. Body, testis, epididymis, seminal vesicle and prostate weights were reduced in all LAS-treated groups. The percentage of males mating, numbers of implants and viable fetuses (determined at GD14) were reduced in the two highest dose groups. In contrast, epididymal sperm concentration and motility and epididymal, vesicular and testis histopathology were unaffected by LAS at any dose.

In summary, no NOAEL was determined, and none of the effects displayed an NMDR.

A.2.d Arzoxifene - ARZ

This chemical is considered to be a third generation SERM that has enhanced specificity and potency as compared to early SERMS like tamoxifen. The studies reviewed herein have examined the effects of ARZ using the ovariectomized female rat model. While some traditional toxicology studies have been executed with some older SERMS (as the one of the major target populations is postmenopausal women) more studies are done using the ovariectomized female rat model of osteoporosis. Specifically, ovariectomized rats have been used for bone efficacy studies of estrogens and SERMs.

A.2.d.1 Sato et al. (1998)

Body weight, uteri, serum cholesterol and bones were shown previously *in vivo* to be sensitive to circulating levels of estrogen, as well as to synthetic SERMs. In the Sato *et al.* (1998) study, 6-month-old, ovariectomized rats were orally dosed with 0.1, 1, 10, 100, 1,000 or 10,000 µg/kg/d ARZ or the positive control EE2 at 100 µg/kg/d for 5 weeks.

ARZ prevented the ovariectomy-induced increase in body weight and serum cholesterol levels of treated rats, and it lowered them to below sham levels in a dose dependent manner similar to EE2. In the uterus, ARZ had marginal effects on uterine weight compared to ovariectomized controls, and histological examination of uterine endometrial epithelial cell height showed little to no stimulatory effect as compared to sham controls or EE2 treated females. ARZ also prevented loss of bone after ovariectomy.

In an uterotrophic assay, ARZ acted as an antiestrogen, rather than an estrogen, antagonizing the estrogen-induced elevation in uterine weight down to vehicle-dosed control.

All dose related effects in the current study were monotonic; no NMDRs were detected.

A.2.e Raloxifene - RAL

A.2.e.1 Buelke-Sam et al. (1998)-

This paper summarizes a number of reproductive and developmental studies with RAL in male and female rats. This includes studies of male and female fertility, effects on preimplantation, a Segment II study, and a Segment II/III study that examined the reproductive performance in the P0 and F1 generations (results summarized in Table 1 of Buelke-Sam et al. (1998).

Male rats were insensitive to RAL as compared to females, and no effects on sperm production, testis weight or histopathology were noted with oral exposure at 10, 30 or 100 mg/kg/d. However, when RAL was administered orally by gavage at doses of 0.1, 1, or 10 mg/kg/d to pregnant rats RAL produced effects including disrupted implantation, reduced litter size, and increased gestation length.

F1 offspring were also affected in all treated groups. PPS was not affected in males, but vaginal patency in females occurred about 2 days earlier than controls in females from the 10-mg/kg group. Estrous cycles of the F1 females were not affected during the first two weeks after VO, but they were disrupted at 12 to 14 weeks of age in the 10-mg/kg group. These females showed poorer mating and fertility indices, and litter size was reduced in the few pregnant females.

Histologically, reproductive organs were not affected in males at any age or in females at PD 21. At PD 60, vaginal mucification occurred in females from the 0.1- and 1-mg/kg groups. At PD 140, the only finding was a high rate of uterine hypoplasia in the 10-mg/kg group, and this finding occurred in the absence of any concomitant ovarian or vaginal changes.

In addition, dose-related decreases in spleen cellularity and thymus weights occurred in both sexes, but immune system function, (as measured by splenic natural killer cell activity and antibody response to SRBC), was not affected. In summary, of the >50 measurements, none displayed an NMDR.

A.2.e.2 Hoyt et al. (1998)

In this study, adult female rats were given oral gavage doses of RAL at 0, 0.1, 1, or 10 mg/kg/d for 4 weeks at which time females were mated with untreated males for up to 3 weeks. The females were allowed to deliver and rear their offspring until PND 21. Doses >1mg/kg caused disruptions in estrous cycles. Females in the 10-mg/kg immediate-cohabitation group had slightly increased gestation lengths and smaller litter sizes. These effects did not display any NMDRs.

A.2.e.3 Byrd and Francis (1998)

RAL is a nonsteroidal, SERM developed primarily as a therapeutic agent for postmenopausal osteoporosis. In the Bird and Francis (1998) study, pregnant rats (25/group) and rabbits (20/group) were dosed once daily by oral gavage with 0, 0.1, 1, or 10 mg/kg on GD 6 through 17 and 7 through 19, respectively. Animals were necropsied on GD 20 and GD 28 for rats and rabbits, respectively, to evaluate fetal viability, weight, and morphology.

In rats, maternal body weight, body weight gain, and food consumption were reduced in all RAL groups. Fetal viability was depressed in the 10-mg/kg group and was often associated with signs of hemorrhaging from the vagina. Fetal growth retardation was indicated in the 1- and/or 10-mg/kg groups by increased incidences of fetal runts and the developmental deviations, wavy ribs and kidney cavitation. There was no evidence of RAL-induced malformations.

In rabbits, depressions in body weight gain and food consumption occurred in the 10-mg/kg group, and a single abortion occurred in the 1-mg/kg group. Fetal viability and weights were not affected in any of the raloxifene treatment groups. The overall proportions of fetuses with malformations, deviations, or variations were not affected by treatment with raloxifene; however, one fetus each from the 0.1-, 1-, and 10-mg/kg groups had incomplete closure of the interventricular septum. Therefore, maternal and fetal NOELs were not obtained in this study of raloxifene. All dose related responses were monotonic; no NMDR were reported.

A.2.e.4 Black et al. (1994)

RAL was administered orally for 5 weeks at 0.1, 0.1, 1 and 10 mg/kg/d to ovariectomized female rat. RAL increased bone mineral density and reduced serum cholesterol levels in an estrogen-like manner as compared to untreated females at doses of 0.1 mg/kg/d and above.

Body weight gain resulting from ovariectomy was decreased to intact control values. RAL increased uterine weight at 0.1 mg/kg/d and above, but the effect was greatly attenuated as compared to that seen with EE2 or uterine weights in intact control females. RAL did not affect any of the four uterine histological parameters.

All dose related responses were monotonic; no NMDR were reported.

A.2.f FC1271a (FC)

A.2.f.1 Qu et al. (2000)

Qu et al. (2000) conducted several *in vitro* and *in vivo* studies in various tissues of female rats to determine if FC behaved in an estrogenic or antiestrogenic manner. The effects of FC on bone structure and strength, on adult and immature uterus, as well as on cholesterol metabolism were studied, and the effects were compared with those of EE2 and estradiol, as positive controls. In one of the experiments, five different doses of FC were given orally for four weeks to ovariectomized female rats at 0, 100, 300, 1,000, 3,000 or 10,000 µg/kg/d.

FC behaved like an estrogen, reducing body weight gain and serum cholesterol, LH and FSH, and it improved seven different bone parameters in ovariectomized rats. In the uterus, FC had a small stimulatory effect on uterine weight in ovariectomized and intact immature female rats. In another experiment, 4 weeks of oral treatment with FC inhibited the growth of 7, 12-dimethylbenz[a]anthracene (DMBA)-induced mammary carcinoma growth.

All responses were monotonic dose-related effects.

A.3 Aromatase Inhibitors (AI: block androgen to estrogen synthesis)

Aromatase is a P450 enzyme that converts androgens to estrogens by aromatizing the A ring of the steroid molecule. It is found in the ovary, brain and other tissues in the body. As a result of the structural similarity of this enzyme to other P450 enzymes in the steroid synthesis pathway (among other pathways), AIs also can affect the synthesis of other hormones and disrupt multiple signaling pathways. In this regard, pharmaceutical companies continue to search for AIs with high potency and greater specificity, reducing untoward side effects when these are used as drugs in women for the treatment of diseases like breast cancer. In general, the target population for such drugs is adult and often postmenopausal women and use during pregnancy is counter indicated.

Few multigenerational animal studies with continuous exposure were found for this mechanism of toxicity. The aromatase inhibitors all appear to induce dystocia and delay delivery due to inhibition of estrogen synthesis at this critical stage of pregnancy. In order to avoid the induction of dystocia, multigenerational studies terminate dosing several days before the end of pregnancy in the rat and reinitiate dosing at birth of the pups.

A.3.a Fenarimol

There are several pesticides like fenarimol that inhibit aromatase, and low dose multigenerational studies have been executed and submitted to EPA and other regulatory agencies (IPCS, 1995) for use in risk assessment. For fenarimol, the critical effects included delays in delivery and reduced F1 litter sizes at relative low dosage levels. In continuously exposed male rats, mating behavior and fertility is reduced, likely due to the inhibition of estrogen synthesis in the brain (Hirsch et al., 1987; Hirsch et al., 1986) which drives male rat sexual behavior.

A.3.a.1 Unpublished multigenerational studies

Several long – term multigenerational and cancer studies have been conducted with fenarimol in rats and mice, and at least one of these used dosage levels that could be considered low dose; that is, at least one dose below 1 mg/g/d.

In this study, P0 female rats (30 of each sex in the control group) were fed fenarimol in the diet at concentrations of 0, 12.5, 25, or 50 ppm, (equivalent to 0, 0.625, 1.25, or 2.5 mg/kg/d). Exposure started 56-71 days before the first mating, and it was continued over three generations. Two P0 females in the high dose group died during parturition with signs of dystocia. Fertility in F1 animals and the proportion of females with copulatory plugs was reduced at 50 ppm, indicative of a lack of male mating. There also was a statistically significant, dose-related reduction in litter sizes at 25 and 50 ppm after the second F1 mating. IPCS (1995) determined that the NOAEL for reproductive effects was 12.5 ppm, equivalent to 0.625 mg/kg/d. The NOAEL for general systemic toxicity was 25 ppm, equivalent to 1.25 mg/kg/d.

The summaries indicate that this low dose effect requires long-term, continuous exposure; however, similar effects occurred in other studies with shorter exposure durations at higher dosage levels.

Since none of the multigenerational long-term studies are published we are unable to examine the dose response data in any detail.

A.3.a.2 Gray and Ostby (1998)

FEN was continuously administered orally by gavage at 0, 4, 8, 17, 35 or 70 mg/kg/d from weaning until necropsy at 9 months of age. When tested at full maturity male rats displayed a dose-related suppression of mating behavior when paired with a fully receptive female rat. In the high dose group males failed to mount receptive females, whereas males exposed to lower dosage levels had long latencies to mount. In contrast, serum LH, FSH, prolactin and T, testicular and epididymal sperm counts and sperm morphology and motility were unaffected by FEN at these dosage levels. No NMRDs were detected in this study.

A.3.b Exemestane (EX)

A.3.b.1 Beltrame et al. (2001) – Reproductive toxicity in male and female rats

The Beltrame et al. (2001) describes a large number series of dose response developmental toxicity as well as male and female reproductive toxicity studies of EX with doses ranging from 2 to 1000 mg/kg/d among the studies (details below). As noted in the paper, this drug is used only in postmenopausal women, and it is contraindicated for use by pregnant or lactating women.

Table A.1 Reproduced from Beltrame *et al.* (2001)

Table 1
Fertility studies

Species (treated sex)	Number of animals per group	Treatment period	Oral doses[a] (mg/kg/d)	Volume (mL/kg)
Rat (M)[b]	8	63 d premating and throughout 14 d of cohabitation until sacrifice	125, 250, 500, 1000	10
Rat (F)[b]	8	14 d premating to Day 7 postpartum	2, 5, 10, 40, 200	10
Rat (F)	30	- Cesarean section subgroup: 14 d premating to Day 20 of pregnancy - Delivery subgroup: 14 d premating to Day 15 of pregnancy; Days 1 to 21 postpartum	4, 20, 100	10

[a] Expressed as 100% of test article active ingredient.
[b] Preliminary experiments.

The main study in female rats was designed to determine the effects of oral administration of EX on the following: ovarian function, estrous cycling, mating behavior, conception rate, early and late stages of pregnancy, parturition, and lactation; and effects on the progeny exposed *in utero* or via the milk. The dosing schedule is in Table E. 1 above. EX was given to female rats at doses of 0, 4, 20, or 100 mg/kg/day, 14 days prior to mating; dosing was continued until day 20 of pregnancy for half of the females per group, which were assigned to cesarean sectioning. In the other half dosing was continued until day 15 of pregnancy for the remaining females, which were allowed to deliver naturally and rear their young to weaning. In this latter group, treatment was resumed on day 1 postpartum and continued up to day 21 of lactation. Note that the dosing regimen of postnatal group does not include days 15 to delivery (about 22.5 days pc) which excludes much of the period of sexual differentiation of the reproductive tract; the withdrawal of treatment was put in place because doses from 5 to 200 mg/kg/day prolonged gestation and interfered with parturition. In the Beltrame et al. (2001) studies, the tables contain data for >100 individual dose response curves.

High dose treatment with EX (125 to 1000 mg/kg/d for 63 days prior to and during mating) affected fertility only at the two highest dosage levels and body weight gain during dosing was reduced in the high dose group.

Reproductive performance in female rats was more sensitive to the adverse effects of EX than in male rats. When EX- treated females were mated with untreated males, doses of 4 mg/kg/d and above lengthened gestation and impaired delivery of the F1 litters, resulting in maternal and perinatal mortality near-term. In addition, F1 female AGD at birth (but not at 21 days of age) was increased in the high dose group and placental weight was significantly increased in all EX-treated groups including 4 mg/kg/d and above. None of these effects displayed an NMDR. There were no effects on F1 ages at puberty in males or females, and neither fertility nor fecundity was reduced.

In two developmental toxicity studies (using high dose levels of EX including 0, 30, 70, 90, 270, 810 mg/kg/d and 0, 10, 50, 250 and 810 mg/kg/d) and placental weight was increased statistically significantly in all dose groups in a monotonic fashion ; this was the considered low dose effect in the developmental toxicity studies. The male/female sex ratio displayed an NMDR with an increased percentage of males in two mid-dose groups in one cohort of one of one the two studies but not the other three cohorts. When F1 were mated to produce the F2, a sex ratio also displayed an NMDR with a reduced sex ratio in a midrange dose group.

A few NMDRs were seen in the current study at doses above that which produced adverse effects. These were not consistent from study to study, and, given the large number of measurements taken in this project, there is a high probability of such random variations in the mean values to appear to be significantly different from the control value.

A.3.c Anastrazole and Fadrazole A.3.c .1 Dukes et al. (1996)

Dukes et al. (1996) exposed mature adult male pigtail monkeys (M. *nemstrina*) to anastrazole and fadrazole at six dose levels (3, 10, 30, 100, 300 and 1000 µg/kg/d) twice a day for 7 days, following which plasma hormone concentrations were measured. Anastrazole and fadrazole both inhibited estradiol levels and increased T levels in the serum in a monotonic manner. In addition, fadrazole-treatment induced a monotonic dose related in the ratio of DOC to cortisol in the serum.

All responses were dose-related and monotonic; no NMDRs were detected.

A.3.d Letrozole

A.3.d.1 Tiboni et al. (2008)

Tiboni et al. (2008) exposed rats to letrozole in the drinking water at 0, 0.01, 0.02, or 0.04 mg/kg during the period of major organogenesis (GD 6-16). Developmental endpoints, including intrauterine mortality, fetal growth and *incidence* of structural abnormalities, were evaluated near the end of gestation on GD20. Treatment-related effects included a dose-dependent increase in post-implantation loss, which reached 47.2% following exposure to 0.04 mg/kg letrozole; there were minor vertebral anomalies affecting 32.2, 29.3 and 42.2% of fetuses exposed to 0.01, 0.02 and 0.04 mg/kg, respectively. All responses displayed a monotonic dose response. The authors concluded that gestational exposure to doses of letrozole that are equal to or lower than the daily recommended human dose has toxic effects on prenatal development in rats.

All responses were dose-related and monotonic; no NMDRs were detected.

A.3.d.2 Kafali et al. (2004)

Kafali et al. (2004) treated young adult female rats with letrozole at 0, 0.1, 0.5 or 1 mg/kg/d orally for 21 days. Estrous cycles were monitored daily and after necropsy uterine and ovarian weights and histology were examined and serum hormone levels were measured including estradiol, T, LH and FSH. Letrozole treatment induced histological changes in the ovary at all dosage levels with the severity increasing in a monotonic dose related manner (hyperplasia of theca cells, decreased number of corpora lutea, incomplete luteinization, capsular thickening and subscapular follicular cysts). Letrozole treatment also reduced serum estradiol and progesterone levels, and increased serum T, LH and FSH levels, also in a monotonic dose related manner.

A.3.d.3 Schieweck et al. (1993)

Schieweck et al. (1993) examined the anti-tumor and endocrine effects of three nonsteroidal AIs on estrogen-dependent DMBA-induced mammary tumors. The AIs studied included CGS 20 267 (0, 3, 10, 30, 100, and 300 µg/kg/d), CGP 45 688 (0, 3, 10, 30, 100, 300, 100 and 3000 µg/kg/d), and CGP 47 645 (0, 3, 10, 30, 100, and 300 µg/kg/d). These AIs were administered daily by oral gavage to adult female rats with estrogen-dependent DMBA-induced mammary tumors for 6 weeks, after which tumor volume, serum hormones and uterine weight were measured. All three AIs suppressed tumor volume, uterine weight and estrous cyclicity in a monotonic manner, with CGP 45 688 being the least potent of the three. CGS 20 267 suppressed serum estradiol levels and increased serum LH levels in a monotonic manner (these endpoints were not measured with the other two AIs.

In summary, none of the effects in the Schieweck et al. (1993) displayed an NMDR.

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