REVIEWER'S APPENDIX TO THE WHITE PAPER ON SPECIES/STOCK/STRAIN IN ENDOCRINE DISRUPTOR ASSAYS

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DISCLAIMER: The White Paper on Species/Stock/Strain in Endocrine Disruptor Assays was prepared in response to an Interim Recommendation from the Endocrine Disruptor Methods Validation Subcommittee (EDMVS) and in support of further discussion on this topic by the EDMVS. It does not represent Agency findings, determinations, or policy.

The White Paper was prepared by a contractor to the EPA and represents the contractor's best effort to review and evaluate the scientific literature on species, stock, and strain considerations that affect endpoints under consideration for inclusion in the Endocrine Disruptor Screening Program. As part of the preparation of the White Paper, the contractor submitted a draft for review by an external reviewer. The reviewer requested that his comments be posted along with the White Paper. Although this is an unusual procedure, the Agency agreed to do so. The Agency regards both the White Paper that responds to an EDMVS request and the Reviewer's Appendix as contractor products, not as Agency products that establish an Agency position.

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FOREWORD

The US EPA White Paper on "Species/Stock/Strain in Endocrine Disruptor Assays" dated July 25, 2003, was distributed to the Endocrine Disruptor Methods Validation Subcommittee (EDMVS) and posted on the US EPA web site: http://www.epa.gov/scipoly/oscpendo/docs/edmvs/strainswhitepaper072503.pdf . The White Paper was reviewed by Dr. Jimmy L. Spearow. Since Dr. Spearow disagreed with several points in the White Paper, as it was presented, this appendix was written by Dr. Spearow and is offered for additional consideration in the selection of appropriate mammalian animal models for the Endocrine Disruptor Screening Program (EDSP). The EPA sponsored the preparation of this appendix through EPA Contract 68-W-01-023 to Battelle Memorial Institute.

1. BACKGROUND

The 1996 US Food Quality Protection Act and the amended US Safe Drinking Water Act mandate the Environmental Protection Agency (EPA) to test a multitude of pesticides and other chemicals for endocrine disruptor activity in the Endocrine Disruptor Screening Program (EDSP). This EDSP will use *in vitro* cell culture assays and *in vivo* animal models in toxicological and ecotoxicological assays to screen a multitude of chemicals for their ability to disrupt reproductive development and function.

The sensitivity/susceptibility of the animal model(s) to the chemicals being tested is a critical factor in these mammalian toxicological screening assays. Studies have shown that strains of mice and rats differ in susceptibility to endocrine disrupting chemicals (Inano et al. 1996; Steinmetz et al. 1997; Apostoli et al. 1998; Steinmetz et al. 1998; Nakai et al. 1999; Roper et al. 1999; Spearow et al. 1999; Long et al. 2000; Spearow et al. 2001a; Putz et al. 2001a). Outbred lines most commonly used in EPA reproductive toxicology assays to date include CD-1 mice, LE rats, and Sprague Dawley (SD)-derived CD rats lines, which were previously selected for robust reproduction. However, several of these highly fecund outbred lines have been shown to be more resistant than other strains to the disruption of several reproductive endpoints by estrogenic chemicals (Inano et al. 1996; Steinmetz et al. 1998; Spearow et al. 1999; Long et al. 2000; Putz et al. 2001b).

With this background, the Biological Factors and Study Design subpanel at the NTP/NIEHS Endocrine Disruptor Low Dose Peer Review, October 2000, determined that the genetic susceptibility of strains used in endocrine disruptor screening assays was of concern (Melnick et al. 2002). They stated that strains used in previous toxicological assays had been chosen on the basis of familiarity and convenience rather than through a deliberate thought process of selecting the most appropriate animal model(s). The subpanel asserted that historical control data for CD-1 mice and SD rats may be compromised by genetic drift and/or selective breeding, and that isogenic strains such as B6C3F1 mice might provide less variable responses. Because of observed strain and species differences in sensitivity, this subpanel recommended that animal model selection be based on responsiveness to known (positive control) endocrine active agents of concern, rather than on convenience and familiarity (Melnick et al. 2002). The subpanel also recommended development of a core of historical data characterizing reproductive endpoint responses to known endocrine-disrupting chemicals of inbred and outbred mouse and rat strains.

The Endocrine Disruptor Methods Validation Subcommittee (EDMVS) recommended in December 2001 that the EPA prepare a White Paper reviewing the literature on the issue of variation between strains in the endocrine control of reproductive development and their responses to endocrine-active chemicals. The EDMVS also recommended that the White Paper provide a rationale for the selection of strains to be used in the EDSP. Because the EPA did not adequately address the reviewer's critiques of the draft White Paper, the reviewer's appendix is presented for consideration with the White Paper.

2. SUMMARY

The White Paper on Species/Stock/Strain in Endocrine Disruptor Assays considers several genetic, reproductive endocrine, developmental and toxicological aspects pertinent to the selection of appropriate animal models for the EDSP. The rodent models discussed include: 1) the use of mice versus rats; 2) the use of highly reproducible inbred strains or F1 of inbred strains, versus genetically heterogenous outbred strains; and 3) the use of a single strain versus multiple inbred strains with diverse genetic backgrounds to better ensure that effects on sensitive genotypes are not underestimated. The reviewer identified the significant issue that outbred strains of rodents including Sprague Dawley (SD)-derived CD rats, Long-Evans (LE) rats, and CD-1 mice most commonly used by the EPA for reproductive toxicology studies have been selected mainly for large litter size or high prolificacy and vigor, e.g., healthy, growthy pups at weaning. Due to reports in the literature, there was concern that such large litter size selected strains were less sensitive to estrogenic and certain other endocrine compounds.

In the course of technically reviewing the draft EPA White Paper, the reviewer raised a number of concerns that were not addressed in the White Paper. These include the following:

- Data on observed strain differences in susceptibility to Endocrine Disrupting Chemicals (EDCs) including estrogen, coumestrol, atrazine, lead, and the antiandrogenic fungicide, Vinclozolin, were not accurately reported in the text summaries and/or tables.

- Data were not included in the text and/or tables of the White Paper which showed that SD or CD strain rats were more resistant or less responsive at specific endpoints than other strains examined to DES, Bisphenol A, and p,p'-DDE.

- There were errors in the section describing the developmental origin of the prostate. Available data showing that prostate hypertrophy is much more common in aging Lewis rats than in Wistar rats, and does not occur in SD rats (Naslund et al. 1988), were not included.

- Other recent data not included showed that SD-derived CD rats were much less affected than LE strain rats by exposure to phthalate.

- Data were omitted from the text and/or tables describing the well documented lack of responsiveness of CD-1 mice, compared to other strains, to the effects of estrogen, DES, soybean isoflavones, and ethylene glycol monomethyl ether (EGME) on specific reproductive endpoints.

These errors or omissions in the White Paper result in an under-representation of the magnitude and direction of strain differences in susceptibility to endocrine disruption. The white paper also grouped strain comparisons by reproductive/endocrine endpoints rather than by endocrine agent mechanistic class, making strain differences in susceptibility to endocrine agents harder to discern.

Strain and/or species differences in susceptibility to the induction of oocyte meiotic errors by Bisphenol A (BPA) were not considered. While the White Paper mentioned that BPA (0.02 to 0.1 mg/kg/day) and/or polycarbonate plastic breakdown products induced meiotic errors in several strains/stocks of mice (Hunt et al. 2003), it did not consider that BPA exposures as high as 50 mg/kg/day did not affect the related litter size endpoint in CD IGS strain rats (Tyl et al. 2002).

Given that similar meiotic errors result in miscarriages and Down's syndrome in humans, consideration should be given to ensure that animal models and endpoints are sensitive to the detection of EDC-induced meiotic errors.

The White Paper did not consider strain differences in the timing of puberty, and that precocious puberty can confound detection of EDCs in immature uterotrophic assays. Use of later maturing strains should be considered as a means of improving the reliability and sensitivity of uterotrophic assays.

A major problem is that the selection history of the SD rat was not complete. The White Paper addressed the evidence that the SD rat strain was developed starting in 1925 from an exceedingly narrow genetic base of 2 rats, by intensive linebreeding, inbreeding, and selection for high lactation/vigor, growth, docility, and prolificacy. However, the White Paper did not mention that **SD rats were also selected for "high resistance to arsenic trioxide***"* (Poiley 1953; Lindsey 1979). SD and SD-derived CD rats have been used extensively as mammalian toxicology models, and CD strain rats have been used to validate the vast majority of the mammalian *in vivo* assays in the EDSP. Yet this strain's history of selection for high resistance to arsenic does not seem to have been considered. A key piece of information would be how factors associated with arsenic resistance impact susceptibility to endocrine disruption. Direct comparisons of the reproductive toxicity of arsenic in SD rats with that of other strains are needed. The mechanisms of arsenic toxicity are yet to be fully delineated, but include disrupting vicinal thiols, inducing reactive oxygen species (ROS), inducing oxidative stress, altering gene expression, and disrupting the expression and function of steroid/nuclear hormone receptors.

While not mentioned in the White Paper, oxidative stress is a major factor contributing to endocrine disruption, infertility, epididymal sperm function, reproductive disease and cancer in mammalian species including humans (Agarwal et al. 2003). Estrogen downregulates several oxidative stress protection enzymes in reproductive tissues, thereby increasing susceptibility to oxidative damage (Ansell et al. 2004). Since many agents have been reported to further deplete and overwhelm oxidative defenses leading to oxidative damage, there is potential for interaction between estrogenic agents and oxidative stress-inducing agents. If SD rats also show upregulated defenses to oxidative stress in reproductive tissues as they do for hepatic tissues (Binda et al. 2001), this strain is likely to be resistant to many EDCs, which act through or interact with oxidative stress mechanisms of toxicity. The Safe Drinking Water Amendments (SDWA) of 1996 mandate the development of a comprehensive plan for studying the health risks of arsenic (and drinking water disinfection byproducts) and implementation of a plan for studies of subpopulations at greater risk of adverse effects. Given these congressional instructions, additional studies are needed to understand the implications of using an animal model in EDSP screening assays which has been previously selected for high resistance to arsenic and for high prolificacy. This includes not just mammalian *in vivo* screening assays, but also the *in vitro* sliced testis steroidogenesis assay, which has been validated only using testes from SD-derived CD rats.

The White Paper did not consider the evidence for dramatic genetic differences within human populations and between murine (mouse and rat) strains in the activity and endocrine regulation of Phase I and II enzymes involved in the metabolism of steroids and xenobiotics. Human genetic polymorphisms in Phase I and II metabolism have been associated with susceptibility to breast, ovarian, and/or prostate cancer/hypertrophy. Even though the EPA did not plan to examine Phase I or II metabolic enzymes in the EDSP, such strain and allelic differences need to be considered in the selection of animal models for endocrine disruptor screening since poor and extensive metabolizer phenotypes respond differentially with respect to the rate of activation, detoxification, clearance, and toxicity of many xenobiotics.

The White Paper did not consider evidence that environmental agents disrupt enzymes involved in estrogen metabolism, especially estrogen sulfotransferase, thereby leading to endocrine disruption due to excessive endogenous estrogens (Kester et al. 2000; Kester et al. 2002). Given that murine strains differ in estrogen sulfotransferase activity (Spearow et al. 2001a), the potential for differential strain susceptibilities to EDCs through disruption of estrogen metabolism needs consideration in the EDSP.

The White Paper did not mention that the role of genetic diversity in susceptibility to endocrine disruption needs to be considered in EDSP Tier I and Tier II studies. The results of Tier II studies may supersede results of the Tier I studies, may be used for risk assessment, and may play a major role in determining the allowable environmental releases of a xenobiotic. The use of a single strain which is less sensitive, and especially a strain which is insensitive to a given EDC in the Tier II studies, will clearly underestimate risk to susceptible species, populations, and individuals. Thus, issues of genetic diversity and sensitivity also need to be addressed in EDSP Tier II assays. In addition, while the White Paper focused on mammalian *in vivo* assays, the EDSP should be aware that the strain used as a tissue or cell source has a major effect on the endocrine responses.

There were also topics for which the reviewer and the White Paper authors disagreed on genetic principles or their interpretation. The reviewer does not agree with the following statements, claims, or implications in the White Paper:

1. That relaxation of selection for large litter size in CD® IGS rats is likely to restore this strain's litter size and sensitivity to EDCs to that of the original population

2. That outbred strains show a broader range of "responsivity" (with a greater likelihood of detecting an effect) and may be more appropriate [as a single strain animal model]

3. That since strains differed markedly in their sensitivity to endocrine disruption by different endocrine agents, (e.g. strain x endocrine agent interactions are highly significant) and because we cannot identify the most sensitive animal model to all endocrine agents, that we can ignore the multiple strain issue and instead focus on choosing a single strain. Instead, the reviewer contends that these highly significant strain x endocrine agent susceptibility interactions are why the EDSP needs to use multiple strains.

The reviewer agrees that isogenic F1 crosses are unsuitable for multigeneration toxicology studies due to increased variability due to segregation of genes at heterozygous loci in the F2 and F3 generations. However, in contrast with F1 crosses, isogenic inbred strains are fully suitable for multigeneration toxicology studies, since all the animals within a strain are homozygous for the same allele, and there is no increase in variability due to segregation of genes in the F2 and F3.

The White Paper did not consider the potential for direct genetic selection for resistance to a reproductive toxicant during the course of multigeneration reproductive toxicology studies in outbred strains. Any time an EDC exposure causes adult infertility, and especially embryonic/fetal/neonatal loss of susceptible genotypes in a genetically variable strain/population, there will be potential for genetic selection for EDC resistance. Such selection for EDC resistance will confound the results of multigeneration reproductive toxicology studies, underestimating effects on susceptible genotypes. The use of isogenic inbred strains in multigeneration toxicology experiments eliminates this problem with differential fertility/viability resulting in unrecognized selection against sensitive genotypes, since all the animals of a given inbred strain have the same

genotype, i.e., are isogenic. The use of multiple inbred strains enables establishment of genetic models for EDC susceptibility, as well as determining the effect of the EDC on reproductive development and function.

The reviewer agrees with the White Paper's statement in the conclusion that "*In endpoints like fertility and gestational parameters, the SD rat appears less sensitive than the F344 rat to several chemicals, suggesting that the F344 rat may be a better strain for assessing the effects of chemicals at these endpoints*." However, this could have been better documented by inclusion of additional published data sets showing strain differences in susceptibility to the disruption of gestation, litter size, or fertility.

Of major concern to the reviewer are the executive summary and the final summary (after Table 3) of the White Paper which do not accurately portray strain differences in susceptibility to specific EDCs. The reviewer disagrees with several statements in the final summary, including the statement on page 73: "*There were no clear patterns indicating the optimal strain for detection of effects due to the majority of endocrine-active compounds tested*." The data actually show that for the majority of EDCs tested, the SD strain is relatively insensitive in comparison to the F344 strain.

Another issue is the lack of consideration of feeding high levels of dietary phytoestrogens and antioxidants, such as found in Purina 5002, on EDSP endpoints. Feeding a soy-based diet combined with using an oxidative stress-resistant animal model is likely to mask the effects of weak environmental estrogens, and underestimate the endocrine disrupting effects of oxidative stress-inducing EDCs on humans and wildlife eating less than optimal diets.

Since strain x endocrine agent susceptibility interactions were found in many experiments, and since no one strain was most sensitive to all EDCs, multiple, preferably isogenic strains need to be used in the EDSP. Toxicological testing with multiple, genetically divergent isogenic strains rather than a single outbred strain increases the sensitivity of screening assays by increasing the likelihood that all of the strains tested are not resistant to the toxicant. It also increases the precision, sensitivity, and reproducibility of endocrine disruption bioassays by using isogenic strains (Festing 1979; Festing 1987; Festing 1993; Festing 1995). The use of several isogenic strains with factorial statistical designs is an efficient means of toxicity testing and can be conducted with essentially the same total number of animals per experiment (Festing 1995; Festing et al. 2001; Festing et al. 2002).

The EDSP should use known sensitive strains, rather than those that have been selected for traits associated with resistance to EDC. Ideally, a panel of: 1) Fisher 344; and 2) an additional two to three diverse isogenic strains such as: Wistar Furth, Lewis, Lewis x BN F1, DA, Dahl Salt Sensitive (DSS) , RHA, MNS, Copenhagen, INR, and/or M520 would provide better genetic diversity for the EDSP. However, the response of several of the latter strains to positive control EDCs is unknown and would need to be examined. The inclusion of a sensitive mouse strain such as the C57BL/6J or B6C3F1 would add assurance that species differences were not missed. Use of SD-derived CD IGS rats alone or in combination with Crl:LE rats as proposed in the White Paper is not suitable, since both are Wistar-derived crosses, both have been selected for large litter size at CRL, and questions remain about the implications of using an animal model previously selected for high resistance to arsenic and high fecundity in toxicological screening assays. However, the reviewer realizes that economic considerations may prompt considering a less than optimal range of genetic diversity in EDSP screening assays. For example, inclusion of the LE outbred strain rather than one of the later mentioned strains in the panel might retain sufficient diversity, but would be far less than optimal due to the segregating nature of this strain and previous selection for high fecundity. The use of an unselected outbred strain such as the Wistar Hanover, might be preferable to a high fecundity selected outbred strain.

Only by using multiple, preferably isogenic, strains as animal models, and including known sensitive strains such as the F344 strain rat, will the EDSP be able to better ensure that effects of EDCs on susceptible genotypes are not underestimated. Such an approach will also provide the needed information on the nature of genetic variation in susceptibility to EDCs, and enhance future studies to identify susceptibility loci and protect human and other animal subpopulations at greater risk of environmentally induced disease.

3. GENETIC CONSIDERATIONS OF MURINE (MICE AND RAT) ANIMAL MODELS FOR MAMMALIAN ENDOCRINE DISRUPTOR ASSAYS

3A. CHOICE OF RODENT SPECIES

The White Paper addresses some of the considerations in the choice of rodent species for EDSP screening assays. The EPA has used both outbred rats and mice in reproductive toxicological screening assays, especially Sprague Dawley (SD)-derived CD strain rats, LE strain rats, and CD-1 strain mice. The EPA generally prefers rats in toxicological screening assays, in part, due to the large amount of historical data on outbred SD rats. However, historical genetic drift, non-reproducibility of individual genotypes, and elevated resistance to certain classes of endocrine agents negate such advantages of outbred SD or SD-derived CD rats. While inbred rat strains are a clear option, the use of mice as animal models for endocrine disruptor assays would be more economical, and offer much greater genetic resources. Mouse models offer adequate sensitivity for the measurement of most reproductive endpoints. However, the small blood volumes limit analysis of hormone levels, particularly on repeated blood samples. Such mouse genetic resources include a much greater set of inbred strains, congenic strains, recombinant inbred strains, and transgenic strains. Not mentioned in the White Paper is that mouse models uniquely offer many different targeted gene deletions, e.g., knockouts, that are powerful tools to determine the role of specific genetic deficiencies in susceptibility to xenobiotics. Such deficiency animal models will be critical for determining the effects of many known mutations in endocrine signal transduction pathways as well as phase I and II enzymes involved in steroid and xenobiotic metabolism on susceptibility to endocrine disruption.

3B. INBRED OR ISOGENIC STRAINS VERSUS OUTBRED STRAINS

The White Paper also addresses several of the considerations involved in the use of genetically heterogeneous outbred strains versus isogenic, highly inbred strains or F1 crosses.

Highly inbred strains have been developed by many generations of full sib matings, and are homozygous at essentially all genetic loci. Inbred strains are isogenic, e.g., all individuals of highly inbred strain have essentially identical genotypes, and show extremely low genetic drift across generations (Festing 1979; Festing 1993). Isogenic inbred strains offer the clear advantage to toxicology testing programs of providing highly uniform, essentially genetically identical animal models that are highly reproducible across individuals and over generations (Festing 1987). The National Toxicology Program uses inbred F344 rats as a reproducible isogenic animal model for many toxicological assays. Toxicologists should treat genetics like other variables and control it by utilizing isogenic strains (Festing 1995). The genetic map and genome wide sequence of C57BL/6J (B6) strain mice and Brown Norway (BN) strain rats are available (Ensembl 2004). Additional information is available on toxicological and other phenotypic characteristics of F344, LEW and

other inbred strains (RatMap 2004). The mouse and rat genome and phenome databases curate and integrate genetic, genomic, and phenotypic data to support research using genetically defined inbred mice and rat strains as genetic models for the study of human disease (Rat Genome Database 2004a; Mouse Phenome Database 2004; Mouse Genome Informatics 2004). Such bioinformatic resources as well as many well defined, inbred, inbred congenic, and recombinant inbred strain genetic resources provide powerful tools for mapping, identifying, and determining the mechanism of action of genes conferring susceptibility to endocrine disruption/toxicogenetic traits.

F1 crosses of inbred strains also offer highly reproducible isogenic strain animal models. Such F1 crosses are uniformly heterozygous at all loci differing between parental strains. Since they are isogenic, inbred strains and their F1 crosses offer highly reproducible animal models with highly uniform genotypes within each strain or F1 that can be repeated to test additional chemicals and doses. The National Toxicology Program also uses inbred B6C3F1 mice as a reproducible isogenic animal model for many toxicological assays. Depending on their sensitivity to mechanistic classes of EDCs, such isogenic F1 cross animal models would be highly appropriate for single-generation toxicology assays, including essentially all of the pubertal and adult assays. If inbred parents were dosed during gestation or lactation, F1 animal models could also be appropriate for assays involving gestational and or lactational exposure of the F1 pups. However, the segregation of many genetic polymorphisms in the F2 and F3 generations makes F1 animal models inappropriate for multigeneration toxicological assays due to increased variability in the F2 and F3 generation, which would decrease assay precision and assay sensitivity.

Outbred strains: The White Paper discussed several features of outbred strains that have been commonly used as animal models in reproductive toxicology assays. Outbred strains are maintained by avoiding closely related matings to help maintain heterozygosity and genetic diversity. While claimed to be more representative of outbred human populations, in actuality, many outbred strains have a relatively narrow genetic base and were inbred early in their development. Such laboratory animal "outbreds" show much less genetic variation than typical outbred populations that have not undergone a major genetic bottleneck. Analysis of DNA and biochemical markers shows that the genetic diversity within outbred rat strains was much less than among commonly available inbred rat strains (Festing 1995; Canzian 1997). For many traits, these commercial "outbred" strains show nowhere near the variability found in genetically heterogeneous populations such as an F2 cross between inbred strains. Such highly linebred, "outbred" strains with limited genetic diversity cannot represent the range of biodiversity found in natural populations, and thus cannot represent the range of susceptibility to EDCs in populations to be protected.

Nevertheless, outbred strains are segregating at many loci, resulting in within-strain genetic and phenotypic variation that is neither repeatable nor predictable. In essence, the use of an outbred strain entails sampling of genotypes segregating in a strain. This makes it impossible to repeat the genotypes and therefore the conditions used for testing EDCs for reproductive toxicity over time. Such genetic segregation in outbred strain populations typically results in higher phenotypic variation and therefore decreased precision in the estimation of phenotypic trait means. Outbred strains are also subject to genetic drift between suppliers and across generations. Such genetic drift further decreases the reproducibility of experiments over time and between laboratories.

Most outbred animal models do have the benefit of higher prolificacy, due to previous selection for large litter size and due to heterozygosity at non-additive loci controlling litter size/fecundity. However, as will be discussed, several studies suggest that historic selection for high reproduction and other traits in several outbred stocks tends to result in increased resistance to certain classes of EDC. Furthermore, most studies with fecund outbred strains reduce or standardize

the number of pups shortly after birth to avoid nutritional stunting related effects (Hellwig et al. 2000; Tyl et al. 2002), somewhat reducing the fecundity advantage of outbred strains.

The White Paper discussed the importance of genetic quality control to ensure that a strain has not been contaminated by inadvertent crossing or has diverged due to genetic drift. However, the White Paper did not mention that molecular markers are not available for even distinguishing SD/CD outbred rats from Wistar outbred rats. Genetic quality control within outbreds mainly consists of monitoring genetic drift at a few loci and is not well suited for detecting strain contamination due to inadvertent crossing with a related strain. In contrast, thousands of well-defined genetic markers are available for reliable genetic quality control on highly inbred strains of rats or mice (Rat Genome Database 2004b; Broad Institute 2004).

Benefits of isogenic strains over outbred strains for toxicology assays: Isogenic inbred strains and isogenic F1 crosses offer a clear advantage to toxicology testing and research by providing highly consistent, essentially genetically identical animal models that are highly reproducible across individuals and generations (Festing 1987). The high degree of isogenicity of a given highly inbred strain or an F1 of highly inbred strains enables the testing of many different chemicals and doses on essentially the same genotype. Therefore, the use of isogenic strains in toxicology testing minimizes phenotypic variances, enabling more precise and repeatable estimates of trait means. Since toxicity testing usually involves the calculation of dose-response curves, the increased precision of isogenic strains maximizes the sensitivity of toxicological assays (Festing 1987; Festing 1995). Nevertheless, the use of multiple inbred strains is needed to better ensure that at least one of the strains is sensitive to a given toxicant.

3C. GENETIC ORIGINS OF MOUSE STRAINS

The White Paper discussed some of the genetic origins of inbred mouse strains (Silver 1995). Many diverse inbred mouse strains, including *Mus musculus, Mus domesticus, Mus castaneous,* and *Mus spretus* subspecies, are available. Numerous congenic inbred, recombinant inbred strains of mice, as well as strains with transgenes and targeted gene mutations, are also available. Extensive information on their genetics, genome, physiology, immunology, cancer biology, and other traits are available (Mouse Genome Informatics 2004).

While many outbred strains of mice are available, the ICR and CD-1 outbred strains trace back to two male and seven female "Swiss mice" that were selected for high productivity and growth rate by Dr. T.S. Hauschka at the Institute for Cancer Research (Taconic 2004). Following Caesarian derivation from ICR outbreds in 1959 by CRL, CD-1 mice were once again selected mainly for large litter size with some selection for vigor through the early 1990s (Parady and Mirley 2003; 2004). The result of well over 100 generations of selection has been the large, vigorous, highly prolific, high lactating CD-1 outbred strain, which is widely used in biomedical research.

3D. ASPECTS OF THE GENETIC ORIGINS OF RAT STRAINS

Many laboratory stocks of Norway rats *(Rattus norvegicus)* descended from the fancier derived breeding colony established at the Wistar Institute in 1906. At least 40% of the 111 strains of rats available in the late 1970s descended directly from, or were crossed to, Wistar stock (Lindsey 1979). Direct descendents of Wistar commercial stock include the Lewis (LEW) inbred strain developed by Margaret Lewis, and many Wistar inbred and outbred substrains (Lindsey 1979). Several albino Wistar females were crossed with a single wild gray male in 1915 and then

used to develop the outbred Long Evans rat stock (Lindsey 1979). The Sprague-Dawley strain was also derived from a Wistar strain cross.

While the Wistar and Wistar-derived strains are all related, many other inbred strains originated from other breeders, including the Fisher 344 (F344), August, Copenhagen (COP), Zimmerman, Marshal and ACI inbred strains, as well as the wild-derived Brown Norway (BN) inbred strain (Lindsey 1979). Thus, use of multiple highly inbred strains such as F344, LEW, Wistar Furth or DA would provide greater genetic diversity than found within or between Wistar and/or Wistar Cross-derived outbred strains.

3E. EFFECT OF HISTORIC SELECTION IN OUTBRED STRAINS ON STRAIN SUSCEPTIBILITY TO EDCS

One of the greatest concerns for EDSP assays is the use of an outbred strain animal model which is highly resistant to EDCs due to previous selection for traits associated with resistance to EDC. Animal breeders have long recognized that selection for one trait can result in correlated responses in other traits with similar or overlapping mechanisms of action (Eisen et al. 1981; Falconer 1989; Haley et al. 1990). For example, estrogen negative feedback on pituitary gonadotropin release is a factor regulating estrous cyclicity, ovulation rate, and litter size. For a high ovulation rate/litter size selected line to mature and ovulate more follicles per cycle, such selection needs to find gene combinations that decrease sensitivity to estrogen negative feedback, increase sensitivity to gonadotropins, and/or decrease estrogen production per developing follicle (Spearow 1985). Otherwise, the increased estrogen produced by an increased number of follicles would feed back to inhibit gonadotropin secretion, and ovulation rate would not be increased. While there are several potential physiological-genetic mechanisms regulating ovulation rate and litter size, lines or breeds selected for large litter size are less sensitive to the inhibition of reproductive function by estrogen (Land 1976; Spearow et al. 1999). Furthermore, selection for large litter size increases embryo and fetal survival (Bradford 1969; Bradford et al. 1980). Of concern is whether such selection increases resistance to many different common low-level pesticide, herbicide, and drinking water disinfectant by-product exposures associated with embryonic mortaility (Greenlee et al. 1999; Bielmeier et al. 2001; Greenlee et al. 2004). Thus, care needs to be taken to ensure that animal models chosen for EDSP assays are not highly resistant to EDCs due to previous selection for high reproduction traits associated with resistance to EDCs.

Strain selection history was not complete: Some of the greatest problems regarding the use of outbred strains as animal models in the EDSP likely result from their breeding and selection history. The White Paper addressed the evidence that the SD rat strain was developed by Robert Dawley starting in 1925 by crossing a half albino "hybrid hooded male of exceptional size and vigor" with what seems to be a Wistar strain albino female. He then backcrossed this hybrid male to his albino daughters for 7 consecutive generations, inbred the lines, and then crossed the best 10 inbred lines to form the SD strain. But the White Paper did not include critical information in a 1946 letter from Sprague-Dawley Inc. to S.M Poiley at the National Institutes of Health also stating that "**Selection was made to retain or acquire characteristics of high lactation, rapid growth, vigor, good temperament, and high resistance to arsenic trioxide**" (Poiley 1953; Lindsey 1979). The implications of such selection for resistance to arsenic will be discussed in Sections 4D and 4E. By selecting for these traits Dawley was able to develop the highly productive Sprague-Dawley (SD) strain. This breeding and selection program resulted in a 70% increase in total litter weights at weaning, due to increased litter size and lactational yield/vigor (Poiley 1953). Such selection,

especially during initial inbreeding, is likely to have purged deleterious recessives limiting reproductive, growth/vigor, and arsenic associated toxicant susceptibility traits commonly found in outbred populations. More recent experiments have shown that inbreeding with selection followed by crossing enhances selection against deleterious recessives, and enables improved selection responses in litter size and other traits controlled by non-additive gene action (Falconer 1971; Eklund et al. 1977; Falconer 1989).

Furthermore, due to seven successive generations of backcrossing daughters to the same hybrid foundation male, over 90% of the genes in this linebred "outbred" strain are anticipated to originate from the single exceptional foundation male. Thus, the SD strain has an exceedingly narrow genetic base and is very unlikely to represent the genetic variation in natural populations that have not undergone an extreme genetic bottleneck and extensive selection for similar traits.

It is unclear when, after 1946, the selection criteria for SD rats changed at Sprague- Dawley Inc., (now Harlan Sprague-Dawley, or HSD). Ownership of the company changed during the 1960s and 1970s, and it was acquired by Harlan Industries, Inc. in 1980. Information about breeder selection criteria in SD rats was not well documented prior to 1980. Harlan representatives report that since 1980, procedures for future breeder selection have been used to avoid selection pressure for large litter sizes or other physical traits. Future breeders are selected from multiparous females that provide breeders that yield average litter sizes. For Hsd:Sprague Dawley (SD) rats, these are litters in the 9 to 12 range. Future breeders are selected from females that have produced litters free of observable phenotypic abnormalities/extremes (McNeelan 2003; 2004).

Charles River Laboratories (CRL) obtained SD rats in 1950 and Caesarian derived them to form the CD® rat strain. The CD strain was then selected mainly for large litter size in their 2nd to 5th litters and vigor, e.g., vigorous pups at weaning until 1991, e.g., for approximately another 80 to 100 generations (Parady and Mirley 2003; 2004). Other reports have also indicated that Harlan selected breeders without regard to the litter size, where as CRL selected for large litter size, and never chose breeders from litters with fewer than 8 pups (Pollock et al. 1998). It would appear that there was considerable deliberate, long-term selection pressure for large litter size in CD® rats. Given this breeding and selection history, the SD strain, and especially the SD-derived CD strain are not outbred populations with anywhere near the genetic diversity and load of deleterious recessives found in humans or "wild" outbred populations of essentially any mammalian species.

Contemporary comparisons show significant differences in several traits between SD substrains. Relative to that of Hsd:SD and/or Tac:SD rats, body weights are increased and longevity is decreased in Crl:CD(SD)BR rats, e.g., CD rats (Klinger et al. 1996; Pettersen et al. 1996; Stanhope et al. 2000). Crl:CD(SD)IGS BR rats showed moderate to extreme adult-onset obesity, while HSD:SD rats did not (Stanhope et al. 2000). Adipocytes isolated from adult CD IGS rats were larger and more resistant to the effects of insulin-induced glucose uptake than that of Hsd:SD strain rats (Stanhope et al. 2000). These strain differences in body weight and responses to insulin could be due to genetic drift but are more likely correlated trait responses to selection for increased vigor/growth in CD rats prior to 1991. CD rats also differ from that of Hsd:SD rats in the effects of nitric oxide synthase inhibition on blood pressure and fetal weight, as well as adrenocorticotropin responses to inflammatory stimuli (Pollock et al. 1998; Turnbull et al. 1999; Buhimschi et al. 2001).

While not contemporary comparisons, the current litter size of Hsd:SD rats is reported to average 10.5 (Harlan 2004c), which agrees with published reports of controls averaging 10.3 $\pm$ 2.9 (Tinwell et al. 2002). In contrast, published reports of untreated CD strain controls showed litter size means of 15.0 $\pm$ 0.7 (Heindel et al. 1994), and 12.8 $\pm$ 2.2 (Daughtrey et al. 1994) for CD® rats and averaging 14.7 (Tyl et al. 2002) for CD®(SD)IGS strain rats. Although contemporary

comparisons are needed, these data suggest that the litter size of CD®(SD)IGS strain rats is considerably greater than that of Hsd:SD rats, e.g., CD®(SD)IGS rats are even more extreme in this trait than Hsd:SD rats. Nevertheless, comments from CRL indicate that the litter size of CD®(SD)IGS rats may be declining (Mirley 2003; 2004), which raises additional concerns about the genetic stability of this population, especially for reproductive traits.

The White Paper also covered how Charles River Laboratories (CRL) has instituted policies to minimize genetic drift, and has crossed several CD rat subpopulations from CRL colonies worldwide to form the CD®(SD)IGS rat. Unfortunately, this process standardized the variability of the population, not the individual CD®(SD)IGS rat. Since these and other outbred strains are segregating at many loci, it is impossible to replicate the susceptibility genotypes and therefore the conditions used to test any toxicant x dose combination in the EDSP. Such unpredictable genetic variation within outbred strains will limit efforts to utilize reproductive toxicological, bioinformatic, genomic, and proteomic approaches to identify EDCs, and efforts to characterize the mechanisms of action and genes controlling susceptibility to specific EDCs. Since many different SD rat substrains have diverged significantly, strain description should always include the substrain designation rather than simply being described as "SD rats."

The White Paper did not mention that even though the Biological Factors and Study Design subpanel at the NTP/NIEHS Endocrine Disruptor Low Dose Peer Review warned against it, the EPA has expressed a de facto preference for using CD® (SD) IGS BR rats as the main, if not the sole, mammalian model in the planned EDSP. This is indicated by the validation of the vast majority of mammalian EDSP assays in the CD® (SD) IGS BR rat strain, and to a much lesser extent, the CRL LE IGS rat strain. Since both of these strains are derived from Wistar crosses, and have a history of selection for high prolificacy at CRL, the EPA is only considering the susceptibility of a narrow range of high fecundity genotypes to EDCs in validating these EDSP assays.

A major problem reported in the White Paper is that the extent of genetic variation in susceptibility to endocrine disruption is poorly appreciated due to relatively few reproductive toxicological assays that have examined more than one strain. The inclusion of more diverse strains including known sensitive isogenic strains in the validation of EDSP protocols would better ensure that effects of EDCs on susceptible individuals and populations are not underestimated.

SD rat resistance to arsenic: The White Paper addressed the evidence that the SD rat strain was developed by intensive linebreeding, inbreeding and selection for high lactation/vigor, growth, docility and prolificacy. However, the White Paper did not mention that **SD rats were also selected for "high resistance to arsenic trioxide"** (Poiley 1953; Lindsey 1979). SD and SD-derived CD rats have been used extensively as a mammalian model for testing the toxicity and reproductive toxicity of many compounds. Yet, correspondence with several government and private toxicology testing organizations indicates this strain's history of selection for high resistance to arsenic does not seem to have been considered. SD-derived CD rats were even used to determine the developmental toxicity of arsenic in the rat (Holson et al. 1999; Holson et al. 2000a; Holson et al. 2000b). Neither these papers nor the TERA toxicological assessment panel, which reviewed these SD rat data along with mouse and rabbit data, considered whether the rat strain used as animal model had been previously selected for resistance to the chemical being tested, e.g. arsenic. If Robert Dawley selected for resistance to arsenic during most of the development of the SD strain, and especially during inbreeding, he is likely to have purged sensitive alleles, and may have fixed resistance alleles. Unfortunately, the reviewer was unable to find direct comparisons of reproductive toxicity of arsenic in SD rats with that of other strains. Thus, this leaves a number of questions

regarding which arsenic susceptibility endpoints have changed during the development of SD rats, and, if they have changed, how they affect susceptibility to other toxicants. The mechanisms of arsenic toxicity are yet to be completely defined, but include disrupting vicinal thiols, inducing oxidative stress, altering the expression of many genes, and disrupting the expression/function of steroid hormone receptors. Oxidative stress is a major factor contributing to endocrine disruption, infertility, epididymal sperm function, reproductive disease, and cancer (Agarwal et al. 2003). The mechanisms of arsenic toxicity overlap somewhat with those of other toxic heavy metals including lead, cadmium, and mercury in that they all cause oxidative stress.

The White Paper did not provide the information needed to understand the implications of using a strain previously selected for high resistance to arsenic and for high fecundity as an animal model for screening a multitude of chemicals for their ability to disrupt reproductive function and development. Thus the mechanisms of reproductive toxicity of arsenic and oxidative stress-inducing EDCs will be discussed in Section 4D. Data showing SD rats to be much more resistant than other strains of rats to disruption of testicular function and spermatogenesis by lead (Apostoli et al. 1998), were reversed in Tables 2 and 3 of the White Paper, thus incorrectly reporting SD rats to be more sensitive than other strains. Since other data, showing SD rats to be more resistant than F344 rats to toxic metals at reproductive and hepatic endpoints, were also not included (Kuester et al. 2002; Dearth et al. 2004), relevant data for genetic variation in susceptibility to EDCs with mechanisms of toxicity overlapping that of arsenic will be discussed in Section 4E.

Before considering the use of SD rats for the EDSP, additional studies are needed comparing SD rats directly with several isogenic rat strains that have not been selected for resistance to arsenic to determine the magnitude of strain variation in reproductive/endocrine, developmental, and systemic toxicity of arsenic. Failure to consider that an animal model has been previously selected for resistance to a heavy metal toxicant may lead to underestimating the risk to human health of arsenic and toxicants with overlapping mechanisms of action.

4. POINTS THAT WERE NOT ADDRESSED OR INCLUDED IN THE WHITE PAPER

The following section involves data on observed strain differences in susceptibility to Endocrine Disrupting Chemicals (EDCs) that were reversed, incorrectly reported, or not included in the White Paper. Given the variation in susceptibility to endocrine disruption between strains of laboratory animals, the Biological Factors subcommittee at the NTP/NIEHS Endocrine Disruptor Peer review recognized that the strains to be used as animal models in the EDSP need to be chosen on the basis of susceptibility to mechanistic classes of endocrine disrupting agents. Strains with known resistance and especially insensitivity to one or more classes of EDCs should be avoided, especially as sole animal models in the EDSP. Otherwise the effects on sensitive genotypes will be underestimated, thereby violating the precautionary principle.

In the course of technically reviewing the draft EPA White Paper, Dr. Jimmy Spearow identified a number concerns that were not addressed. The White Paper grouped strain comparisons of susceptibility to endocrine disruption in the text and especially in Tables 2 and 3 by reproductive/endocrine endpoints rather than by endocrine agent mechanistic classes. This makes strain differences in susceptibility to endocrine agents harder to discern. The reviewer suggests that strain differences in susceptibility to specific endocrine agents generally are much clearer when grouped by endocrine agent and should be considered in that manner. Thus, this appendix will cover endocrine agents primarily by mechanistic class. One exception was regarding susceptibility to EDCs that disrupt gestation, where grouping by endpoint showed that high prolificacy strains were more resistant.

The reviewer generally agrees with the White Paper about species variation in susceptibility to hormonally active compounds; however, the White Paper did not present the large variation between mammalian species in susceptibility to the reproductive and pathological effects of estrogens (Hart 1990). Species differ not only in sensitivity to estrogens, but also in the relative cellular responses of mammary, pituitary, ovarian, uterine, adrenal, hepatic, bone marrow, and blood tissues to estrogens. For example, estrogens mainly elicit ductular growth in the mouse, rat, rabbit, and cat, but also elicit lobular-alveolar growth in guinea pigs, cow, and monkeys (Hart 1990). In contrast, estrogens elicit little mammary growth in the ferret and dog. Estrogens decrease body weight in rodents, but increase body weight in hamsters, guinea pigs, sheep, and cattle. Species differ in uterotrophic responses to estrogens and especially to anti-estrogens. Tamoxifen has been reported to be mainly an estrogen antagonist in the rat, but an agonist in the mouse, guinea pig, and dog. Clomiphene is anti-uterotrophic in the rat, but uterotrophic in the ferret. Susceptibility to estrogens generally declines in the order of cat, ferret, rat and mouse, and dog, but depends on the trait examined.

4A. STRAIN DIFFERENCES IN SUSCEPTIBILITY TO ESTROGENIC AGENTS IN MALES

Several studies reviewed in the White Paper showed that males from strains of rats and mice previously selected for high fecundity and/or resistance to arsenic were much more resistant to the disruption of reproductive development by estrogens. Unfortunately, several sections of the White Paper, including the executive summary, Tables 2 and 3, and the final summary, incorrectly report or omit certain data leading the reader to a different conclusion, namely that while strains differ, there are no clear patterns. The reviewer disagrees.

A conclusion of the White Paper stating "*Sensitivity to the effects on male reproductive organ weights was found in most rat strains and chemicals studie[d] except for F344 (low dose E2)…*" is especially significant, misleading, and disturbing. The original data show that SD rats

were more resistant than Wistar/MS rats, and much more resistant than F344 rats to decreases in testes and seminal vesicle weights and serum testosterone by adult exposure to DES (Inano et al. 1996). Implantation of 3 mg DES pellets from 3 to 15 months of age did not significantly reduce testes or seminal vesicle weights in SD rats, whereas 3 mg DES resulted in a 45% reduction in testes weight, and a 79% reduction in seminal vesicle weight in Wistar/MS rats (P's<0.01). DES also resulted in an 85% reduction in testes weight, and a 95% reduction in seminal vesicle weight in F344 rats (P's<0.01). SD rats were also much more resistant than Wistar/MS and F344 rats to decreases in testicular 17 alpha hydroxylase and 17B-hydroxysteroid dehydrogenase activity following adult exposure to DES (Inano et al. 1996). Reference to the findings of Inano et al. (1996) were omitted from Tables 2 and 3, despite the reviewer's request that these data be included.

Data of Putz et al. (2001b) on the susceptibility of SD and F344 rats to disruption of male reproductive organ weights by neonatal E2 exposure were accurately reported earlier in the text of the White Paper, but strain sensitivities to low-dose E2 were reversed and misrepresented and in Tables 2 and 3 and the conclusion of the White Paper. Actually, the paper by Putz et al. (2001b) examined the responses of SD rats at PND 35 and 90 to a 7-log order neonatal dose range of estradiol benzoate (0.015 to 15000 microgram/kg). Where as the response of F344 rats was only examined at PND 90 to 0.15, 15, and 1500 microgram/kg estradiol benzoate. The dose (0.015 microgram/kg/day) that produced low-dose effects in SD rats was not given to F344 rats. To say that the F344 was insensitive or less sensitive at low dose is incorrect; it was not examined. This paper clearly states (page 1510) "F344 rats were far more sensitive to the estrogen treatment than were SD rats (Putz et al. 2001)." The authors of this paper, Dr. Oliver Putz and Dr. Gail Prins, also agree with the reviewer that the data show F344 rats to be more sensitive than SD rats to the inhibitory effect of neonatal E2 exposure on reproductive organ weights, e.g., reduction of testes, epididymal, seminal vesicle and coagulating gland weights (Prins 2003; Putz 2003). The conclusion in the White Paper stating that F344 rats were insensitive to low-dose E2 is highly misleading and incorrect. While additional studies are needed, available direct strain comparisons of reproductive organ weights show SD rats to be much less sensitive than F344 or Wistar/MS rats to E2 or the synthetic estrogen, DES.

Furthermore, as will be discussed later, the statement in Table 3 that Eldridge et al. (1994) found SD rats to be sensitive and F344 rats to be less sensitive/insensitive to prolactin (Prl) responses to E2 treatment is also in error in many regards.

The White Paper correctly reported that highly fecund CD-1 mice are much more resistant than C57BL/6J (B6) strain mice to the disruption of reproductive development and spermatogenesis by estradiol (Spearow et al. 1999). Effects of strain, E2 dose, and the interaction of strain and E2 dose on testes weight and spermatogenesis were all highly significant (P<0.0001). Spermatid maturation was eliminated by low doses of E2 in B6 strain mice and in randomly selected control inbred C17/Jls strain mice (Spearow et al. 1999). In contrast, mice of the widely used CD-1 line, which has been selected for large litter size, showed little or no inhibition of spermatid maturation even in response to 16-fold higher doses of E2. This study raised concerns that product safety bioassays conducted with animals selected for high fecundity may greatly underestimate disruption of male reproductive development by estradiol and environmental estrogenic compounds (Spearow et al. 1999; Spearow et al. 2001a; Spearow et al. 2001b). The high susceptibility of B6 strain mice to the disruption of testes development and spermatogenesis by estradiol was confirmed relative to that of highly prolific ICR outbred strain mice (Nagao et al. 2002). ICR was correctly listed as less sensitive/insensitive to effects of E2 on male organ weights in Table 3, but it was mistakenly listed with Reference 39 in the White Paper when it should be with Reference 38 (Nagao, 2002).

The text of the White Paper included additional data showing that CD-1 mice were >195 fold more resistant than B6 strain mice to disruption of testes weight and >41 times more resistant than B6 strain mice to the inhibition of spermatogenesis (Spearow et al. 2003). But these data and data showing that CD-1 strain mice were more resistant to endocrine disruption than wild-derived *Mus spretus* strain mice were not included in the tables. These data demonstrate major differences in susceptibility to endocrine disruption between strains of mice.

The data of Inano et al. (1996), Spearow et al. (1999), Putz et al. (2001a, 2001b), Spearow et al. (2001a), and Spearow et al. (2003) show, in both rats and mice, major differences between strains in sensitivity or responsiveness of the male reproductive tract to estrogenic agents. In several cases the differences were much greater than 10-fold. In each case, the highly fecund strain was most resistant to estrogen at reproductive endpoints. These data show that care needs to be taken in endocrine disruptor screening assays to ensure that the chosen animal model does not underestimate the effect of estrogenic agents on reproductive development of susceptible genotypes (Inano et al. 1996; Spearow et al. 1999; Putz et al. 2001a; Putz et al. 2001b).

Effects on the Prostate: A companion paper to Putz et al. (2001b), which was not cited in the White Paper, also showed that prostate compartments of F344 and SD rats responded markedly differently to neonatal E2 (Putz et al. 2001a). High-dose neonatal E2 "resulted in a suppression of prostate morphogenesis in F344 rats that did not allow distinguishing the dorsal and lateral lobes, making their individual dissection impossible" (Putz et al. 2001a). In contrast, individual lobe dissection was always possible in SD rats even in response to 10-fold higher E2. Thus, strains differed in prostate morphogenesis in response to high-dose E2, but not in the overall prostate growth response.

Errors in the Developmental Origin of the Prostate: The White Paper's description of the developmental origin of the prostate is incorrect. In the section on reproductive tract development on page 39 it states that "*The epididymides, vas deferens, ventral prostate and seminal vesicles are formed from the embryonic structures known as the Wolffian ducts...*" As Dr. Gail Prins has pointed out, the prostate forms from the urogenital sinus, not from the Wolffian ducts (Takeda et al. 1990; Prins, 2003). This difference in embryonic origin may be the basis for the huge differences in cancer rates between the seminal vesicles (rare) and prostate gland (very common).

Prostate Hypertrophy: The White Paper did not include available data showing major strain differences in prostatitis. Prostate hypertrophy with increasing age is a problem affecting essentially all elderly men. Spontaneous nonbacterial prostatitis is much more common in aging Lewis rats (72%) than in Wistar rats (27%, $p < 0.05$), and does not occur in SD rats (Naslund et al. 1988). The incidence of spontaneous prostatitis increases with age in Lewis rats (Naslund et al. 1988). Treatment of young males with E2 increases the incidence and severity of prostatitis in mature Wistar rats, which is further enhanced by treating adults with testosterone (Naslund et al. 1988). The Naslund data show that genetic background, hormonal imbalance, and aging all contribute to the pathogenesis of nonbacterial prostatitis in rats. These data suggest that Wistar and Wistar-derived Lewis inbred rats provide a much better sensitized animal model than prostatitis-resistant SD rats for screening chemicals for their ability to induce prostate hypertrophy relevant to humans.

Section summary: Validation studies need to examine effects of estrogenic agents on males from multiple strains. The available data show major strain differences in susceptibility to endocrine

disruption of male reproductive development and function by estrogenic agents as well as in prostatitis, with the EPA's favored animal model, the SD rat, being much more resistant than F344 and/or Wistar strain rats. Since unselected control strains were not maintained for the SD or CD-derived SD rat, it is unclear how much of the observed differences in susceptibility to disruption of male reproductive-endocrine function by estrogens are due to chance genetic differences in the initial population; to previous selection for traits associated with increased lactation, vigor, or prolificacy; or to previous selection for high resistance to arsenic trioxide.

These data show that use of a multiple strain screening assay, which includes isogenic strains known to be sensitive to estrogenic agents in males, would better ensure that effects of EDCs on sensitive genotypes were not underestimated.

4B. STRAIN DIFFERENCES IN SUSCEPTIBILITY TO ESTROGENS AND ESTROGENIC XENOBIOTICS IN FEMALES

Strain differences in effects of estrogenic agents on uterine weight and histopathology: Uterotrophic assays have historically been used as one of the endpoints to understand the molecular and biochemical mechanisms underlying E2-dependent growth. The White Paper mentioned in the section on "*Results of Controlled Experiments*" that quantitative trait loci (QTL) linkage analysis identified two regions on mouse chromosomes 5 and 11, respectively, that control uterine weight responses to estrogen, and a region on chromosome 10 which interacts to control both uterine weight and leukocyte responses to estrogen (Roper et al. 1999). Thus, E2-dependent responses are genetically controlled and a genetic basis underlies the variation observed in several E2-dependent phenotypes (Roper et al. 1999). Unfortunately these data and their implications to uterotrophic assays were not included in the section on the Uterotrophic assay in the text, or in Tables 2 or 3 of the White Paper.

The data of Steinmetz et al. (1998) on strain differences in the effects of estrogen and BPA on uterine weight and histopathology in OVX SD rats and F344 rats were not adequately reported. The Steinmetz data on these endpoints were not mentioned in the section on uterotrophic assays or anywhere else in the text of the white paper. OVX SD and F344 rats showed similar uterotrophic weight responses to estradiol. However, SD rats were less responsive than F344 rats to estrogen induced epithelial cell height. The Steinmetz et al. (1998) entry for uterine weight in Table 2 of the White Paper should list that F344 was more sensitive to E2 than SD at histological endpoints rather than for uterine weight; however, a section for histopathology endpoints was not included in the table.

The White Paper did not adequately report the findings of Steinmetz et al., 1998 on strain differences in susceptibility to BPA. Treatment of OVX females with 0.3 mg/kg/day BPA for 3 days resulted in increased uterine weight, and uterine epithelial cell height in F344 rats, while SD rats were insensitive (Steinmetz et al. 1998). This paper reported a striking induction of uterine hypertrophy, hyperplasia, and mucus secretion as well as vaginal epithelial hyperplasia and cornification by BPA in F344 rats. However, these stark differences in reproductive tract histopathology endpoints between F344 and SD rats in response to BPA were not reported anywhere in the White Paper. Table 2 mentions that F344 rats were more sensitive to BPA for uterine weight endpoints but did not mention that SD rats were insensitive (Steinmetz et al. 1998). Table 3 did not even mention strain differences in effects of BPA on uterine weight or uterine histopathology observed by Steinmetz et al. (1998, Reference 3). It seems inappropriate for the White Paper to omit these striking data, especially on uterotrophic histopathology endpoints, and

then report on page 73 in the final summary "*In the uterotrophic assay the SD rat is sensitive to many endocrine disruptors, except for EE and BPA in one study, in addition to many other strains*."

The White Paper did include descriptions of a study showing that BPA increased vaginal epithelial proliferation and DNA synthesis with a median effective dose of 37.5 mg/kg body weight in F344 rats, but failed to stimulate DNA synthesis at any dose tested in S-D rats (Long et al. 2000). Strains did not differ in the clearance of (3)H-BPA from blood, the concentration or affinity of the vaginal estrogen receptor, or induction of c-fos, mRNA, showing that strains differ in effects of xenoestrogens downstream of the ER (Long et al. 2000).

The reviewer agrees with the White Paper that the data of (Diel et al. 2001) and Diel et al. (2004) in OVX females showed that the uterine weight response of SD rats to estradiol was less than that of DA/Han or Wistar rats (Diel et al. 2004). All of these strains showed comparable slight uterotrophic responses to 50 and 100 mg/kg genistein and comparable moderate uterotrophic response to 200 mg/kg p-tert-octylphenol. BPA did not stimulate uterine wet weight in Wistar rats, whereas in the SD and Da/Han rats showed a very slight stimulation in response to the highest dose (200 mg/kg) (Diel et al. 2004). Note that the White Paper reported that SD rats did not respond to BPA based on an earlier abstract of this work, whereas the full paper reported a very slight stimulation (Diel et al. 2004). The White Paper accurately reported that Alderly Park rats were slightly more responsive than SD rats to nonylphenol (Odum et al. 1999).

The White Paper did not report the effects on uterine epithelial thickness, for which the Wistar was more responsive to estradiol than DA/Han or SD rats. While none of the strains showed uterine epithelial thickness responses to BPA (5, 50, or 200 mg/kg), Wistar and SD rats were more sensitive than DA rats to genistein and octylphenol (Diel et al. 2004). The reviewer agrees that overall that the study of Diel et al. (2004) shows small to moderate differences in sensitivity between Wistar, SD, and DA rats in uterine weight responses to E2, BPA, genistein, and octylphenol.

The White Paper also cites the multi-study summary paper of Kanno et al., 2001 as showing that the uterine weight response to EE was similar across laboratories, strains, diet, bedding, diet (Kanno et al. 2001). A similar, more recent multi-study summary paper, not mentioned in the White Paper, claimed similar uterotrophic responses to BPA, genistein, methoxychlor, nonylphenol, and o,p –DDT across laboratories, strains, diet, and bedding (Kanno et al. 2003). However, the effects of strain were not actually tested and were confounded with all of the other variables in both of these studies (Kanno et al. 2001; Kanno et al. 2003). Furthermore, there was considerably more heterogeneity between laboratories/strains in the in-uterine weight dose responses to BPA and Nonylphenol than there was to EE in intact immature females (Kanno et al. 2003).

Data were omitted from the uterotrophic section of the White Paper and the tables describing the lack of responsiveness of CD-1 and ICR strain mice, compared to other strains, to the effects of DES, soybean isoflavones on uterine weight endpoints (Farmakalidis et al. 1984a; Farmakalidis et al. 1984b; Farmakalidis et al. 1985).

Subsection Summary: The reviewer agrees that these uterine responses to estrogenic agents demonstrate a strain- and chemical-specific sensitivity in uterine weight and histopathology responses. Comparisons between Wistar, Wistar-derived Alderly Park, Wistar-cross-derived SD, and (in one study) DA strain rats generally show relatively small to moderate differences in sensitivity at uterine weight endpoints to estradiol and the environmental estrogens tested to date. However, other studies that included F344 strain rats showed this strain to be more sensitive than SD strain rats to estrogen and especially to BPA at uterine weight and especially at uterine histopathological endpoints (Steinmetz et al. 1998; Long et al. 2000; McKim et al. 2001; Bailey et al. 2002). As discussed below, these strain differences in susceptibility to estrogenic agents extend

to other phenotypes, including pituitary prolactin secretion and tumorigenesis. The reviewer's concern is that by not including a more diverse set of strains in EDC screening panels, and especially known estrogenic agent-sensitive strains like the F344, the effects of harmful EDCs that are genotype-specific may be missed, e.g., effects of an EDC on sensitive individuals and populations will be underestimated. Thus the use of multiple unrelated strains including known sensitive, isogenic strains in the EDSP would enhance the reliability of the EDSP for detecting EDCs and not underestimating effects on sensitive genotypes.

Precocious puberty confounds the intact immature uterotrophic assay: The section on the uterotrophic assay touches on, but does not directly address, a major problem with using the intact immature uterotrophic assay to detect estrogenic activity. Namely, the intact immature assay is not a robust method for detecting estrogens if the animal model is starting to undergo pubertal processes resulting in elevated ovarian activity, serum estrogen levels, and uterine weights. The White Paper mentions that variability in intact uterotrophic assays in the study of Christian et al. (1998) "*was associated with background spontaneous incidences of abnormally high relative uterine weights possibly due to fluctuations in estrogen occurring between pnd 21 and 25*" in controls. But the White Paper did not squarely address the problem that any time the uteri of control animals are stimulated by endogenous pre- or peripubertal estrogens, the dose response to exogenous estrogenic compounds will be flatter, and thus the sensitivity of the assay reduced. In this regard, Christian et al. (1998) described previous immature uterotrophic assays in Wistar rats in which positive control agents did not significantly increase uterine weights due to controls with high and variable uterine weights (Christian et al. 1998). The White Paper mentions that two different Wistar strains and the Crl:CD rat strains showed differences in means and variability for control uterine weights, and therefore different criteria for "biological outliers" (Christian et al. 1998). Depending upon the exclusion of "biological outliers" from outbred strain data sets is not satisfactory from a genetic perspective, since it will also tend to eliminate consideration of genetic variants remaining in the outbred strain. Recent approaches have attempted to resolve this problem with excessive variability in uterotrophic weight in controls by using very young females shipped at 17 days of age for intact uterotrophic assays (Kanno et al. 2003). Nevertheless, strain means for vehicle control uterine weights differed over 3-fold between laboratories/strains/diets (Kanno et al. 2003), suggesting that using younger animals alone was insufficient to solve the problem with precocious puberty. As discussed in section 4N, high levels of dietary phytoestrogens in some diets may have also contributed to the observed variability in uterine weights.

A recent immature uterotrophic assay, whose data were not included in the White Paper, shows that Alpk and C57BL/6J strain mice responded significantly to 1 µg/kg/day DES, while CD-1 and B6CBF1 strain mice did not (Ashby et al. 2003). All strains showed a significant weight response to 10 µg DES/kg/day. Ashby et al. (2003) hypothesized that the lack of a low-dose response in CD-1 females was related to higher [and more variable] uterine weights in the controls. The reviewer found similar problems with elevated uterine weights in control CD-1 mice confounding detection of s.c. estradiol benzoate (EB) in immature uterotrophic assays (Spearow et al. 2000). Even though CD-1 rats showed high EB-stimulated uterine weights, the dose response to EB was not significant due to greatly elevated control uterine weights (NTP 2001). In contrast, later maturing C57BL/6J, C17/Jls, and *Mus spretus* mice all showed low control uterine weights and highly significant responses to 1 and 10 µg/kg E2 ($P<0.0001$) (Spearow et al. 2000; NTP 2001). The relatively high uterine weights observed in untreated CD-1 controls suggests that this apparent "insensitivity" of CD-1 may be, at least in part, related to control CD-1 females approaching

puberty. Other immature uterotrophic assay data sets also show a relatively high percentage of precocious puberty in control CD-1 females (Markey et al. 2001b).

The White Paper did not mention that murine strains differ in the timing of puberty, and did not consider that using a later maturing strain(s) in intact immature uterotrophic assays offers a simple means of improving the reliability of this assay for detecting estrogenic agents. Age at puberty is heritable. Controlled selection experiments in murine and livestock species show that selection for large litter size, increased ovulation rate, or increased growth rate generally advance the timing or puberty, but not the body weight at which puberty is reached (Rios et al. 1986; Lamberson et al. 1991; Ernst et al. 1999). Control Crl:SD rats show markedly earlier vaginal opening than that of Hsd:F344 rats (~32 versus ~38 days, respectively)(Dearth et al. 2004). Control Wistar-derived Alderly Park (AP) rats also showed earlier vaginal opening than Hsd:SD Olac rats, (33.8 + 1.4 versus 38.9 + 2.6 days, respectively) (Tinwell et al. 2002). The apparent differences in age at puberty between CRL CD and Hsd:SD substrains may also be associated with body weight or prolificacy, and even utilizing a smaller, later-maturing SD or Wistar substrain may improve the reliability of the intact uterotrophic assay. Available data suggest that genes controlling uterotrophic response to estrogens and the timing of puberty are likely important determinants of the response characteristics of uterotrophic assays (Rios et al. 1986; Ernst et al. 1999; Roper et al. 1999). Given the problem with variably elevated uterine weights in Wistar and CD strain controls (Christian et al. 1998), screening additional isogenic strains for timing of puberty and estrogenic dose responses as well as using a later-maturing estrogenic agent-sensitive strain such as the F344 needs to be considered to improve the reliability of EDSP uterotrophic assays.

Strain differences in effects of estrogen and BPA on pituitary prolactin secretion and pituitary tumors: The White Paper reported that F344 strain rats are sensitive while Sprague-Dawley and Brown Norway (BN) strain rats are resistant/insensitive to estrogen- and DES-induced pituitary tumors (Gregg et al. 1996; Wendell et al. 2000). The White Paper also accurately reported that F344 rats were much more sensitive than SD rats to estrogen-induced hyperprolactinemia (Steinmetz et al. 1997). BPA induced prolactin levels 7-8 fold in Fischer 344 rats, but SD rats were insensitive (Steinmetz et al. 1997). These data, showing that SD rats are much more resistant to BPA than F344 rats for many different organ weight, histopathological, and endocrine endpoints, argue against the reliability of measuring additional endpoints as a means of detecting effects in a resistant strain. In addition, serum LH, testosterone, and testicular testosterone production responses to BPA in male LE rats are markedly non-monotonic, e.g. U-shaped, with effects at low but not high doses (Akingbemi et al. 2004b), making interpretation of bioassay results difficult.

Effects of bisphenol A (BPA) on meiotic errors and embryo survival: The White Paper mentioned in the section on confounders, but not in the section on gestational parameters, that Hunt et al. (2003) found a marked increase in the incidence of oocyte meiotic errors in several strains of mice exposed to polycarbonate breakdown products or BPA. Exposure to polycarbonate breakdown products resulted in a marked, e.g., 6- to 18-fold, increase in oocyte meiotic errors in C57BL/6 (B6) inbreds and in animals on a mixed genetic background with chromosome inversions (Table 1 and Fig. 3). A subsequent oral exposure study with normal B6 inbred strain mice detected an increased incidence of oocyte meiotic errors in response to 0.02 to 0.1 mg/kg/day BPA, with the higher dose increasing meiotic errors about 6-fold (Hunt et al. 2003). While some non-geneticists have tried to discredit these data as involving weird mice, with the exception of Table 1 and Fig. 3, all of the data in this paper utilized only B6 inbred strain mice (Hunt 2004). Furthermore,

essentially all of the mice reported in this study were homozygotes, which normally show a low incidence of meiotic errors. Moreover, similar effects were found in B6 inbreds as in other strains.

Humans are exposed to levels of BPA in this dose range, and similar human meiotic errors result in aneuploid conditions of high medical concern, e.g., Down's syndrome and miscarriages. Thus, data showing that EDCs affect the incidence of meiotic errors need consideration. Available data show that the litter size of CD-1 mice in a National toxicology Program (NTP) continuous breeding study was reduced by exposure to ~875 mg/kg/day BPA in feed, but not by lower doses (NTP 1985). Litter size, number of implants, or other specific reproductive toxicities of CD IGS rats in a multigeneration study were not reduced by doses of BPA ranging from ~0.02 to ~50 mg/kg/day (Tyl et al. 2002). While CD IGS rats showed significant reductions in number of implants, and litter size in response to ~500 mg/kg/day BPA, these were mainly associated with systemic toxicities (Tyl et al. 2002). Unfortunately, these CD-1 mouse and CD rat studies did not measure ovulation rate and therefore could not calculate preimplantation losses most pertinent for the BPA-induced aneuploidy phenotype. The observed extreme differences in effects of BPA could be due to many factors including oral versus feed dosing regimes and genetic differences in susceptibility to BPA. CD-1 strain mice are much less sensitive than B6 strain mice to the effects of estrogen on the inhibition of testicular development/spermatogenesis, and disruption of gestation (Spearow et al. 1999; Spearow et al. 2003).

SD rats are far more resistant than F344 strain rats to the effects of BPA on uterine weight and especially on uterine histopathology and pituitary prolactin secretion (Steinmetz et al. 1997; Steinmetz et al. 1998; Long et al. 2000). Thus, a question that remains is whether highly prolific CD strain rats are also more resistant to BPA-induced meiotic errors, e.g., aneuploidy and non-disjunction.

The finding by Hunt et al. (2003) that BPA adversely affects meiotic chromosome segregation needs further investigation. Down's syndrome and miscarriages have high medical and societal costs. If low EDC exposure induces oocyte meiotic errors as the data of Hunt et al. (2003) show for BPA in several strains of mice, it is critical that the EPA ensure that the animal models used in the EDSP are sensitive to the detection of such effects. The EPA would also need to consider endpoints in the EDSP that enable detection of EDC effects on meiotic errors or preimplantation embryo survival.

Mammary histopathology: Marked strain differences in histopathological responses of OVX C57 Black, DBA, or A strain mice to long-term E2 have been known for over 50 years (Silberberg et al. 1951). Strains differed in a number of responses to E2 including developmental sensitivity of ductal mammary, alveolar, vaginal and endometrial responses over time (Silberberg et al. 1951).

The genetically related ACI and Copenhagen (COP) rat strains display diametrically opposed susceptibilities to mammary cancer development when treated chronically with E2 (Harvell et al. 2000). Mammary glands of ACI rats exhibited a significantly greater proliferative response and lobuloalveolar hyperplasia than that of COP rats following treatment with E2 (Harvell et al. 2000). Focal regions of atypical epithelial hyperplasia were observed in ACI, but were not observed in COP rats. These strain differences in response to E2 were not due to differences in circulating E2, progesterone or, prolactin. Two-thirds of the induced mammary cancers in ACI rats showed aneuploidy (Harvell et al. 2000). Thus, there is a correlation between E2 action in the induction of mammary cell proliferation and atypical epithelial hyperplasia and genetically conferred susceptibility to E2-induced mammary cancers (Harvell et al. 2000).

Questions remain as to the optimal assay for detecting medically relevant estrogenic effects. BPA has effects on uterine histopathology/epithelial cell height at dose levels below that needed to detect effects on uterine weight (Markey et al. 2001b). The uterotrophic assay does not detect effects of BPA and/or estrogens on meiotic and or mitotic aneuploidies which have serious health consequences. Since breast cancer is more of a human health problem than uterine hypertrophy, per se, and since mammary proliferation/hyperplasia end points are more sensitive than that of uterine weight, Markey and others have called for the reevaluation of the uterotrophic assay and the use of a more sensitive and medically relevant assay in the EDSP (Markey et al. 2001a; Markey et al. 2001b).

Effects on reproductive tract immune function: The White Paper did not address genetic differences in susceptibility to disruption of reproductive tract immune function. Reproductive tract immune function is critical for reproductive success. The administration of E2 to ovariectomized (Ovx) and sexually immature rodents leads to uterine-specific leukocytic infiltration, cell proliferation, and organ growth (Griffith et al. 1997; Roper et al. 1999). However, in response to E2, Ovx C57BL/6J and B6C3F1 mice showed a greater infiltrating eosinophil and macrophage response as compared to that of C3H/HeJ (Griffith et al. 1997). Genetic linkage analysis mapped Est1, the major locus controlling E2 induced uterine eosinophil infiltration to chromosome 4. These results show that uterine leukeocyte infiltration is regulated by E2, and differs between strains. Given the importance of immune function to reproductive success, major genetic differences in effects of endocrine agents on reproductive immune function should not be ignored in the EDSP.

Effects of Estradiol on susceptibility to common reproductive tract pathogens: The White Paper included data in the text but not in the tables showing that highly fecund CD-1 mice were much more resistant than B6 strain mice to estradiol-sensitized *Candida albicans* infection (Calderon et al. 2003; Clemons et al. In Press) Susceptibility to recurrent vaginal *Candida albicans* infection is an estrogen-induced trait plaguing millions of women. In each of three independent experiments, estradiol treated CD-1 strain mice were found to be markedly more resistant than B6 strain mice to vaginal Candidia infection.

4C. STRAIN DIFFERENCES IN SUSCEPTIBILITY TO PARTIAL ESTROGEN AGONISTS IN MALES AND FEMALES

Partial estrogen agonists are compounds that have some agonist activity, as well as varying degrees of estrogen antagonist activity, e.g., the ability to block estrogen-induced uterine weight gains. Partial estrogen antagonists include tamoxifen, atrazine, and octamethylcyclotetrasiloxane (D4) as indicated by their inhibition of E2-induced uterine weight (Eldridge et al. 1994; McKim et al. 2001). The effects of partial estrogen agonists/antagonists on reproductive development and function differ greatly between and within species (Hart 1990; Nakai et al. 1999; Bailey et al. 2002).

The White Paper did not report that strains of mice differ dramatically in inflammatory responses of the testes and reproductive tract to neonatal tamoxifen exposure (2 µg tamoxifen/day) (Nakai et al. 1999). At 3 months of age, tamoxifen- treated animals of all strains showed significantly smaller testes and seminal vesicles than those of the control groups. Histologically, testes of AKR/J, BALB/cAnN, and FVB/N mice showed no abnormality after neonatal tamoxifen treatment (Nakai et al. 1999). In contrast, the testes of A/J, C3H/HeJ, C57BL/6J, and DBA/ 2J mice were frequently necrotic and highly disorganized, with severe inflammation.

The White Paper accurately reported that SD strain rats were about 10-fold more sensitive than F344 strain rats to the induction of uterine weight and epithelial cell height by 4-OH Tamoxifen (Bailey et al. 2002).

However, there were errors in the reporting of the study by Eldridge et al. (1994). The reviewer agrees that atrazine administration to SD and F344 rats for up to 12 months produced changes in estrous cyclicity in SD rats (increased the number of days of vaginal estrus), increased E2, and increased incidence of mammary tumors in SD rats only, with no significant treatment-related effects in F344 rats. The increased E2 levels found only in SD rats seemed related to atrazine inducing prolonged estrus in SD rats. But the data of Eldridge et al. 1994 does not show an atrazine-related decrease in progesterone as claimed in the text and Table 2 of the White Paper. The White Paper authors may have been confused by the observation that, independent of atrazine dose, plasma progesterone declined with age in SD rats but increased with age in F344 rats. Furthermore, in Table 3 under the endocrine endpoint "Hormone levels" for Eldridge et al. (1994; Ref. 22), the White Paper reports that SD rats were sensitive while F344 rats were less sensitive/insensitive to E2 effects on serum prolactin (Prl). However, the original paper treated these strains of rats with atrazine, not with E2. Furthermore, prolactin levels were not affected by age or atrazine treatment (Eldridge et al. 1994; pg. 33). The reviewer contends that Table 3 of the White Paper should have stated that SD rats were sensitive while F344 rats were less sensitive/insensitive to changes in plasma E2 in response to treatment by atrazine.

However, F344 was sensitive while SD and LE rats were less sensitive or insensitive to the effect of atrazine on embryo survival and gestational losses (Cummings et al. 2000; Narotsky et al. 2001).

As reported in the White Paper, Hsd:LE rats are more sensitive than Hsd:SD rats to the inhibition of estrous cycles and prolactin levels by atrazine exposure (Cooper et al. 2000). Hsd:LE rats were sensitive while Hsd:SD rats were insensitive to effects of 1 to 3 days of atrazine exposure on inhibition of serum LH levels.

The data of McKim et al. (2001) were incorrectly reported. The White Paper reports in the text (page 57) that coumestrol (CE) produced uterine weight responses in SD and F344 rats. However, due to limited availability, CE was administered to only SD rats, and no dose responses were reported in F344 rats (McKim et al. 2001, Figs. 4 and 5). Thus we do not know the relative response of F344 rats to this weak phytoestrogen. The White Paper reported in Table 2 but not in the text or in Table 3 that F344 rats were markedly more sensitive than SD to EE, especially on uterine epithelial cell height endpoints. The reviewer does agree with the White Paper that the study by McKim et al. (2001) found SD rats to be more sensitive than F344 rats to the effects of D4 on uterine weight responses. However, the White Paper did not mention that these strains showed similar uterine epithelial cell height responses to D4.

Cumulatively, the corrected data strongly suggest that SD rats are more sensitive than F344 rats to partial estrogen antagonists, e.g., compounds with some antiestrogenic activity, including tamoxifen, atrazine, and D4. Nevertheless, F344 rats did respond to both tamoxifen and D4 (McKim et al. 2001; Bailey et al. 2002). F344 rats were more sensitive than SD rats to estrogens and especially BPA at uterine weight and especially at histopathological endpoints. Thus, these strains show very divergent responses to estrogens, partial estrogen antagonists, and certain xenobiotics such as BPA.

4D. MECHANISMS OF TOXICITY OF ARSENIC AND OXIDATIVE STRESS-INDUCING EDCS

The White Paper did not provide the information needed to understand the implications of using a strain such as the SD rat that has been previously selected for high resistance to arsenic and for high fecundity as an animal model for screening a multitude of chemicals for their ability to disrupt reproductive function and development. Thus, it is necessary to consider the mechanisms of toxicity of arsenic and oxidative stress-inducing EDCs and evidence for genetic variation in susceptibility to heavy metal and oxidative stress-inducing toxicants.

Oxidative stress is a disruption in the balance between the production of reactive oxygen species (ROS) and antioxidant defenses. It occurs when ROS production overwhelms the antioxidant defense system or when antioxidant defenses decline. Oxidative stress is known to cause DNA damage, mutations of tumor suppressor genes that initiate carcinogenesis, lipid peroxidation, and inactivation of cellular enzymes by oxidizing sulfhydryl or amino groups resulting in disruption of cellular functions (Klaassen 2001; Higuchi 2003). DNA damage and lipid peroxidation can then lead to apoptosis (Higuchi 2003).

Mechanisms of Arsenic Toxicity: The mechanisms of arsenic toxicity are yet to be fully delineated, but include disrupting vicinal thiols, inducing ROS, inducing oxidative stress, altering gene expression, and disrupting the expression and function of steroid/nuclear hormone receptors. Toxic sulfhydryl-reactive metals including arsenic, lead, cadmium, and mercury are widely found in the environment and can disrupt many biochemical and endocrine processes, at least in part, from binding to sulfhydryl groups in proteins, especially vicinal thiols, disrupting antioxidants and enzymes (Pi et al. 2002). These and other toxic metals also induce oxidative stress through the generation of ROS including hydroxyl radical, superoxide radical, and hydrogen peroxide (Klaassen 2001; Nesnow et al. 2002; Harris et al. 2003). These pro-oxidative effects of several toxic metals are compounded by the fact that they also deplete intracellular antioxidant, deplete glutathione (GSH), and inhibit antioxidative enzymes including Glutathione S-Transferase (GST).

Biological mechanisms involved in the protection against ROS include superoxide dismutase (SOD), catalase, glutathione S-transferase (GST), and GSH. GSH is involved in the detoxification of many different xenobiotics, and depletion of GSH enhances the toxicity of arsenic, cadmium, and several other xenobiotics (Klaassen 2001; Pi et al. 2002).

Disruption of Nuclear (Steroid) Hormone Receptor function by Arsenic: The White Paper did not address the evidence that arsenic and other toxic metals disrupt the induction and function of steroid/nuclear hormone receptors and are thus highly pertinent for consideration as EDCs. Low levels of arsenic(III) (0.1 to 3.3 μM) disrupt the ability of steroid hormones to regulate gene transcription through glucocorticoid and estrogen receptors in a biphasic manner (Kaltreider et al. 2001; Chen et al. 2002; Hamilton et al. 2002). Arsenic also inhibits expression of ER alpha in cell cultures (Chen et al. 2002), and suppresses estrogen-mediated induction of vitellogenin gene expression in developing chick liver (Hamilton et al. 2002). Arsenic trioxide [As(III)], chromium(VI), and lead also disrupt the binding of SP1 and other transcription factors to DNA response elements (Hanas et al. 1999; Kaltreider et al. 1999). Oxidation of redox-sensitive cysteine residues in glucocorticoid, estrogen, and other nuclear hormone receptors under conditions of oxidative stress residues eliminates DNA binding and transcription (Webster et al. 2001). Oxidative stress protection mechanisms, especially elevated GSH, can reverse this process and restore receptor function (Webster et al. 2001). Oxidative stress also affects cellular signaling mechanisms (Harris et al. 2003) as well as estrogen receptor expression (Tamir et al. 2002).

Low doses of arsenic alter gene expression of cells *in vitro,* altering the mRNA abundance of genes coding for transcription factors, inflammatory cytokines, and enzymes involved in DNA repair enzymes and protection against oxidative stress (Andrew et al. 2003; Rea et al. 2003).

Susceptibility to Oxidative Stress Mechanisms in the Male. The White Paper did not consider the oxidative stress-related mechanisms of estrogen action. Such consideration is also needed since the main animal model used in the validation of mammalian EDSP assays, the SD rat, has a history of selection for high resistance to an oxidative damage-inducing toxicant, arsenic trioxide. While the evidence for effects on oxidative stress in this appendix will focus on the male, where most information is available, there is also concern regarding oxidative stress effects on breast, prostate, and other reproductive tract cancers, pathophysiology of endometriosis, unexplained infertility, negative IVF outcomes, hydrosalpingeal fluid-mediated embryotoxicity, and poor *in vitro* embryonic development (Agarwal et al. 2003).

Estrogens act on the testis to induce oxidative stress, increase genotoxic 8-oxodG (Wellejus et al. 2002), induce germ cell apoptosis, and cause testicular damage (Blanco-Rodriguez et al. 1997). Estrogen-induced testicular cell oxidative damage is blocked by the estrogen antagonist, ICI 182.780, further indicating that this oxidative stress effect is estrogen receptor-mediated (Wellejus et al. 2002). Estrogen also inhibits antioxidant response element (ARE)-mediated gene expression in mouse uterus, thereby repressing several Phase II oxidative stress protection enzymes, further increasing susceptibility to oxidative stress (Ansell et al. 2004). Testosterone also regulates the transcription of several genes involved in the generation and protection from oxidative stress in rat prostate (Tam et al. 2003). Androgen deprivation via castration induces oxidative stress in rat prostate through increased ROS and diminution of antioxidant detoxification (Tam et al. 2003).

Mammalian sperm are extremely sensitive to oxidative damage-inducing chemicals, and the sperm of infertile men show elevated ROS and oxidative damage (Agarwal et al. 2003; Moustafa et al. 2004). Ethinylestradiol induces oxidative stress in testes of Wistar rats (Wellejus et al. 2002). Diverse EDCs including methoxychlor, nonylphenol, TCCD, and bisphenol A (BPA) have all been shown to induce sperm oxidative stress/damage and/or disrupt sperm fertility parameters in Wistar rats (Chitra et al. 2001; Chitra et al. 2002; Latchoumycandane et al. 2002; Chitra et al. 2003; Latchoumycandane et al. 2003). BPA (0.2 to 20 micrograms/kg body weight orally) also induces oxidative stress in ICR strain mouse testes (Kabuto et al. 2003). These data show that epididymal sperm are especially sensitive to oxidative stress, and that such sperm oxidative stress can be readily induced by known EDCs.

Despite the finding that 25 to 40% of infertile men show sperm oxidative stress (Agarwal et al. 2003; Moustafa et al. 2004), and oxidative stress is induced in epididymal sperm by EDC exposure, the role of oxidative stress has not been adequately considered in the design of EDSP endpoints. Since sperm motility is sensitive to oxidative stress, measuring sperm motility at autopsy may provide adequate assessment of EDC effects on oxidative stress, provided a sensitive strain is used as an animal model. Unfortunately, very few EDC screening studies have included sperm motility data. Since pubertal male studies typically do not include analysis of any sperm parameters, there is risk that EDCs with specific effects on spermatogenesis and especially epididymal sperm function will be missed in the EDSP.

4E. STRAIN DIFFERENCES IN SUSCEPTIBILITY TO ARSENIC, OTHER TOXIC METALS, AND OXIDATIVE STRESS

The EPA has been validating the majority of the EDSP assays only in the SD-derived CD IGS rat strain apparently without realizing that the SD strain was also selected for resistance to arsenic trioxide early in its development. Unfortunately, contemporary comparisons of CD IGS and divergent rat strain susceptibility to arsenic is not available. Thus, it is important to consider if SD/CD rats differ from other strains in susceptibility to endocrine disruption by related toxic metals and chemicals with overlapping mechanisms of toxicity. Even though multiple strain studies are not available, it is important to understand what the available single strain studies show regarding the reproductive toxicity of arsenic, related toxic metals, and oxidative stress-inducing agents.

Arsenic is an endocrine disruptor/reproductive toxicant: Exposure of adult female Wistar rats with levels of sodium arsenite found in drinking water in regions of several countries (0.4 ppm/rat/day) decreased plasma LH, FSH and estrogen; reduced ovarian, uterine, and vaginal weight, and prolonged diestrous phase of the estrous cycle (Chattopadhyay et al. 1999; Chattopadhyay et al. 2003). Treatment of mature male Wistar rats with 5 or 6 mg/kg sodium arsenite (IP) decreased serum FSH, LH, and testosterone; decreased accessory sex organ weights; decreased epididymal sperm counts; and resulted in a massive degeneration of all stage VII germ cells (Sarkar et al. 2003). The effects of arsenic on these endocrine endpoints in SD rats are unknown.

Strain differences in the developmental toxicity of arsenic: Unfortunately, contemporary comparisons of the reproductive toxicity of arsenic in SD/CD strain rats versus that of other strains of rats are not available. Strain comparisons that are available involve different forms of arsenic, duration, and/or routes of exposure. Wistar rats showed dramatic dose-dependent increases in gonadal agenesis, teratogenisis, and embryo toxicity following a single sodium arsenate injection (IP) on gestational day (GD) 8 to 11 (Beaudoin 1974). Swiss-Webster mice injected IP with arsenic also showed dramatic increases in fetal loss and malformations (Hood 1972; Hood et al. 1972). Unfortunately, these studies did not report maternal toxicity.

Recent studies examined the prenatal developmental toxicity of lower oral or inhaled exposures to arsenic trioxide [As(III)] in SD-derived CD rats (Holson et al. 1999; Holson et al. 2000a; Holson et al. 2000b). These studies in CD IGS rats reported no evidence for fetal developmental toxicity at arsenic doses below those causing maternal toxicity, with a developmental no observed adverse effect level (NOAEL) estimate of 5 mg/kg/day arsenic trioxide [As(III)] (Holson et al. 2000b). A separate study from the same laboratory showed that arsenic acid [As(V)] had about a 10-fold higher systemic and developmental toxicity in New Zealand rabbits than in CD-1 mice (Nemec et al. 1998). Unfortunately, several factors limit reliable comparison of the data from these two studies. As(III) is much more potent than As(V), but the latter can be reduced *in vivo* to the more toxic As(III) and many factors regulate this process as well as bioactivation and/or detoxification of arsenic (Nemeti et al. 2002; Patterson et al. 2003). Nemec et al. (1998) dosed rabbits from GD 5 to 18 and mice from GD 6 to 15. In contrast, the study of Holson et al., (2000a) dosed CD rats from 2 weeks prior to mating through gestation, and likely differs in the loading and adaptive responses to arsenic (Andrew et al. 2003; Rea et al. 2003). Since these studies differed in both the form of arsenic and in the duration/timing of dosing, additional data are needed to enable reliable comparisons of the developmental toxicity of arsenic in CD rats with that of other strains of mice and rats. Additional contemporary studies are also needed to

compare the toxicity at other reproductive endpoints in CD rats with that of other strains of rats and mice, especially those that have not been also selected for large litter size.

Strain Differences in Susceptibility to Lead (Pb): Strain differences in susceptibility to lead were incorrectly reported in Tables 2 and 3 of the EPA White Paper, where Sprague-Dawley (SD) strain rats are more resistant—not more sensitive—to lead. This is very misleading to most readers who will tend to focus on data reported in the tables and the summary rather than reading the entire text of the White Paper. SD rats and NMRI mice were far more resistant to testicular toxicity of lead than other strains of rats and mice examined (Apostoli et al. 1998). Apostoli et al. (1998) reported that lead exposure resulted in a dose-related suppression of spermatogenesis or epididymal sperm counts and in seven out of eight different andrology toxicology studies using Wistar rats, but in zero of six different studies using SD or CD rats. Lead did not reduce fertility in SD rats and did not alter testis weight of either strain. The statement in the White Paper (page 47) that concentrations of blood lead > 40 μ[g]/dl decreased male reproductive endpoints is misleading, since this result appears to be the case for sperm counts in humans and Wistar rats but not for SD rats. For example, spermatogenesis and serum testosterone levels were inhibited in Wistar rats by blood lead levels of 30 to 45 μg/dl (Sokol et al. 1991). In contrast, neither spermatogenesis, testes or epididymal weights, or serum FSH, LH, or testosterone were inhibited in SD rats by blood concentrations of lead as high as 124 μg/dl (Nathan et al. 1992). Also the reduction of sperm counts and fertility in Wistar rats in the absence of reduced testes weights shows the need for determining effects of environmental agents on sperm counts and histopathology rather than just male reproductive organ weights. Semen levels of cadmium and/or lead have also been associated with decreased sperm quality parameters in humans (Xu et al. 2001; Pant et al. 2003).

The White Paper did not include recent data showing that F344 rats were sensitive while SD rats were insensitive to the disruption of female puberty and estrogen levels by gestational and lactational exposure to lead (Dearth et al. 2004). Gestational and lactational exposure of F344 strain rats to 12 mg/ml lead acetate delayed the timing of puberty (vaginal opening) ($p < 0.01$), and suppressed serum levels of luteinizing hormone (LH) ($p < 0.001$), and E2 ($p < 0.01$) in 30 day-old female offspring (Dearth et al. 2004). These effects did not occur in SD offspring exposed to either 12 or 24 mg/ml lead acetate. Dearth et al., 2004 reported a substantial strain difference in susceptibility to endocrine disruption of puberty and serum E2 concentrations by levels of lead known to be of concern for humans.

Strain Differences in Susceptibility to Cadmium: The White Paper accurately reported strain differences in reproductive toxicity of cadmium among strains of mice and rats, and between species (mice, rats, and Syrian hamsters) (Rehm et al. 1988). Syrian hamsters were most susceptible to CdCl2-induced ovarian hemorrhagic necrosis. DBA/2NCr mice were sensitive to CdCl2 induced ovarian hemorrhage, while Balb/c, C57Bl/6N and NFS/N strain mice were resistant (Rehm et al. 1988). Rats showed dose- and age-dependent toxicity of the ovaries, uterus, cervix, and liver. Cadmium induced uterine lesions in mature F344 rats, but not in Wistar Furth rats.

The White Paper did not mention that strains of mice differ dramatically in susceptibility to the disruption of testis function by cadmium (Nolan et al. 1986; Bhave et al. 1988; Liu et al. 2001). Microarray analysis showed mRNA levels of several oxidative damage protecting enzymes were greater in B6 resistant strain testes than in sensitive 129 strain testes (Liu et al. 2001). While both F344 and SD rats showed hepatotoxicity in response to 3mg/kg cadmium chloride, hepatic injury was 18-fold greater in F344 rats as indicated by plasma alanine aminotransferase activity (Kuester

et al. 2002). Additional research is needed to determine if the increased hepatic resistance to cadmium of SD rats over that of F344 rats extends to reproductive endpoints.

Strain Differences In Susceptibility To Oxidative Stress: While strain differences for reproductive endpoints are unknown, SD rat hepatocytes have higher antioxidants, higher antioxidant enzyme levels, and ability to resist acetaminophen-induced oxidative stress than that of WKY strain rats (Binda et al. 2001). SD rats are much more resistant than F344 rats to several oxidative stress-inducing toxicants including 1,2,-Dichlorobenzene (DCB) and the herbicide diquat (Gupta et al. 1994; Younis et al. 2000). SD rat liver showed much lower oxidative stress, loss of GSH, and lipid peroxidation than F344 rats in response to DCB (Younis et al. 2000). F344 and Wistar rats also differ in intestinal and hepatic antioxidant defense enzymes, some of which change with age (Jang et al. 2001). These findings clearly indicate strain differences in susceptibility to oxidative stress-inducing toxicants.

Strains selected divergently for sensitivity (ALS/Lt) as opposed to resistance (ALR/Lt) to Alloxan, a potent generator of superoxide and hydroxyl radicals commonly used to destroy pancreatic beta-cells, differ markedly in antioxidant enzyme levels and antioxidants status (Mathews et al. 1999). Genetic linkage analysis mapped a small number of genes controlling susceptibility to oxidative stress, including Suppressor of superoxide production, (*Susp*) to mouse chromosome 3 (Mathews et al. 2002). These studies show that a small number of highly heritable loci have major effects on susceptibility to oxidative stress-inducing chemicals. The finding that selection was effective in increasing the resistance of ALR/Lt strain mice to oxidative stress raises questions as to whether the increased resistance of SD rats to oxidative stress-inducing chemicals may have resulted from early selection for high resistance to arsenic trioxide rather than chance. If SD rats also show better detoxification and oxidative stress defense mechanisms in reproductive tissues, this strain is likely to be more resistant not just to estrogens and toxic metals such as lead but also to many reproductive toxicants with effects on oxidative stress.

The combination of metal-induced oxidative stress with other mechanisms of toxicity induced by organic toxicants is also of concern (Carpenter et al. 2002). Lead exposure interacts with the testicular toxicity of chlordane, markedly decreasing testes weight, as well as Sertoli and germ cell number in Balb/c strain mice (Al-Omar et al. 2000). Since the mechanisms of metal toxicity and the mechanisms of estrogen action interact, considering the implications of endocrine disruption through oxidative stress mechanisms, and especially the implications of using animal models previously selected for high resistance to arsenic in EDC screening assays needs to be a priority.

Implications: Additional research is needed to determine if the increased resistance of SD rats to the reproductive toxicity of lead and cadmium over that of F344 rats extends to other reproductive traits and endpoints. Compared with other strains examined, SD rats are clearly more resistant to the disruption of testicular function and female pubertal development by lead. SD rats are relatively resistant to estrogens at several endpoints. If oxidative damage protection systems are up-regulated in reproductive tissues of SD rats as they have been shown to be in the liver of this strain, it is likely to be more resistant to many other chemicals that act or interact via oxidative stress mechanisms.

Unfortunately, the issue of endocrine disruption through, or in association with, oxidative stress mechanisms has not been considered in the selection of animal models for the EDSP. Thus, it is essential to determine the magnitude of strain differences in susceptibility to the disruption of several different reproductive endpoints, and especially the sensitive testicular and epididymal

spermatogenic endpoints by estrogens, toxic metals, and other oxidative damage-inducing chemicals. Such studies need to compare the susceptibility of SD rats with that of diverse isogenic rat strains such as F344, LEW, DA, etc. at oxidative stress sensitive endpoints. Since we do not know the relative genetic susceptibility of SD rats at such endpoints, this should be done before proceeding to use an animal model that has been previously selected for resistance to a nuclear hormone receptor-disrupting, oxidative stress-inducing toxicant, like arsenic.

This section has focused on oxidative stress mechanisms of genetic susceptibility. Nevertheless, we should realize that there are many other potential mechanisms of genetic susceptibility including altered gene expression, as well as genetic differences in uptake, metabolism, and excretion of arsenic and other toxic metals. Any one of these could also mediate strain differences in susceptibility to arsenic and related toxic metals. Humans also show genetic variation in susceptibility to arsenic, with individual humans and human ethnic groups differing in the methylation and detoxification/excretion of inorganic arsenic (Vahter 2000; Loffredo et al. 2003). Defining the mechanisms of reproductive toxicity of arsenic and other EDCs in animal models will ultimately help to identify other potential mechanisms of genetic susceptibility in humans as well.

4F. SPRAGUE-DAWLEY RATS USED FOR TIER I *IN VITRO* SLICED TESTIS STEROIDOGENESIS ASSAYS

The White Paper focused on factors involved in the choice of strain for mammalian *in vivo* EDSP screening assays. However, the *in vitro* sliced testis steroidogenesis assay has been validated with, and calls for using testes from, 11 to 15 week old Crl:CD® (SD)IGSBR strain rats, e.g., CD IGS rats (Battelle 2003). Luteinizing Hormone (LH) induces Leydig cell testosterone synthesis via LH receptor/G-Protein/Adenylate Cyclase/cAMP/Protein Kinase A mechanism resulting in increased synthesis of steroidogenic acute regulatory (STAR) protein and other steroidogenic enzymes. Strains of mice differ markedly in LH stimulated Leydig cell testosterone production, as well as in 3β-Hydroxysteroid Dehydrogenase (3β-HSD) and P450 side chain cleavage enzyme (CYP11A) activity (Stalvey et al. 1984; Nolan et al. 1990). Strains of mice also differ in susceptibility to the inhibition of Leydig cell 3B-HSD activity by androgens *in vitro* (Stalvey et al. 1992) Testicular and Leydig cell steroidogenesis, e.g., progesterone and testosterone production, is inhibited by ROS, including free radicals and hydrogen peroxide, in part by inhibition of STAR protein levels (Diemer et al. 2003; Tsai et al. 2003). The inhibition of testicular steroidogenesis by such ROS was recognized in the prevalidation study of the EDSP sliced testis assay (Battelle 2003). As previously discussed, the finding that SD rats were selected for high resistance to an oxidative stress inducting agent, e.g., arsenic, and the finding that this strain is resistant to a variety of EDCs raises concern that testis slices from this SD-derived CD rats will underestimate effects on susceptible genotypes. Additional studies are needed to understand the implications of using an animal model in the *in vitro* sliced testis steroidogenesis assay which has been previously selected for high resistance to arsenic and for high prolificacy.

4G. STRAIN DIFFERENCES IN SUSCEPTIBILITY TO PHTHALATES

The White Paper was accurate in reporting that CD-1 mice were sensitive while JCL:ICR mice were resistant to DEHP-induced seminiferous tubule atrophy (Oishi 1993). The White Paper also reported that CD-1 mice were sensitive while F344 rats were resistant to developmental

toxicity of gestational exposure to DEHP (Tyl et al. 1988). These data show major strain and species/strain differences in susceptibility to the reproductive toxicity of phthalates and mandate increased attention to strain selection. The White Paper did not include data presented at the December 2003 EDMVS meeting showing that testes weight and seminal vesicle weights of CD IGS (SD) rats were inhibited much less by 1000 mg/kg DiButyl Phthalate than those of CRL LE rats (Strain x Treatment interaction $p<0.01$) (Gray 2003). Since direct comparisons of phthalate susceptibility of SD or LE rats with that of F344, Wistar, or other unselected strains of rats were not included in the White Paper, the magnitude and nature of genetic differences in susceptibility to phthalates is unknown.

Phthalates are a major, persistent environmental peroxisomal proliferator, and a human body burden contaminant. One of the most common environmental phthalates, di-(2-ethylhexyl) phthalate (DEHP), disrupts multiple male rodent reproductive endpoints including decreasing spermatogenesis and fertility, increasing serum LH, and inducing Leydig cell hypertrophy and testicular cancer (Akingbemi et al. 2001; Akingbemi et al. 2004a). DEHP disrupts steroid hormone synthesis through an androgen receptor independent mechanism, namely via the inhibition of the peripheral-type benzodiazepine receptor (PBR) synthesis, which is required for cholesterol transport across the mitochondrial membrane (Gazouli et al. 2002). DEHP and its metabolite MEHP induce testicular and epididymal germ cell apoptosis and testicular atrophy through mechanisms that involve oxidative stress, depletion of glutathione, induction of mitochondrial injury, and cytochrome c release, which induces germ cell apoptosis and testicular atrophy (Richburg et al. 2000; Kasahara et al. 2002).

Given that sperm oxidative stress is a major contributor to human infertility (Agarwal et al. 2003), the finding that reproductive organ weights of SD rats are less affected by 1000 mg/kg DBP than those of LE rats is very concerning. This finding adds support to the hypothesis that previous selection of SD rats for high resistance to arsenic trioxide increased the resistance of this strain to oxidative damage-inducing toxicants. Thus, it is essential to compare the susceptibility to endocrine disruption by phthalates of SD rats with that of more diverse strains including F344, an unselected isogenic Wistar such as LEW, and/or DA rats before using an animal model that has been previously selected for resistance to an oxidative damage-inducing toxicant.

4H. STRAIN DIFFERENCES IN SUSCEPTIBILITY TO ANDROGEN DISRUPTORS

The reviewer agrees with the presentation of data on strain differences in susceptibility to androgen disruptors on pages 40-41 of the White Paper. The White Paper correctly reported that SD-derived CD rats were much less sensitive than LE rats to the effects of gestational day (GD) 14 to 18 exposure to p,p'-DDE (a DDT metabolite) on male Anal Genital distance (AGD) (You et al. 1998). In contrast, male SD rats were more sensitive than LE rats to nipple retention (You et al. 1998).

The reviewer does not agree with portions of the White Paper's report of the difference in susceptibility to post-pubertal exposure to p,p'-DDE between CD and LE strain rats (O'Connor et al. 1999). Endpoints that changed in response to increasing doses of p,p'-DDE in each strain were accurately reported as a table on page 56, which is followed by the statement: "*These data demonstrate strain-sensitive differences in response to an endocrine-disrupting chemical. CD rats were much less sensitive to the effects of p,p'-DDE than were LE rats*." However, there was no mention that CD rats were insensitive to effects of p,p'-DDE (300 mg/kg/day) on seminal vesicle and prostate weights. The only responses in reproductive tract weights were for the epididymis, and strains showed modest responses in opposite directions. There was no mention that the described *in*

vivo male battery using CD rats did not identify p,p'-DDE as an endocrine active compound (EAC), whereas the same *in vivo* male battery using LE rats did identify this compound as an EAC (O'Connor et al. 1999). The insensitivity of CD rats to effects of p,p'-DDE on seminal vesicle and prostrate weights was also omitted from both Tables 2 and 3 and from the summaries. Table 3 did not even list O'Connor et al. (1999) (reference 13 from Table 2) under Male reproductive organ weights. Thus, the insensitivity of CD rats to p,p'-DDE was under-reported in the White Paper.

The reviewer agrees with some parts but not others of the White Paper's coverage of the effects of developmental exposure (gd14 to pnd 3) to Vinclozolin in LE and Wistar rats (Hellwig et al. 2000). The reviewer agrees that the data of Hellwig et al. (2000) show LE strain rats to be somewhat more demasculinized than Wistar strain rats at the 12 mg/kg dose of Vinclozolin. The reviewer also agrees that overall there were more similarities than differences between strains, with disruption of several histopathology endpoints in Wistar rats and even more in LE rats. The 200 mg/kg dose of Vinclozolin markedly disrupted many developmental endpoints in both strains of rats, including reduced neonatal viability; reduced AGD; nipple and areolae retention; hypospadias; penile hypoplasia or development of the vaginal pouch; and reduced function and chronic inflammation of the epididymides, prostate, seminal vesicles, and coagulating glands (Hellwig et al. 2000). A minor point is that the Hellwig data (Table 8) do not show a reduction in seminal vesicle weights in LE strain rats in response to 12 mg/kg Vinclozolin as claimed on page 42 and in Table 2 of the White Paper.

More importantly, the summary of the White Paper below Table 3 distorts the data of Hellwig et al. (2000) by stating: "*Sensitivity to the effects on male reproductive organ weights was found in most rat strains and chemicals studie[d] except for F344 (low dose E2), and Wistar (Vinclozolin)*." While LE rats were more sensitive to lower doses on some endpoints, the White Paper did not mention that Wistar rats (Table 7) showed a slightly greater inhibition of prostate and seminal vesicle weights than LE rats in response to gestational exposure to 200 mg/kg Vinclozolin (Hellwig et al. 2000). Also not mentioned was that Wistar rats were sensitive to effects of Vinclozolin on testes weight, whereas LE rats were insensitive.

One aspect of this study not mentioned related to the fact that a large number of pups in both strains were stillborn or died soon after birth in response to gestational exposure to 200 mg/kg Vinclozolin (Hellwig et al. 2000). The Wistar rats and LE rats used in this study were both outbred and related (LE rats were derived from a Wistar x Wild rat cross). Thus, it is not possible to determine if the elevated neonatal mortality in the high Vinclozolin treatment was totally environmental, e.g., due to the Vinclozolin treatment, or was also associated with a polymorphism segregating within each strain conferring susceptibility to this xenobiotic. Any time a xenobiotic reduces the viability of individuals with a susceptible allele segregating in an outbred strain, the effects of the xenobiotic on endocrine disruption endpoints in susceptible individuals will be underestimated, e.g., reproductive endpoints are not measured on the inviable susceptible offspring. Had this study used multiple inbred strains from different genetic backgrounds, it would have revealed the effects of this anti-androgen on reproductive development as well as revealing the effects of inbred strain genotype on susceptibility to Vinclozolin-induced endocrine disruption and to neonatal mortality.

Metabolism of Androgen Disruptors: One important strain difference in susceptibility to androgen disruptors reported was that of 6- to 8-fold higher serum p,p'-DDE levels in LE rats than in SD rats (You et al. 1998). The White Paper authors correctly attributed this to potential strain differences in pharmacokinetic characteristics, e.g. xenobiotic half-life/metabolism. As will be discussed in section 4J, such dramatic strain differences in xenobiotic metabolism emphasize the

need for including multiple strains in endocrine disruptor screening assays to better ensure that effects on poor metabolizer strains/individuals are not underestimated. Since, the Phase I or II metabolic enzymes responsible for the observed strain differences in serum p,p'-DDE levels are unknown, we do not know what other xenobiotics will also be metabolized more extensively in SD than in LE or other strains of rats.

The White Paper did include the andrology data of Wilkinson et al. (2000), comparing untreated poor CYP2D1/2 metabolizer DA strain males with that of untreated extensive CYP2D1/2 metabolizer Wistar and SD strain males. But comparisons were only available for effects on untreated DA males. The White Paper did not include data on the responses of DA males to EDCs metabolized by CYP2D1/2. Nor did the White Paper evaluate dose responses to androgen disruptors in strains known to be poor metabolizers for other more pertinent Phase I or II metabolism enzymes. For example, animal models for poor UGT metabolizer phenotypes such as the Gunn rat strain (Low UGT1A) and LA Wistar rat strain (low UGT2B2) might be highly applicable. Animal models for genetic deficiency in CYP3A4 would also be especially applicable, since the activity of this enzyme differs over 10-fold in activity between poor and extensive metabolizer phenotype humans (Dai et al. 2001). Furthermore, CYP3A4 metabolizes and is regulated by many endocrine and other compounds being screened in the EDSP (Klaassen, 2001). The same is true for genetic variation in endocrine response mechanisms including androgen receptors, 17 alpha-hydroxylase, 5 alpha reductase, and other mechanisms controlling sensitivity/susceptibility to androgens.

Data presented in the White Paper suggest that SD, LE, and/or Wistar strain rats show substantial differences in susceptibility to endocrine disruption by androgen receptor antagonists, flutamide, Vinclozolin, and p,p'-DDE. Unfortunately, these strain comparisons, as well as the Therimmune studies comparing only SD and LE rats, have examined only a small portion of the genetic variation among rats stains in susceptibility to androgen disruptors, and only in Wistar and Wistar cross-derived strains. Since the studies reviewed in the White Paper have not included comparison with more diverse strains of rats such as F344, LEW, Wistar Furth, DA, or poor Phase I and II metabolizer phenotype strains, the magnitude of variation in susceptibility to androgen disrupting agents between strains of rats is unknown. Inclusion of multiple strains from diverse genetic background in the EDSP would decrease chances of underestimating effects on susceptible genotypes.

4I. STRAIN DIFFERENCES IN SUSCEPTIBILITY TO THYROID HORMONE AND RELATED DISRUPTORS

The White Paper considered the effects of thyrotropin-releasing hormone TRH on TSH, T3, and T4 levels in F344 and SD strain rats (Fail et al. 1999). The White paper reported that both strains showed responses in TSH and T4 levels to TRH challenge, whereas F344 rats showed increased T3 levels but SD rats did not, in part due to small sample size and greater inter-animal variability in SD rats. But the White Paper did not mention that F344 rats were more sensitive than SD rats to TRH-induced TSH levels, with effective dose 50 (ED50) of TRH of 61 ng in F344 rats and 78 ng in SD rats.

The White Paper accurately reported that CD rats were insensitive to the effects of p,p'-DDE on TSH levels, while LE rats were sensitive (O'Connor et al. 1999). Both strains showed declines in T4 levels in response to p,p'-DDE. These data demonstrate that there are considerable strain-sensitivity differences to p,p'-DDE exposure. The White Paper did not mention that the *in*

vivo male battery of tests using CD rats did not identify p,p'-DDE as an EAC, whereas the same battery using LE rats did identify this compound as an EAC.

The White Paper also reported differences between LE and Wistar/Han strain rats in susceptibility to the of TSH and T4 levels by TCCD (Pohjanvirta et al. 1989). Given the few studies that have been conducted on thyroid disruptors, the inclusion of strain comparisons for such environmentally relevant thyroid disruptors as perchlorate is warranted.

The White Paper omitted data showing that F344 and Wistar Furth strain rats differed dramatically in the endocrine regulation by thyroid hormone and Phenobarbital of many hepatic cytochrome P450 Phase I xenobiotic metabolizing enzymes, including CYP2B1, CYP2B2, CYP3A1, CYP2A1, CYP2C6 and/or epoxide hydrolases, as well as Phase II metabolizing enzymes, including uridine diphosphate-glucuronosyl transferase (UGT2B1), PB-inducible aldehyde dehydrogenase, and/or glutathione transferases Ya1 and Ya2 (Larsen et al. 1995; Ganem et al. 1998; Ganem et al. 1999). Thyroid hormone suppressed the induction of these Phase I and II xenobiotic metabolism enzymes in Wistar Furth but not F344 strain females. These major strain differences in the endocrine regulation of hepatic Phase I and II xenobiotic metabolism will clearly affect the rate of activation, detoxification, and excretion of diverse xenobiotics and steroid hormones. Thus, thyroid hormone disruptors have markedly different effects on steroid and xenobiotic metabolism and depends on the strain.

4J. POLYMORPHISMS IN ENDOCRINE FUNCTION AND PHASE I AND II ENDOCRINE/XENOBIOTIC METABOLISM IN HUMANS AND MURINE STRAINS

Since strains and species of laboratory animals differ markedly in susceptibility to pharmacological, toxicological, endocrine, and carcinogenic agents, choosing the most appropriate rodent species and strains as animal models for testing environmental chemicals for endocrine disrupting activity is critical. The use of a strain more sensitive than humans as the animal model could overly restrict the use of relatively benign chemicals. In contrast, the choice of a highly resistant strain(s) as animal model would risk underestimating effects on sensitive genotypes and populations. This would result in disruption of reproductive development and function as well as reproductive disease and infertility.

Animal models selected for toxicological screening assays would ideally reflect the sensitivity of human and wildlife populations to be protected. However, the White Paper did not consider the genetic variation within and between human populations in susceptibility to endocrine agents or to endocrine disruption. While considering human variability was not the charge of the White Paper, it is nevertheless necessary to consider the range of genetic susceptibility to endocrine disruption and/or endocrine and xenobiotic metabolism found in human and other populations to be protected. If humans are very homogenous and consistent in susceptibility to EDCs, then the EPA can focus on protecting the population average. On the other hand, if individual humans differ greatly in susceptibility to EDCs, we need to consider the range of genetic susceptibility and the mechanisms by which they differ, so that animal models do not underestimate risk of susceptible individuals. If humans differ greatly in susceptibility, efforts also need to be made to protect the most sensitive as well as the average individuals from endocrine disruption. The objective of protecting humans from endocrine disruption is complicated by evidence for genetic polymorphisms in xenobiotic metabolizing enzymes and endocrine receptor/signal transduction systems, and a broad range of sensitivities to endocrine-active chemicals in human populations.

Studies of whether exposure to environmental chemicals is reducing sperm counts in human populations (Swan et al. 1999) are also complicated by observed variability in susceptibility to

endocrine agents within human populations. Individual men differ dramatically in susceptibility to the inhibition of spermatogenesis by testosterone contraceptive exposures (Behre et al. 1995; Handelsman et al. 1995). In contrast, a portion of oligospermic men show improved sperm counts following treatment with the anti-estrogen tamoxifen (Kotoulas et al. 1994; Adamopoulos et al. 1997), and increased sperm counts and fertility treatment following treatment with a combination of testosterone and tamoxifen (Adamopoulos et al. 1997; Adamopoulos et al. 2003). These and other data suggest that humans vary considerably in susceptibility to the disruption of reproductive development by endocrine agents and that considering susceptibility genotype will improve the ability to detect and evaluate environmentally induced disease.

Individual genetic variability in estradiol synthesis, metabolism, and receptors has been shown to be a significant contributor to susceptibility to disease and hormonal responses with variations depending on ethnic background. Polymorphisms in 17 alpha-hydroxylase (CYP17), which converts progestins into androgens, and/or polymorphisms in aromatase (CYP19), which converts androgens into estrogens, have been associated with serum steroid hormone levels, response to hormone replacement therapy, and endometrial, prostate, and breast cancer and/or prostate hyperplasia (Azzouzi et al. 2002; Huber et al. 2002). Polymorphisms in estrogen receptor-alpha (ERα) have been associated with azoospermia in men, response to hormonal stimulated *in vitro* fertilization protocols, and response to estrogen replacement therapy (Georgiou et al. 1997; Suzuki et al. 2002; Tempfer et al. 2004). The available evidence shows that estrogen-metabolizing genes are strong hereditary determinants of the susceptibility to benign and malignant conditions (Huber et al. 2002). Polymorphisms in 5 alpha-reductase have been associated with susceptibility to androgen contraceptives (Anderson et al. 1996). Common polymorphisms in human androgen receptors have been associated with risk of prostate carcinoma, benign prostate hyperplasia, and prostate response of hypogonadal men to testosterone replacement therapy (Mononen et al. 2002; Zitzmann et al. 2003).

Human polymorphisms in estrogen metabolism that have been associated with breast, prostate, endometrial, and/or ovarian cancer risk include those in: CYP1A1 and CYP1B1, controlling formation of genotoxic catechol estrogens (Hanna et al. 2000; Huber et al. 2002; Sasaki et al. 2003); and, Catechol-O-methyltransferase (COMT), controlling methylation of catechol estrogens (which greatly reduces their genotoxicity, and potential for causing oxidative damage via redox cycling) (Dawling et al. 2001; Huber et al. 2002). Polymorphisms at phenol-sulfotransferase 1A1 (SULT1A1), controlling the sulfation of xenobiotics and estrogens, as well as the bioactivation of heterocyclic amines and polycyclic aromatic hydrocarbons, have been associated with increased risk of breast and other cancers (Zheng et al. 2001; Saintot et al. 2003). Effects of human polymorphisms at estrogen sulfotransferase (SULT1E1) are presently unknown but strains of mice differ in testicular estrogen sulfotransferase activity (Spearow et al. 2001a).

The pharmacokinetics of ethinyl estradiol differs dramatically between individual women (Goldzieher 1990; Baumann et al. 1996). The observed major inter-individual variation in the pharmacokinetics of estradiol may be explained, at least in part, by the observed variation in estrogen sulfotransferase and estrogen UDP-glucuronosyltransferase (UGT) activities between women (Fisher et al. 2000). Estrogen UGT activity differs 30-fold between individual women and is associated with common allelic variants in the UGT1A1 promoter (Fisher et al. 2000).

Polymorphisms in several hepatic P450s, including CYP2D6 and CYP3A4, as well as in specific sulfotransferases, UDP-glucuronsyltransferases, glutathione S-transferases, and other Phase I and II metabolic enzymes each result in dramatic differences in the rate of metabolism and the toxicity of many endocrine and environmental compounds of toxicological concern. Glucuronidation is a major pathway of xenobiotic metabolism in mammalian species with the

exception of the cat family (Klaassen 2001). A family of UDP-glucuronosyltransferase (UGT) enzymes conjugates many different steroids and xenobiotics to UDP-glucuronic acid, enabling excretion of the glucuronide conjugate in the urine or bile. Bisphenol A is metabolized by conjugation to UDP-glucuronic acid by UGT2B1 and perhaps other UGT and excreted mainly in the bile (Yokota et al. 1999; Elsby et al. 2001). Polymorphisms in the promoter region as well as in coding exons result in wide genetic variation in UGT activity. The low androsterone (LA) Wistar rat has a defect in UGT2B2, which enabled this enzyme to be identified as the main enzyme responsible for glucuronidation of androsterone and triiodothyronine (T3) in rats. In contrast, a single alternately spliced UGT1A gene codes for a family of UGT1A enzymes with different substrate specificities. Mutations in a constant region of UGT1A, such as that found in humans with Crigler-Najjar syndrome and Gilbert's Syndrome, or in Gunn or RHA/jj strain rats, can result in deficiencies of the entire UGT1A family. Gilbert's syndrome is due to homozygosity for a 7 TA repeat in the UGT1A promoter. The frequency of 7/7 homozygotes ranges from <3% in Asians up to 23% in Africans (Burchell et al. 1999; Burchell et al. 2000). This and other polymorphisms in UGT1 and UGT2 isozyme coding regions result in decreased UGT activity and increased toxicity to drugs normally metabolized by specific UGT (Burchell et al. 2000). Common polymorphisms in prostate UGT2B15 also differ 2-fold in Vmax for glucuroniation of androgens (Burchell et al. 2000).

The only mention of a polymorphism or strain difference in Phase I metabolism in the White Paper was that the Dark Agouti (DA) strain rat has a deficiency in CYP2D1. This was mentioned just after appropriately reporting that untreated DA strain rats showed lower absolute and relative testes weight and more variability in sperm counts, but did not differ in other andrology endpoints relative to untreated outbred Wistar or SD strain rats (Wilkinson et al. 2000). However, the statement in the White Paper that DA rats are deficient in CYP2D1 activity, and several P450 cytochromes may also be absent, may be dated. DA rats do have a much lower hepatic metabolism of debrisoquine (an antihypertensive drug), which seems to be mainly due to a 30- to 40- fold lower CYP2D2 in DA females, and 6- to 8-fold lower CYP2D2 in DA males, than that of SD or Wistar rats (Schulz-Utermoehl et al. 1999). More importantly, the White Paper did not consider that the DA rat strain is widely used as an animal model for the human CYP2D6 poor metabolizer phenotype, while SD or Wistar rats are used as models for the extensive metabolizer phenotype. This P450 enzyme is involved in the metabolism of several pharmacological and toxicological compounds, including desbrisoquine, tamoxifen, propranolol, haloperidol, codeine, etc. (Klaassen 2001). In the discussion of the White Paper at the August 2003 EDMVS meeting, it was suggested that the use of the DA strain rat would be inappropriate since they are deficient in several CYP enzymes. However, so are many humans. The frequency of this CYP poor metabolizer phenotype differs between ethnic groups, and is approximately 5 to 10% in Caucasians, but less than 1% in Japanese (Klaassen 2001). Nevertheless the CYP2D6 poor metabolizer phenotype is of considerably greater concern to the toxicity of pharmacologically and neurologically active agents. Thus, more appropriate strains to be used in endocrine disruptor screening assays are those that are poor, or very high metabolizers, for compounds to be examined in the EDSP.

OVX DA/Han strain rats differ only slightly to moderately from Wistar or SD rats with respect to uterine weight and uterine epithelial thickness responses to ethinylestradiol, Genistein, BPA, or p-tert-octylphenol (OCT) (Diel et al. 2004). While this may add some confidence that CYP2D1/2 rat or CYP2D6 poor metabolizer humans are unlikely to be more susceptible to ethinylestradiol, Genistein, BPA, or OCT, this was not suspected to be the case, since this enzyme is not known to metabolize these compounds. Diel et al. (2004) used the DA rat for a different reason,

namely they had developed an endometrial adenocarcinoma cell line from this inbred rat strain, enabling comparative *in vitro* studies (Diel et al. 2004).

Human CYP3A4 metabolizes many physiologically, clinically, and toxicologically important compounds, including cortisol, ethinylestradiol, testosterone, flutamide, retinoic acid, tamoxifen, theophylline, and warfarin (Klaassen 2001). It is induced by many compounds, including phenobarbital, dexamethasone, phenytoin, and inhibited by many compounds, including ethinylestradiol and ketoconazole (Klaassen 2001). Many compounds that are substrates, inducers, or inhibitors for CYP3A4 will be screened for endocrine disruptor activity in the EDSP. The expression of CYP3A4 varies 40-fold in individual human livers, and metabolism of CYP3A4 substrates varies at least 10-fold *in vivo* (Dai et al. 2001). Human CYP3A4 polymorphisms differ dramatically in the metabolism of testosterone and especially the pesticide chlorpyrifos (Dai et al. 2001). The genetic variation in toxicity of compounds metabolized by this enzyme alone can be much greater than the 10-fold within-species safety factor.

Humans also show polymorphisms in oxidative damage protection enzymes, including (NAD(P)H:quinone oxidoreductase (QR) and several glutathione s-transferase (GST) isozymes. Polymorphisms in QR and or GST isozymes have been associated with susceptibility to several different cancers as well as susceptibility to benzene poisoning (Wan et al. 2002), oxidative damage in term pregnant women (Hong et al. 2002), and sensitivity to thimerosal (Westphal et al. 2000). Depending on ethnic background, 22 to 100% of humans have null alleles at GSTM1, e.g., lacking GSTM1 activity; and 11 to 58% of have null allele genotypes at GSTT1 (Klaassen 2001). Individuals homogozyous for null alleles at both GSTM1 and GSTT1 are at much greater risk of thimerosal sensitization and several cancers. However, high metabolizers may be at greater risk for xenobiotics, such as dichloromethane, that are activated by GST (Klaassen 2001).

The White Paper did not consider that murine strains differ dramatically in the endocrine/xenobiotic regulation of Phase I or II metabolic enzymes. Strains of rats differ markedly in the regulation by gonadal steroids and thyroid hormone of CYP and epoxide hydrolase enzymes involved in xenobiotic metabolism (Larsen et al. 1994; Larsen et al. 1995; Ikegwuonu et al. 1996). F344 and WF strain rats also differ in the induction of several CYP enzymes by phenobarbital, HCB, and o,p-DDD, in part due to suppressive effects of thyroid hormone in WF but not in F344 strain rats (Ganem et al. 1999). Strains of rats also differ dramatically in the endocrine regulation and disruption by TCDD of hepatic CYP1A1 (Jana et al. 1998).

The point is that the toxicity of many compounds can differ dramatically between humans with poor metabolizer phenotypes (with low activity of a specific enzyme) and extensive metabolizer phenotypes (with high enzyme activity). The same is true of laboratory animals with poor versus extensive metabolizer phenotypes, and this was not considered in the White Paper. The variation in xenobiotic metabolism/toxicity due to genetic differences in Type I and II metabolism ranges from a few-fold to much greater than the 10-fold within-species safety factor proposed for use in the EDSP and has strong genetic interactions with detection of reproductive and other toxicities. Yet little is known regarding how such polymorphisms affect susceptibility to the multitude of chemicals to be tested in the EDSP. Even though the EPA did not plan to consider the effects of polymorphisms in the activity or endocrine regulation of Phase I or II metabolic enzymes in the EDSP, such genetic differences need to be considered in the selection of animal models for endocrine disruptor screening, since genotypes respond differentially with respect to the rate of activation, detoxification, clearance, and toxicity of many xenobiotics.

This presents a quandary. Screening for EDCs in a single strain will clearly not suffice. On the other hand, screening all EDCs in a full set of more than a dozen animal models for poor or extensive metabolizer phenotypes found in humans would be impractical and would impede the

screening of the many compounds that need to be examined in the EDSP. Furthermore, screening for the effects of a single poor metabolizer locus is insufficient since several poor metabolizer phenotypes interact mechanistically to result in a much greater susceptibility to EDCs. The use of multiple, sensitive strains from diverse genetic backgrounds in the EDSP would reduce chances of underestimating effects of xenobiotics on such susceptible individuals. Nevertheless, there is a need to consider conducting validation studies with positive control EDCs using poor metabolizer animal models for common polymorphisms that play critical roles in the metabolism, detoxification, and/or action of steroids and/or xenobiotics to be screened in the EDSP. This could be done, for example, by comparing the susceptibility to positive control EDCs of wild type strains with that of rats with poor UGT metabolizer mutations or mice with targeted deletions of specific Phase I and II metabolism or endocrine response genes, the results of which would address whether such mutant strains are needed for routine EDSP screening assays or whether the EDSP should use a greater safety factor.

The reviewer recognizes that evaluating the genes involved in human and other species susceptibility to EDCs is likely to extend well beyond the EDSP. Nevertheless, the EDSP should at least be aware of, and make an effort to consider, the effects of major poor metabolizer phenotypes on susceptibility to EDCs. Selection of multiple reproducible isogenic strain animal models from diverse genetic backgrounds for the EDSP would greatly aid the present effort and enable more efficient identification of the genes controlling susceptibility to environmentally induced disease in future studies. Choice of outbred strain(s) for the EDSP will generally decrease the precision and therefore the sensitivity of the EDSP assays, and will not establish a reproducible framework for future studies to efficiently identify the genes underlying susceptibility to EDCs.

4K. ADDITIONAL MECHANISMS OF EDC ACTION

Endocrine disruption via inhibition of sulfotransferase: The White Paper did not consider evidence that several environmental pollutants can cause endocrine disruption by inhibition of enzymes involved in estrogen metabolism, especially estrogen sulfotransferase. The White Paper mentioned that strains of mice differ dramatically in susceptibility to endocrine disruption by estrogenic agents, and testicular estrogen sulfotransferase activity (Spearow et al. 2001a). However the White Paper did not consider how the observed strain differences in sulfotransferase activity are likely to affect susceptibility to endocrine disruption.

The White Paper did not consider evidence that environmental agents disrupt enzymes involved in estrogen metabolism, especially estrogen and phenol sulfotransferases, thereby leading to endocrine disruption due to excessive endogenous estrogens (Harris et al. 2000; Kester et al. 2000; Kester et al. 2002). PCBs and other polyhalogenated aromatic hydrocarbons (PHAHs) are persistent environmental pollutants, which disrupt reproductive endocrine function in birds, fish, reptiles, and mammals (Kester et al. 2000; Kester et al. 2002). Even though hydroxylated PHAHs and PCBs show low affinity for the ER, several exert their estrogenic effects at sub-nanomolar concentrations by inhibiting E2 metabolism, especially the sulfation of E2 by estrogen sulfotransferase (SULT1E1) (Kester et al. 2000; Kester et al. 2002). Since this is an important pathway for E2 inactivation, inhibition of SULT1E1 leads to an increased bioavailability of estrogens (Kester et al. 2002). Crystal structure analysis confirms that OH-PCB occupies the estrogen binding site of human estrogen sulfotransferase in a twisted manner (Shevtsov et al. 2003). In addition, short chain 4-n-alkylphenols are substrates for the phenol-sulfating enzymes (SULT1A1/2), while long chain 4-n-substituted alkyl phenols act as inhibitors of SULT1A1/2 (Harris et al. 2000). By acting either as substrates or inhibitors of SULT1A1/2, alkylphenols can

influence the sulfation, and thus the excretion, of estrogens and other phenol sulfotransferase substrates in humans (Harris et al. 2000). This new paradigm needs to be considered in explaining the endocrine disrupting potential of environmental chemicals that have low or no binding affinities for steroid hormone receptors (Song 2001).

Testicular estrogen sulfotransferase activity of highly fecund CD-1 mice is higher and more resistant to disruption by estrogen than that of B6 strain mice (Spearow et al. 2001a; Spearow et al. 2003). Estrogen sulfotransferase converts estradiol to estradiol-3-sulfate, which eliminates binding to the estrogen receptor and is excreted in to the urine on the first pass through the kidney. Thus, the elevated estrogen sulfotransferase activity in CD-1 testes should protect the gonads of this strain from excessive estradiol, even in the face of increased xenobiotics that inhibit estrogen sulfotransferase. Given that murine strains differ markedly in estrogen sulfotransferase activity (Spearow et al. 2001a), the potential for differential strain susceptibilities to EDCs through disruption of estrogen metabolism needs consideration in the EDSP.

Nevertheless, the sulfate can be removed by the action of estrogen /steroid sulfatase converting it back to estradiol. Steroid sulfatase activity in liver and reproductive tissues also differs dramatically between strains of mice and rats (Erickson et al. 1983), illustrating another potential mechanism for strain differences in sensitivity to estrogens.

Genetic differences in xenobiotic mechanisms and other EDC mechanisms of action: The design of the EDSP has mainly focused on mechanisms of endocrine disruption involving direct agonist or antagonist activity via ligand-receptor interactions, especially those involving estrogen, androgen, and thyroid hormone (EAT) receptors. The EDSP should not ignore the evidence that a number of xenobiotics act through signal transduction mechanisms other than classic EAT receptors to disrupt reproductive function and development.

The effects of partial estrogen agonists/antagonists on reproductive development and function differ greatly between and within species (Hart 1990; Nakai et al. 1999; Bailey et al. 2002). One of the main mechanisms of action of the partial estrogen agonist, tamoxifen, is likely via the inhibition of estrogen receptor (ER). Nevertheless, additional non-ER-mediated signaling mechanisms include the involvement of oxidative stress, PKC, calmodulin, transforming growth factor-beta (TGFbeta), and the proto-oncogene c-myc, mitogen-activated protein kinases (MAPK), and caspases (Mandlekar et al. 2001). Tamoxifen and several other environmental estrogens act through an ER- dependent mechanism to alter GST Ya ARE-dependent gene expression known to regulate Phase II detoxification enzymes critical for protecting against oxidative damage (Ansell et al. 2004). The protein kinase C (PKC)-mediated mechanism of tamoxifen action can be inhibited with anti-oxidants (Gundimeda et al. 1996). Thus effects of tamoxifen and other partial estrogen receptor agonists are likely to interact with dietary anti-oxidant intake and genetic differences in levels of oxidative damage protecting enzymes.

There are several examples in which strain differences in response to estrogens cannot be accounted for simply by differences in ER. The White Paper reported that SD strain rats were more sensitive than F344 rats to tamoxifen, and that the levels of uterine ER expression did not differ between these strains (Bailey et al. 2002). But the White Paper did not report that the expression of receptor co-regulator CARM1 was higher in SD than in F344 (Bailey et al. 2002), indicating a likely strain difference in transcriptional regulation.

In contrast, SD rats are insensitive, while F344 strain rats are sensitive to BPA-induced vaginal epithelial DNA synthesis (Long et al. 2000). But strains did not differ in the clearance of (3)H-BPA from blood, ER concentration or affinity, or the induction of the immediate early gene, c-fos (Long et al. 2000). Since F344 and S-D rats differ dramatically in sensitivity to the effects of

BPA on vaginal epithelial cell proliferation, these strains must differ in signal transduction mechanisms downstream of the estrogen receptor (Long et al. 2000).

Methoxychlor is well recognized as an estrogenic pesticide. The White Paper did not mention that recent gene expression studies, and studies in animals with estrogen receptor knockouts, have shown that this estrogenic xenobiotic can also act, at least in part, through estrogen receptor independent signal transduction mechanisms (Ghosh et al. 1999; Waters et al. 2001).

These studies are particularly significant since they indicate that measuring effects on classic ER receptor binding is not sufficient to monitor effects of environmental estrogen agonists and antagonists across environmental estrogens or across strains, e.g., genotypes. These studies, as well as observations of dramatic differences in effects of partial estrogen agonists/antagonists on reproductive development between species (Hart 1990), strongly suggest that the EDSP needs to consider a wider set of genetic and signal transduction mechanisms by which EDCs can disrupt reproductive development and function Since the EDSP plans to conduct the vast majority of initial Tier I screens only *in vitro* studies, care is needed in designing *in vitro* screening assays to avoid missing EDCs that disrupt reproductive development through genetically variable or non-classical estrogen/androgen/thyroid hormone signaling mechanisms. Of particular concern is the use of CD IGS rat testes as the animal model for the *in vitro* sliced testis steroidogenesis assay. Additional studies are needed to understand the implications of using an animal model in the *in vitro* sliced testis steroidogenesis assay which has been previously selected for high resistance to arsenic and for high prolificacy. Using multiple strains from diverse genetic backgrounds in the mammalian *in vitro* and *in vivo* screens will help to avoid the problem of missing genotype-specific EDC.

4L. SUSCEPTIBILITY TO THE DISRUPTION OF FERTILITY AND GESTATION

The reviewer agrees with the White Paper's statement in the conclusion that "*In endpoints like fertility and gestational parameters, the SD rat appears less sensitive than the F344 rat to several chemicals, suggesting that the F344 rat may be a better strain for assessing the effects of chemicals at these endpoints.*" However, this could have been better documented by inclusion of additional published data sets showing strain differences in susceptibility to the disruption of gestation, litter size, or fertility.

Human epidemiological evidence has associated exposure to low-dose bromodichloromethane (BDCM) a common drinking water disinfection by-product, with increased pregnancy loss in humans (Waller et al. 1998). The White Paper did report that gestational exposure to 75 mg/kg BDCM resulted in full litter resorption in 62% of Hsd:F344 rat litters, but 0% full litter resorption in Hsd:SD rat litters in response to 75 or 100 mg/Kg BDCM (Bielmeier et al. 2001). Thus SD rats are insensitive to BDCM-induced full litter resorption. The White paper also reported data showing that SD and LE rats were more resistant than F344 rats to atrazine induced full-litter resorptions (Narotsky et al. 2001). The White Paper accurately reported in the text that F344 and Holtzman rats were sensitive to atrazine-induced embryo loss, while SD and LE were not significantly affected, e.g., they were insensitive (Cummings et al. 2000). However, in Table 3 of the White Paper, F344 rats were inappropriately listed along with SD and LE rats as less sensitive to atrazine at fertility/gestational loss endpoints.

The White Paper also omitted data showing that highly prolific CD-1 strain mice were more resistant than C57BL/6J (B6) and especially C3H strain mice to disruption of litter size and fertility by exposure to ethylene glycol monomethyl ether (EGME) (Chapin et al. 1993).

The White Paper also excluded data showing that highly prolific CD-1 strain mice were markedly more resistant than B6 strain mice to disruption of gestation and full litter resorptions by

gestational exposure to increasing doses of estradiol (Spearow et al. 2003). This study found highly significant effects of Strain and E2 Dose on litter size, e.g., number of pups born ($P<0.0001$). CD-1 strain female mice were about 16 times more resistant than B6 strain mice to gestational E2-induced fetal mortality. Another experiment examined effects on embryo/fetal survival at gestational day 16 and found no full-litter resorptions in zero dose control mice of either strain. Implantation with body weight-proportional 2.5 to 3.25 µg E2 implants resulted in 0% and 42% full-litter resorptions in CD-1 and B6 mice, respectively ($P<0.005$). The CD-1-derived CD10 inbred strain was also resistant to estrogen-induced gestational losses. These findings raise further concern that the use of prolific strains of laboratory animals in the EDSP may underestimate effects of estrogenic agents on the disruption of fetal survival and gestation (Spearow et al. 2003).

Selection for large litter size in mice increases ovulation rate, embryo survival, and litter size (Bradford 1969). Embryo survival is under genetic control. Selection for high or for low embryo survival produced strains of mice differing markedly in embryo survival, litter size, and serum hormone levels during pregnancy (Bradford 1969; Barkley et al. 1979). While polymorphisms in Factor V Leiden and prothrombin have also been associated with recurrent pregnancy loss in humans (Tempfer et al. 2004), whether women with low embryo survival are more susceptible to environmental chemicals has not been determined. Since many humans also show a high incidence of embryo mortality, the estimation of risk of pregnancy disruption in humans by environmental agents may be better modeled by strains that have not been selected for large litter size, and high, robust embryo survival.

Given the instructions from Congress in the SDWA and FQPA, it would seem inappropriate to use a highly prolific strain such as the SD rat, that has been shown to be insensitive to the disruption of gestation, e.g., full-litter resorptions, by the drinking water chlorination by-product, bromodichloromethane (BDCM).

4M. GENETIC DIVERSITY IN TIER I AND II EDSP ASSAYS

The White Paper did not mention that the role of genetic diversity in susceptibility to endocrine disruption needs to be considered in EDSP Tier I and Tier II studies. The results of Tier II studies may supersede results of the Tier I studies, may be used for risk assessment, and may play a major role in determining the allowable environmental releases of a xenobiotic. The use of a single strain which is less sensitive, and especially a strain which is insensitive to a given EDC in the Tier II studies, will clearly underestimate risk to susceptible species, populations, and individuals. If anything, it is even more critical not to rely on the use of strains that are less sensitive to the detection of EDCs in Tier II studies, since the results will be used for establishing allowable environmental exposures. Thus, issues of genetic diversity and sensitivity need to also be addressed in EDSP Tier II assays.

4N. DIETARY CONFOUNDERS

High levels of dietary phytoestrogens and antioxidants confound the detection of estrogenic agents, protect against endocrine disruption through oxidative stress, and interact with strain differences in susceptibility to endocrine disruption. The White Paper did not consider that feeding a diet with high levels of phytoestrogens and antioxidants, and using an oxidative stress-resistant animal model, is likely to underestimate the endocrine disrupting effects of oxidative stress-inducing EDCs on humans and wildlife eating less than optimal diets.

High-phytoestrogen diets such as the EPA's preferred diet (Purina 5002) can mask the effects of endocrine active agents on reproductive development and function (vom Saal et al. In Press). Available diets differ dramatically, in phytoestrogen content, ranging from near zero in Teklad 2016 Soy and Alfalfa Free diet, to very high levels of phytoestrogens in Purina 5001 and 5002, with soy as the second ingredient. Such high phytoestrogen levels, and other factors, affect reproductive development of Wistar-derived Alpk rats similar to that of weak estrogenic agents (Odum et al. 2001). Thus, Odum et al. (2001) argue for the use of a soy-free or low-soy diets such as Teklad Global 2016 or RM1, respectively, since these diets give low control uterine weights, thus reducing chances of these diets confounding endocrine toxicity studies. The issue of dietary phytoestrogens is even more pertinent to the issue of strain selection, since strains of mice differ in sensitivity to the effects of dietary phytoestrogens on uterine weight (Farmakalidis et al. 1984b; Farmakalidis et al. 1985). Strain differences in the induction by phenobarbital of several Phase I CYP enzymes involved in xenobiotic metabolism also interact with diet (Larsen et al. 1994), and this was not considered in the White Paper.

It was not discussed in the White Paper that several dietary antioxidants, including vitamins, trace metals, and phytochemicals, can protect against oxidative stress, while malnutrition and certain dietary imbalances can result in oxidative stress and disease (Fang et al. 2002; Furst 2002; Park et al. 2003). Phytoestrogens and other phytochemicals can also scavenge reactive oxygen species (ROS), thereby protecting against oxidative stress (Fang et al. 2002; Furst 2002). Consumption of flavanols and procyananidins reduce 8-oxodG in SD rat testes (Orozco et al. 2003).

Antioxidants including Vitamin C and Vitamin E have been shown to inhibit or block the endocrine disruption/reproductive toxicity of methoxychlor, arsenic, and mercury. Vitamin E protected Wistar rats against the disruption of epididymal weight, sperm counts, sperm motility and epididymal oxidative stress by Methoxychlor (Latchoumycandane et al. 2002). Vitamin E protected Wistar male rats from inhibition of sperm counts, sperm motility, serum testosterone, mating rate, and fertility by mercury (Rao et al. 2001). Vitamin C reduced or prevented the inhibition of ovarian and uterine weight, ovarian steroidogenic enzymes, plasma gonadotropins and estrous cyclicity by arsenic (Chattopadhyay et al. 2001). Thus, human and wildlife populations that are exposed to many dietary imbalances and/or deficiencies likely to perturb redox status may be more subject to oxidative stress related endocrine disruption than laboratory animals fed an optimized diet rich in oxidative damage protecting antioxidants and phytoestrogens.

In addition, murine strains differ in susceptibility to oxidative stress, and oxidative stress-inducing toxicants (Sams et al. 2000; Binda et al. 2001; Mathews et al. 2002) and such genetic differences may interact with the diet. SD rats were selected for resistance to arsenic, and are more resistant to the reproductive toxicity of lead, cadmium, and estrogenic agents at several endpoints in females and especially in males. If the resistance of SD rats to endocrine disruption by such EDCs involve upregulated oxidative stress defense mechanisms, as data for liver would suggest (Younis et al. 2000; Binda et al. 2001), the combination of genetic resistance to oxidative stress and high levels of dietary antioxidants may make this strain even more resistant to endocrine disruption. In any event, the White Paper did not consider whether dietary phytoestrogens and antioxidants interact with strain differences in susceptibility to endocrine disruption.

The White Paper also did not consider that the published isoflavone content of soybeans differs 6-fold between varieties and growth conditions (Fletcher 2003). QTL or genes controlling soy phytoestrogen content have been mapped and are being used to rapidly develop commercial soybean varieties, some with much lower and others with much higher phytoestrogen content (Meksem et al. 2001), greatly increasing the potential for long-term variability in dietary phytoestrogens in high soy diets like PMI 5002. Thus, the warnings of Odum et al. (2001)

regarding diets used in endocrine disruption assays should be heeded. Odum argued that: 1) a diet with low levels of phytoestrogens should be utilized; and, 2) a diet containing even small amounts of soybean meal of varying phytoestrogen content could produce experimental variation in endocrine disruptor assays. The independent finding of vom Saal, that Purina 5002 high soy diet masks the effects of prenatal DES exposure on reproductive development in CD-1 mice, further heightens these concerns (vom Saal et al. In Press).

4O. ANIMAL MODELS FOR MULTIGENERATION REPRODUCTIVE TOXICOLOGY STUDIES

Use of isogenic strains for multigeneration toxicology experiments: The following statement on page 20 of the White Paper is misleading:

> *"However, in any study requiring generation of offspring, such as the two-generation reproductive toxicity study, the advantages of utilizing isogenic strains is lost since use of F1 parents will produce F2 offspring which are segregating at many loci with differing genotypes and phenotypes."*

This statement is true for the use of isogenic F1, crosses but false for the use of isogenic inbred strains. Apparently the White Paper authors do not realize that there are two kinds of isogenic strains, isogenic inbred strains and isogenic F1 crosses of inbred strains. The reviewer agrees that isogenic F1 crosses are unsuitable as parents for multigeneration toxicology studies due to increased variability due to segregation of genes at heterozygous loci in the F2 and F3 generations. However, isogenic inbred strains are homozygous at all of their loci and will not show increased variability in successive generations, and will thus be suitable for single- as well as multigeneration toxicology experiments. Furthermore, if inbred parents were crossed and dosed during gestation or lactation, the isogenic F1 cross offspring would be appropriate as animal models for assays involving gestational and or lactational exposure of the F1 offspring.

Selection for resistance to reproductive toxicants in multigeneration toxicology experiments: The White Paper did not consider the potential for direct genetic selection for resistance to a reproductive toxicant during the course of multigeneration reproductive toxicology studies in outbred strain animal models. Such selection will confound the results of multigeneration reproductive toxicology studies but has been inadequately addressed. The response to increasing doses of a reproductive toxicant in an inbred strain represents environmental effects, e.g., effects of the chemical being tested. In contrast, the response of a reproductive toxicant in a genetically heterogenous outbred strain involves a combination of genetic and environmental sources of variation.

Outbred individuals resistant to a reproductive toxicant will tend to leave more offspring than sensitive individuals, depending on the proportion of the phenotypic variance that is genetic, i.e., the heritability of the trait. Any time a toxicant exposure results in differential reproduction or fitness between mating pairs, and especially infertility, there is potential for selection for resistance to the reproductive toxicant. Additionally, if an EDC causes embryonic, fetal, or neonatal loss, and the genotype of the conceptus contributes to its own survival, there may also be selection at the level of the conceptus for higher genetic resistance to the EDC under analysis. In essence, any alleles in the outbred strain providing resistance to the EDC/reproductive toxicant and maintaining fitness will increase in frequency.

Since reproductive traits generally have a medium to low heritability, it might seem as though the magnitude of genetic change would be quite small over the course of a typical 2- to 3-generation ("multigeneration") reproductive toxicology experiment. However, in an outbred strain with high nominal fecundity it is likely that an individual's susceptibility to a reproductive toxicant will be the main determinant of fitness. If a susceptibility trait shows a medium to high heritability, and/or is controlled by a small number of loci with major additive effects that are segregating in the outbred population, 2 to 3 generations of selection could markedly increase the frequency of resistant alleles in EDC-treated groups, e.g., sublines. In other words, after 2 to 3 generations of selection for EDC resistance, the treated groups may be genetically more resistant to the EDC. In contrast, the (zero dose) control group/subline will not have been selected for resistance to the EDC. Such selection for EDC resistance will confound the results of multigeneration reproductive toxicology studies, underestimating effects on susceptible genotypes. The magnitude of the response to selection depends on the heritability of the trait, the selection differential and the number of generations of selection (Falconer 1989). Heritability is the percentage of the total phenotypic variance which is additive genetic, and also depends on the frequency of susceptible alleles segregating in the population. Since several selection experiments have reported changes in pharmacological or toxicological traits in response to fairly short-term selection experiments (Wise et al. 1993; Rasile et al. 1995; Stino et al. 1998), the potential for selection confounding results of multigeneration selection experiments in outbred strains cannot be ignored.

Differential fertility indicates a potential problem. Unfortunately, EDCs with the greatest effects on fertility/fecundity of mating pairs or embryonic/fetal/neonatal viability are the most subject to selection-related bias. Furthermore, since many of the parameters needed to estimate selection differentials for fecundity/fertility/fitness traits are not reported, most multigeneration experiments in outbred strains will not even recognize the possibility of genetic change and selection confounding the results. Moreover, it will be impossible to estimate selection differentials arising from differential embryo/fetal/neonatal survival. An example of this problem is seen in the data of Hellwig et al. (2000), where a large number of LE and Wistar outbred pups showed neonatal mortality in the 200 mg/kg Vinclozolin gestational exposure groups.

The problem of selection within the multigeneration experiment can be reduced by minimizing selection differentials via selecting an equal number of progeny from each mating pair and randomly mating for the next generation. Nevertheless, genetic selection for resistance to the EDC will occur in outbred strains if a chemical results in infertility of any mating pairs or increased mortality of sensitive embryo/fetal/neonatal genotypes. Minimizing the number of generations in multigeneration experiments will tend to reduce the potential for genetic change and confounding of EDC effects as well as reducing costs.

The use of highly inbred, isogenic strain animal models avoids the potential problem of selection confounding the results of multigeneration reproductive toxicology studies. The use of multiple inbred strains enables establishment of genetic models for EDC susceptibility, as well as determining the effect of EDCs on reproductive development and function. The main limitations for the use of inbred strains of moderate fecundity for multigeneration toxicology experiments are the smaller number of offspring available per litter and variability in percentage of inbred strain mating pairs reproducing over a short interval. This may limit the precision of estimating effects of EDCs on breeding performance endpoints in multigeneration toxicology assays. The EDSP would be aided by screening for inbred strain models with good breeding performance and reasonable litter size that are not resistant to classes of EDCs.

5. SUMMARY OF STRAIN DIFFERENCES IN SUSCEPTIBILITY TO ENDOCRINE DISRUPTION

Of major concern to the reviewer are the executive summary, Tables 2 & 3, and the final summary (after Table 3) of the White Paper. Section 4 of this Appendix discussed several errors in the reporting of strain differences in sensitivity to EDCs in the text and tables of the White Paper. Several statements in the final summary do not accurately portray the direction or magnitude of strain differences in senstitivity/susceptibility to EDCs shown in the original data.

The way in which "EDC-sensitive" strains are categorized and used in the White Paper makes it difficult to discern which strains are suitable for EDC screening assays. The White Paper categorizes strains as being "sensitive," "less sensitive," or "insensitive" to a given EDC and reproductive endpoint. However the categories of "less sensitive" and "insensitive" strains were then pooled in a single column in Table 3, making it impossible to determine from this table which strains actually failed to respond to a given EDC. Then in the summaries and elsewhere, strains that showed "less sensitive" responses to an EDC were described as being "sensitive" because they responded. The reviewer contends that this is not appropriate and that it under-reports the magnitude of strain differences in susceptibility to EDCs. A "less sensitive" strain should be described as "less sensitive."

As discussed in Section 4 of this Appendix, in several cases the text and/or tables did not clealy indicate when a strain failed to respond to an EDC, e.g., was insensitive. For several other EDCs, the sensitivity of strains was incorrectly reported. For example, strain differences in sensitivity of males to lead (Pb) and low-level estrogen were reversed, in both cases incorrectly reporting SD strain rats to be the most sensitive strain (Apostoli et al. 1998; Putz et al. 2001b). The reviewer contends that this confuses and hinders the selection of robust, sensitive animal models for reliably detecting EDCs in the EDSP.

Importance of considering which strains were less sensitive and/or insensitive to an EDC in Tier I and II Assays: It is important to consider the EDCs and endpoints for which each strain was less sensitive or insensitive (and where other strains responded). Depending on less sensitive strains to detect EDCs in Tier I assays is ill advised since they are less likely to detect effects of weaker environmentally endocrine-active chemicals, and larger numbers of less sensitive strain animals will be required to achieve statistical significance. In other words, the use of less sensitive animals in Tier I assays will result in less robust and dependable screening assays. Depending on insensitive strains to detect EDCs in Tier I assays is totally inappropriate, since they will fail to detect EDCs that are harmful to sensitive individuals, thus excluding them from further consideration as EDCs. In essence, the use of less sensitive or insensitive strains increases the likelihood that a harmful chemical will be missed in the EDC screen.

Strains that are less sensitive or insensitive to EDCs will also underestimate effects of EDCs on sensitive genotypes and individuals in Tier II studies. Since the Tier II assay results will be used in establishing allowable environmental releases, the use of strains that are either less sensitive or insensitive to EDCs in Tier II assays will result in higher allowable environmental levels of EDCs, that will be detrimental to the reproductive development, function, and health of sensitive species, populations, and individuals. The following is a list of the EDC and reproductive endpoints for which a given strain was shown to be less sensitive or insensitive. Note that the reviewer would have preferred to use a table but this was not allowed by the EPA.

EDCs and reproductive endpoints for which F344 strain rats were less sensitive or insensitive: Relative to that of SD rats, inbred F344 strain rats were less sensitive to effects on uterine weight and uterine histopathology by tamoxifen (Bailey et al. 2002) and effects on uterine weight by D4 (McKim et al. 2001). F344 rats were also insensitive to disruption of estrous cyclicity and serum E2 (and resulting mammary tumors) by atrazine (Eldridge et al. 1994). These three compounds are all partial estrogen agonists. Nevertheless, F344 rats did respond to tamoxifen and D4, and were more sensitive than SD rats to the effects of atrazine on gestational losses (Cummings et al. 2000; Narotsky et al. 2001). F344 was also less insensitive to flutamide inhibition of glans penis weights in a Hershberger assay, where as other endpoints did respond. (Yamasaki et al. 2001). Thus, there were four EDCs for which F344 rats were less sensitive or insensitive, and then only at some endpoints.

EDCs and reproductive endpoints for which SD or CD strain rats were less sensitive or insensitive: In contrast, relative to F344 rats, highly fecund SD or CD strain rats were less sensitive or insensitive to: decreases in testes and seminal vesicle weights and serum testosterone by adult exposure to DES (Inano et al. 1996); decreases in reproductive organ weights, e.g., reduction of testes, epididymal, seminal vesicle and coagulating gland weights by neonatal estrogens (Putz et al. 2001b); reduction of lateral and dorsal prostate weights by neonatal estrogens (Putz et al. 2001a); disruption of female reproductive tract histopathology by estrogen (Steinmetz et al. 1998; Bailey et al. 2002); disruption of pituitary weight and pituitary prolactin secretion by estrogen (Schechter et al. 1987; Steinmetz et al. 1997); induction of pituitary tumors by estrogen and DES (Gregg et al. 1996; Wendell et al. 2000); disruption of uterine weight, female tract histopathology, vaginal DNA synthesis, and prolactin levels by BPA (Steinmetz et al. 1997; Steinmetz et al. 1998; Long et al. 2000); disruption of gestation (full-litter resorptions) by bromodichloromethane (BDCM) (Bielmeier et al. 2001), disruption of embryo survival or gestation by atrazine (Cummings et al. 2000; Narotsky et al. 2001), disruption of estrous cycles by feed restriction (Tropp et al. 2001); decline in spermatogenesis by lead (Sokol et al. 1991; Nathan et al. 1992; Apostoli et al. 1998), and disruption of female pubertal development by gestational and lactational lead exposure (Dearth et al. 2004).

While effects on reproductive tissues are unknown, SD rats were also more resistant than F344 rats to hepatotoxicity of cadmium (Kuester et al. 2002); GSH depletion and hepatotoxicity by 1,2-Dichlorobenzene (1,2-DCB), acetaminophen, and the herbicide diquat (Gupta et al. 1994; Younis et al. 2000). SD rats were also insensitive to induction of T3 levels by TRH, although TSH and T4 did respond. The latter are needed for endocrine challenge assays to detect thyroid hormone disruptors (Fail et al. 1999). For some classes of EDCs, such as androgen and thyroid hormone disruptors, very few contemporary comparisons between SD and F344 rats are available. So the actual list of strain differences is likely to be even greater.

Relative to Wistar or Wistar-Derived Alderly Park rats, SD/CD rats were less sensitive or insensitive to: the decrease in testes and seminal vesicle weights and testosterone levels by DES (Inano et al. 1996), delay of vaginal opening by BPA, (Tinwell et al. 2002); inhibition of sperm count and daily sperm production by BPA (Tinwell et al. 2002); induction of uterine weight and epithelial cell thickness by estradiol (Diel et al. 2004); inhibition of spermatogenesis or epididymal sperm counts by lead (Apostoli et al. 1998); and slightly less sensitive to effects of p-nonylphenol on uterine weight (Odum et al. 1999). While aging Wistar and Wistar-derived Wistar inbred rats were susceptible to prostatitis, SD rats were not (Naslund et al. 1988).

Relative to LE strain rats, SD/CD rats were less sensitive or insensitive to: inhibition of testis and seminal vesicle weights by dibutyl phthalate (Gray, 2003); p,p'-DDE (a DDT metabolite) on male anal genital distance (AGD) (You et al. 1998); insensitive to effects of p,p'-DDE (300 mg/kg/day) on seminal vesicle and prostate weights and TSH levels (O'Connor et al. 1999); disruption of estrous cyclicity, LH and prolactin levels by atrazine (Cooper et al. 2000). Following gestational treatment with an aromatization inhibitor, relative to LE rats, SD rats were also less sensitive to induction of lordosis by estrogen and progesterone (Whalen et al. 1986).

Once the errors of reporting strain sensitivity in the White Paper are corrected, a much clearer picture emerges showing that, for a majority of EDCs tested, SD or CD strain rats are strikingly more resistant than F344 rats to endocrine disruption by many EDCs. SD/CD rats are also more resistant to several EDCs than Wistar or LE rats. Collectively, these data show that F344 rats would be suitable, while SD rats would not be suitable, for screening for estrogens, xenoestrogens such as BPA, toxic metals compounds, and agents that disrupt pregnancy. In contrast, SD rats might be suitable for screening partial estrogen angonists, e.g., antiestrogens.

EDCs and reproductive endpoints for which other strains of rats were less sensitive or insensitive: Relative to F344 rats, LE rats were more resistant to induction of full-litter resorptions by atrazine (Narotsky et al. 2001); embryo loss by atrazine (Cummings et al. 2000); and inhibition of testes weight by vinclozolin (Hellwig et al. 2000).

Relative to F344 rats, Wistar strain rats were less sensitive to the disruption of testis, and seminal vesicle weights by DES (Inano et al. 1996); induction of pituitary tumorigenesis by estrogens (Yin et al. 2001); and induction of uterine lesions by cadmium rats (Rehm et al. 1988).

Relative to Wistar rats, F344 rats were less sensitive to the inhibition of glans penis weight by flutamide in the Hershberger assay (Yamasaki et al. 2001); to the induction by thyroid hormone of many different Phase I and II enzymes involved in xenobiotic metabolism and detoxification (Larsen et al. 1995; Ganem et al. 1998; Ganem et al. 1999).

Of special note is that the White Paper asserts that strains do not differ substantially in sensitivity to estrogenic compounds for uterine weight endpoints in the uterotrophic assay. These assertions are made regarding comparisons involving Wistar, Wistar-derived ALKP, and SD rats, and in one study DA rats. However, these comparisons did not include more diverse inbred strains such as F344, or ACI rats, which are known to be sensitive to estrogenic agents. Furthermore, in some studies involving Wistar, Wistar-derived, and Wistar cross-derived strain rats, e.g., SD, the effects of strain were confounded across laboratories with all the other variables, and the effect of strain was not actually tested (Kanno et al. 2001; Kanno et al. 2003).

EDCs for which strains were insensitive up to the highest dose of the EDC tested: It is important to consider the EDC and endpoints for which each strain was insensitive to up to the highest dose tested (and where other strains did respond). Depending on such insensitive strains to detect EDCs in Tier I assays is inappropriate, since they will fail to detect EDCs that are harmful to sensitive individuals, thus excluding them from further consideration as an EDC. The use of insensitive strains in Tier II assays is also inappropriate since it will result in allowable environmental releases and levels of EDCs that will be detrimental to the reproductive development, function, and health of sensitive species, populations and individuals. Thus, the following list shows strain x EDC endpoints where one strain was insensitive up to the highest EDC dose tested while another strain examined responded.

EDCs and reproductive endpoints for which F344 strain rats were insensitive: As pointed out in Section 4 of this Appendix, F344 strain rats responded to the vast majority of the EDCs administered in the studies reported in the White Paper, with the exception of long-term atrazine exposures on estrous cyclicity, serum E2, and mammary tumors (Eldridge et al. 1994). F344 was also less insensitive to flutamide inhibition of glans penis weights in a Hershberger assay, where as other endpoints did respond. (Yamasaki et al. 2001). The insensitivity to atrazine also appears to be endpoint-specific, since F344 strain rats were more sensitive than both SD and LE rats to atrazine-induced gestational losses (Cummings et al. 2000; Narotsky et al. 2001). Thus, F344 rats were insensitive to two chemicals, atrazine, and flutamide at some, but not all, endpoints.

EDCs and reproductive endpoints for which SD or CD strain rats were insensitive: In several experiments in which other strains did respond to an EDC, SD or CD rats failed to respond up to the highest dose administered. SD or CD strain rats failed to show an effect of: BDCM (a drinking water disinfectant by-product) on gestational losses (full litter resorption) (Bielmeier et al. 2001); atrazine on embryo/fetal loss (Cummings et al. 2000); DES on decreases in testes and seminal vesicle weights (Inano et al. 1996); BPA on prolactin levels (Steinmetz et al. 1997); BPA on uterine weight and especially on uterine histopathology (Steinmetz et al. 1998); BPA on vaginal cell proliferation and DNA synthesis (Long et al. 2000); BPA on sperm counts in males and age at vaginal opening in females (Tinwell et al. 2002); lead on spermatogenesis (Apostoli et al. 1998); lead on disruption of female pubertal development (Dearth et al. 2004); food restriction on estrous cyclicity (Tropp et al. 2001); and, p,p'-DDE (300 mg/kg/day) on seminal vesicle and prostate weights, and TSH levels (O'Connor et al. 1999). Thus, SD/Cd rats are insensitive to several positive control EDCs including estrogens, estrogenic xenobiotics, lead, a anti-androgen, and chemicals that disrupt gestation.

These studies show that the effect of EDCs was significantly different between strains, and indicate that strain x EDC interactions are very important. Since strain, e.g. genetic background, was found to interact with susceptibility to endocrine agents in many experiments, since no one strain was most sensitive to all EDCs, and since many strains were insensitive to several EDCs, these data show that multiple, preferably isogenic strains need to be used in the EDSP to reduce chances that all the animal models will be resistant to the EDCs being screened. Unfortunately, a uniform set of strains has not been screened for susceptibility to a set of control EDC.

6. DISAGREEMENTS ON GENETIC PRINCIPLES OR INTERPRETATION

6A. RELAXED SELECTION

The reviewer does not agree with the statement in the White Paper that relaxation of selection for large litter size in CD IGS rats is likely to restore this strain's litter size and sensitivity to EDCs to that of the original population. Data from several experiments show that SD/CD rats are currently highly resistant to several different EDCs at most endpoints. For several reasons, the reviewer considers it highly unlikely that simply relaxing selection for large litter size in the CD IGS strain will restore the litter size and sensitivity of this strain to that of the initial population.

The White Paper states:

"Since selection for large litter size was relaxed since the formation of the CD IGS rat population, this population may be restored to its original litter sizes, but not necessarily the original genotype. However, if the restoration of original litter size resulted from changing the genes which control litter size, these genes may also control response of other endocrine-sensitive endpoints (i.e. a pleiotropic effect, whereby a single gene controls a number of parameters/responses). Therefore recovery of original litter size may also change the sensitivity of the strain to the [pleiotropically]-related endpoints (e.g. FSH or LH levels, number of eggs ovulated, responsivity to E2, or estrogen-like compounds, etc.) back to where it was, whether or not the original genotype was recovered" (page 19).

The reviewer contends that this statement is misleading in several regards and is not supported by available scientific information. First of all, as previously discussed, the SD rat was developed from an exceedingly narrow genetic base with intense inbreeding and with selection for high lactation/reproduction/vigor and high resistance to arsenic trioxide (Poiley, 1953). Such selection, especially with inbreeding, is very likely to have eliminated deleterious recessives commonly found in outbred populations (Falconer 1971; Eklund et al. 1977; Falconer 1989). Even by 1946, SD strain rats showed "tremendously increased vigor and reproductive power, so that the weight of young produced by each mother during the nursing period was increased more than 70%, partly accounted for by increased size of litters and partly by increased weight of each young." (Poiley 1953). SD-derived CD rats were then selected by Charles River Laboratories (CRL) for an additional ~80 generations, mainly for large litter size and for increased vigor (Parady and Mirley 2003; 2004) and appear to be more prolific than Hsd:SD rats. Thus the litter size of CD rats would have to decline to well below the means of the Hsd:SD rats to recover the original litter size. Due to the early inbreeding and long-term selection purging deleterious recessives affecting reproduction, it would be essentially impossible to restore the initial genotypes. New mutations occurring in large outbred populations are likely a significant source of variation.

Secondly, CRL has gone to extensive efforts in the IGS program to avoid genetic drift, and reports using random selection in the IGS stocks, which should minimize genetic change in this outbred population. Thus, it is unlikely that the litter size of CD IGS strain will decline to that of the Hsd:SD rat, much less to that of the "initial population" unless there was downward selection pressure for smaller litter size. Nevertheless, since growth rate/body size is genetically correlated with ovulation rate and litter size, selection for decreased body size/vigor may also reduce litter size (Baker et al. 1975; Eisen et al. 1980; Barria et al. 1981). However, if the litter size of CD rats is declining, as implied by the White Paper authors and as claimed by CRL technical service personnel, the newly formed CD IGS rat may be undergoing genetic change in reproductive traits

that would raise even greater questions about the use of this outbred strain for many reproductive toxicology screening assays over time.

The reviewer agrees that selection for high or low litter size will have pleiotropic effects on other traits, especially reproductive traits with overlapping endocrine signal transduction control mechanisms. The reviewer also agrees that this strain's sensitivity to endocrine compounds is also likely to change if its litter size changes. Such changes in endocrine sensitivity would be of serious concern to the suitability of this outbred animal model for long-term reproductive toxicology assays. **However, the reviewer does not agree with the White Paper that the sensitivity of the CD IGS strain to endocrine compounds will change back to that of the original population.** Correlated trait responses are not 100%. Some of the genes controlling ovulation rate/embryo survival/litter size involve estrogen response mechanisms, while others are largely independent of such mechanisms. Correlated trait responses depend on which genes are segregating and respond to selection, the mechanism of action of a given gene, and its effects on different phenotypes. Mutations in several genes affecting estrogen metabolism, feedback, or signal transduction pathways are likely to affect ovulation rate/litter size, as well as sensitivity to natural and environmental estrogens. But there are also many genes controlling gonadal function, ovulation rate, fertilization rate, and embryo/fetal survival, e.g., the components of litter size, which do not involve estrogen actions. Deleterious mutations in such estrogen-independent genes are likely to decrease litter size, but have little or no effect on the response of a given tissue to estrogen. Other mutations that control litter size act through signaling pathways that are largely independent of estrogen action. For example, mutations in TGFβ-related family members' bone morphogenetic protein 15 (BMP15), and bone morphogenetic protein 1B Receptor (ALK-6) alter follicular development, ovulation rate, and litter size through intraovarian paracrine signaling mechanisms, and do not affect reproductive function of males (Galloway et al. 2000; Wilson et al. 2001).

Data from several experiments show that SD / CD rats are currently moderately to highly resistant to several different EDCs at several endpoints. The reviewer considers it highly unlikely that simply relaxing selection for large litter size will restore the sensitivity of CD IGS rats to that of the initial population.

6B. RESPONSIVITY OF OUTBRED STRAIN MODELS

The reviewer disagrees with the claims of the White Paper that outbred strains have a higher "*responsivity*" and therefore a greater likelihood of detecting an effect. The authors of the White Paper argue that "*the confidence interval for an outbred strain is wider due to the variability, so that use of an outbred strain with greater variability and therefore less precision, would be better because of its variability, than using a very precise isogenic strain if it were not the most sensitive one to the specific chemical or for the specific endpoints*" (page 20). Then, in the final assertion of the White Paper, the authors state: "*If selecting a single strain for endocrine disruptor screens, outbred strains have more genetic variability, exhibit a broader range of responsivity (with a greater likelihood of detecting an effect), and may be more appropriate*" (page 75).

The reviewer does agree that using a single inbred or outbred strain is highly problematic since it may be resistant to the chemical being tested, and thus effects on sensitive genotypes will be underestimated. Nevertheless, the reviewer contends that since the objective of the EDSP is to detect chemicals with endocrine disruptor activity, the best way to do this is with precise, sensitive, and reproducible endocrine disruptor assays that can be best provided by isogenic inbred strain or isogenic F1 cross animal models. The high degree of genetic uniformity of isogenic strains minimizes variation within treatment groups, enabling precise estimates of treatment means, and

minimizes the number of individuals needed per treatment group to detect a significant effect (Festing 1987; Festing 1995).

In contrast, the White Paper authors argue for a more variable, less precise assay using a more variable outbred strain. Then they argue that since it has more variability in its responses, it has a greater likelihood of detecting an effect. The problem with such assumptions are many-fold:

1. The use of genetically more variable outbred stocks reduces the precision of toxicology dose-response curves and therefore the sensitivity of bioassays (Festing, 1979).

2. The authors assume that genetic variability remaining within an outbred strain will provide sufficient diversity to detect an effect. However, if a toxicant susceptibility allele is segregating at a low frequency in an outbred strain, the variability within toxicant treated groups will increase dramatically. But if most individuals have resistant genotypes, the mean is unlikely to decline significantly, and effects on the few susceptible individuals will be missed. Regardless of whether susceptible alleles are segregating at a low or a moderate frequency, the increased variability in trait responses decreases assay precision and decreases the likelihood of detecting a toxicant effect.

3. The common practice of excluding "outliers" (Tyl et al. 2002) will also tend to exclude the low-frequency genetic variants within a segregating strain, further reducing the chances of detecting an effect on low-frequency susceptible alleles.

4. As discussed previously, many outbred stocks have been selected long-term for high fecundity traits associated with increased resistance to estrogenic agents and compounds that disrupt pregnancy. SD rats were also selected for high resistance to arsenic, the effects of which have yet to be fully elucidated.

5. As shown in Sections 4 and 5 of this Appendix, the results of many experiments show that outbred stocks, such as the SD rat, are less responsive than inbred strains, such as the F344, to many positive control EDCs (with the exception of antiestrogens). Thus, the use of outbred strain(s) generally decreases sensitivity in EDC screening assays and masks the detection of EDC effects on low-frequency susceptible alleles and individuals. Nowhere else in biology when there is a specific concern about assay sensitivity and reproducibility would one choose to use a less precise, less sensitive, less reproducible assay, when more precise, sensitive, and reproducible assays are available at comparable or only slightly higher overall cost. Why should the EDSP?

6C. TREATMENT OF MULTIPLE VS. SINGLE STRAIN ISSUES

The reviewer disagrees with the implication of the White Paper that since strains differed markedly in their sensitivity to endocrine disruption by different endocrine agents, and because we cannot identify the most sensitive animal model to all endocrine agents, then we can ignore the multiple strain issue and instead focus on choosing a single strain. Instead, the reviewer contends that these are among the very reasons why multiple strains are needed in EDC screening assays.

The data from several experiments show that effects of several EDCs differ markedly between strains, e.g., many strain x EDC interactions were observed. While some strains were sensitive to specific EDCs, other strains were insensitive to the disruption of reproductive

development/function, even by the highest dose administered. Unfortunately, relatively few strain comparisons of susceptibility to EDCs have been made, and those have involved different combinations of strains. Thus, we do not know the relative susceptibility or insensitivity of many strains for many different EDCs. Nevertheless, the currently available comparisons show that SD/CD strain rats are less sensitive or insensitive to a substantial proportion of EDCs tested to date, making them unsuitable as a sole animal model. While fewer comparisons have been made with LE and Wistar rats, they also show strain-specific EDC insensitivities. The F344 rat strain is sensitive to a high percentage of compounds tested, but insensitive to atrazine at some endpoints while more sensitive at others. While the F344 rat would be suitable as one of several animal models for the EDSP, the reviewer does not consider the F344 as a suitable single-strain animal model for the EDSP, since several features of its reproductive endocrinology differ from those of other strains. The problem with genetic background effects on traits of interest is not unique to the environmental reproductive toxicology field. Due to strain-specific features associated with pituitary function and mammary carcinogenesis, questions have also been raised regarding the wisdom of examining mouse transgenic mammary tumor models on a single genetic background (Wakefield et al. 2003).

Need for multistrain EDC screening assays: Given the frequent finding of strain x EDC interactions, conducting EDSP assays with multiple strains on different genetic backgrounds, and including known sensitive strains, would better ensure that all the animals tested are not resistant to the EDC being analyzed. Otherwise, the use of a single strain that is genetically resistant to a given chemical or class of chemicals risks missing a deleterious chemical as an EDC and underestimating effects on sensitive genotypes (Festing 1979; Festing 1987; Festing 1993). As Narotsky et al. (2001) concluded, "thus, routine toxicity tests that use only a single strain may be unreliable since the outcome may hinge on choice of strain." Of even greater concern is using a single strain such as the SD-derived CD rat in toxicological screening assays, since it has been selected for traits associated with resistance to EDCs and is known to be resistant to several positive control EDCs.

A multistrain design would allow the use of several isogenic strains from diverse genetic backgrounds and known EDC sensitivities. The number of animals at each dose would be equally divided between strains, and the total number of animals per experiment would be similar to that used for single-strain assays. The statistical precision of a multistrain assay is far improved over that of an assay using a single resistant strain, particularly if one uses factorial designs and blocks (Festing 1995; Festing et al. 2001; Festing et al. 2002). While one degree of freedom is lost for each additional strain, and additional strain x dose treatment group, the use of multiple strains in factorial experimental designs remains a sensitive method of detecting chemicals with endocrine disrupting activity. Such a multistrain approach with a factorial experimental design would require a similar total number of animals, would be highly repeatable, would give high statistical precision, and would provide a better basis for extrapolation to humans than the use of a single stock. The results of the most sensitive strain should be considered as the indicator strain for the establishment of allowable environmental release levels.

The benefits of such a multistrain experimental design far outweigh the disadvantages. The disadvantages include a higher cost per animal for isogenic strains and the potential for mixing up strains, which can be avoided by using strains differing in coat color or other easily scored phenotypic markers (Festing, 1979). The main benefits of using multiple genetically divergent isogenic strains are: increased sensitivity and reliability of EDSP assays by increasing the likelihood that all of the strains tested are not resistant to the toxicant; and increased precision, sensitivity, and reproducibility of endocrine disruption bioassays by using genetically defined isogenic strains.

For example, strains of mice differ quantitatively and qualitatively in hematological responses to chloramphenicol succinate (CAPS). Four inbred mouse strains (C3H/He, CBA/Ca, BALB/c, and C57BL/6) show greater susceptibility than an outbred CD-1 stock ($P<0.01$) (Festing et al. 2001). A simulation with a sampling of data showed that using a total of eight mice equally divided at each dose level between strains CD-1, CBA, BALB/c, and C57BL/6 would have given a more sensitive experiment than the use of the same total number of CD-1 mice alone (Festing et al. 2001). Moreover, it would also have shown that the response is strain dependent.

Another example of the advantage of multistrain assays can be seen from an experiment involving a 4-week pubertal exposure of four strains of mice to increasing doses of E2 via subcutaneous implants (Spearow et al. 2003). The highly fecund outbred CD-1 strain showed no significant reduction in relative testis weight (testes weight/body weight ratio) in response to increasing doses of E2 provided as SC implants. In contrast, increasing doses of E2 resulted in a highly significant reduction in relative testes weight in each of three other strains tested ($P<0.0001$). Factorial analysis of combined data from all four strains by two-way ANOVA, fitting the effects of strain, E2 dose, and strain x E2 dose interaction showed that all of these effects were highly significant ($P<0.0001$). Thus, the factorial analysis of multiple strain data revealed that relative testes weight was significantly affected by E2, and that the effects of E2 differed according to strain. The use of only CD-1 strain mice would have missed these effects.

Since susceptibility to EDCs varies considerably between strains, the use of robust, multistrain assays in screening chemicals for endocrine disrupting activities will better ensure that at least one of the strains is sensitive to a reproductive toxicant and that effects on sensitive genotypes are not underestimated. Such multistrain assays need to include known sensitive strains and isogenic strains from diverse genetic backgrounds that have not been selected for traits associated with resistance to EDCs. The use of only two different strains like outbred CD and LE rats from CRL, which were both derived from Wistar crosses and which were both selected for traits associated with resistance to EDC, i.e., increased fecundity, cannot be considered a robust multistrain assay.

An additional advantage of testing with multiple isogenic strains is that identification of strain differences enables an additional genetic resource to determine the mechanisms of EDC toxicity (Narotsky et al. 2001). Once parental strains are shown to differ in a toxicological susceptibility trait, the same reproductive endocrine toxicological responses can be measured across congenic, consomic, and recombinant inbred (RI) strains and used to map, characterize, and identify genes controlling EDC susceptibility traits (Matin et al. 1999; Cowley et al. 2001; Williams et al. 2001; Liang et al. 2002). Thus, in addition to being more sensitive, reliable, and reproducible for detecting EDC, the use of multiple genetically defined inbred parental strains in the EDSP has the added benefit that it will greatly enhance the identification of genes controlling susceptibility to endocrine disruption. Other genetic principles with which the reviewer disagreed are presented in Section 4O of this Appendix regarding the use of Isogenic Strains for Multigeneration Toxicology Experiments.

7. CONGRESSIONAL INSTRUCTIONS FOR CONDUCTING THE EDSP

The Food Quality Protection Act (FQPA) of 1996 and the Safe Drinking Water Amendments (SDWA) of 1996 include several Congressional instructions for conducting the EDSP, which were not mentioned or addressed in the White Paper. The EPA is conducting the EDSP under the instruction of these two Acts of Congress. The SDWA specifically instructs the Administrator to develop a comprehensive plan for studying the health risks of arsenic and disinfection byproducts in drinking water; to propose and promulgate a national primary drinking water regulation for arsenic; and to implement a plan for studies of subpopulations at greater risk of adverse effects. The FQPA instructs the Administrator to consider:

- The validity, completeness, and reliability of the available data from studies of a pesticide/chemical
- Available information concerning the cumulative effects of such residues and other substances with common mechanisms of toxicity
- Variability in sensitivity of major consumer subgroups
- Whether the pesticide chemical has an effect in humans similar to that of estrogen or other endocrine effects
- Safety factors recognized as appropriate for the use of animal experimentation data.

Given these specific instructions from Congress, as well as the evidence that arsenic and other toxic metals have endocrine effects, and may have greater than additive interactions with other toxicants, it would seem inappropriate to use a strain in the EDSP such as the SD rat that has been selected for high resistance to arsenic with out thoroughly investigating this issue. For similar reasons it would seem inappropriate to use a strain such as the SD rat, that has been shown to be insensitive to the disruption of gestation, e.g., full-litter resorptions, by the drinking water chlorination by-product, bromodichloromethane (BDCM).

8. ADDRESSING CONCERNS RAISED AT THE AUGUST 2003 EDMVS MEETING

At the suggestion of U.S. EPA, the following responses are provided to answer concerns raised by members of the EDMVS following the presentation by Dr. Jimmy Spearow at the August 2003 EDMVS meeting.

Comment #1: One member of the EDMVS asked if there was any evidence that Sprague-Dawley/CD/outbred rats miss identifying EDCs when tested up to the Maximum Tolerated Dose. Jim Kariya of U.S. EPA commented that since Tier 1 assays will not be used to set dose-response relationships, it only matters that these assays be able to identify interaction with the endocrine system. Since Tier 1 testing generally goes up to the MTD, the SD/CD/outbred rats would need to be shown to be insensitive at high doses in order to cast serious doubt on their utility in Tier 1 assays.

Reviewer's Reply #1: In Section 5 of this appendix the reviewer listed the EDCs and endpoints for which SD/CD rats were less sensitive/insensitive and for which they were insensitive up to the highest dose tested. These data show that F344 rats were insensitive to two compounds, (flutamide and atrazine) at certain endpoints but were sensitive to flutamide and more sensitive to atrazine at other endpoints. In contrast, SD/CD rats were insensitive to several compounds

including: DES on decreases in testes and seminal vesicle weights; BPA on prolactin levels, uterine weight, and especially on uterine histopathology; BPA on vaginal cell proliferation and DNA synthesis; BPA on sperm counts in males and age at vaginal opening in females; lead on spermatogenesis; lead on disruption of female pubertal development; p,p'-DDE on seminal vesicle and prostate weights, and TSH levels; food restriction on estrous cyclicity; and BDCM (a drinking water disinfectant by-product) on gestational losses (full litter resorption). SD/CD rats were also insensitive to atrazine induced embryo/fetal loss in one study, and less sensitive to atrazine-induced embryo loss in another study. Consideration of strain differences in susceptibility to EDCs will be important for Tier I as well as for Tier II assays. A strain that is less sensitive in detecting known positive controls is also likely to be less sensitive in detecting weaker environmental chemicals with similar mechanisms of action. Thus, using a less sensitive strain in Tier I assays is more likely to miss an EDC than using a more sensitive strain. If a weak EDC is missed in Tier I, it will not be considered in Tier II assays. This will result in a chemical that is harmful to sensitive individuals not being regulated.

The use of a strain that is genetically less sensitive or insensitive to a given EDC in Tier II assays will clearly underestimate effects on sensitive genotypes, individuals, and populations. As discussed in Section 4J, several genetic differences in individual Phase I and II metabolism enzymes involved in steroid and xenobiotic metabolism are greater than the 10-fold within-species safety factor. The use of a less sensitive or insensitive strain in Tier II assays will clearly establish NOAELS, LOAELS, and environmental release rates that underestimate effects on sensitive genotypes and put them at risk for endocrine disruption and reproductive harm.

The Biological Factors and Study Design subcommittee at Endocrine Disruptor Peer Review advised that the strains used in EDSP assays be based on known sensitivities to EDCs (NIEHS 2000; NTP 2001; Melnick et al. 2002). **This subcommittee did not intend for the EPA to evaluate strain differences in sensitivity and then use the resistant strain.** Since sensitive strains are available for many mechanistic classes of EDCs, there is no excuse for using a genetically resistant strain in either Tier I or Tier II assays. The use of sensitive strains will require fewer animals in screening assays to detect EDCs, and will be more reliable in detecting EDCs. Nowhere else in biology would a less sensitive assay be used when a much more sensitive and reliable assay is available.

The other problem is that different EDCs have different mechanisms of action and a strain that is sensitive to one mechanism of EDC action may be resistant to another mechanism of EDC action. This can clearly be seen from the many different strain x EDC susceptibility interactions. As discussed in sections 4J and 4K of this appendix, there are several other mechanisms of EDC actions including inhibition of estrogen sulfotransferases, oxidative stress, etc. that also differ between strains and have not been considered by the EDSP. Thus, it is critical to include multiple sensitive strains in EDC screens to better ensure that effects on susceptible individuals are not missed.

Comment #2: Some members of the EDMVS seemed to be concerned about the generalizability of conclusions that one strain is better or worse than another for the endpoints under consideration. There seemed to be agreement that certain chemicals and certain endpoints may be more detectable in one strain than in others, but for testing unknown chemicals in assays with a variety of endpoints, it appeared that no systematic prediction could be made about which strain would be most or least appropriate.

Reviewer's Reply #2: The original data and listing of strain sensitivities in Sections 4 and 5 of this appendix clearly show major strain differences in susceptibility to several mechanistic

classes of EDCs. SD/CD rats were more resistant than other strains and especially than F344 rats to many different EDCs. In many cases the SD/CD rats were resistant at multiple endpoints. A strain that is resistant to a given positive control endocrine agent is likely to also be resistant to environmental chemicals that act via the same mechanisms of action. However, EDMVS members are correct that a strain may differ in its sensitivity to other EDCs that act through other endocrine mechanisms. As discussed in the last paragraph of the previous question, this is precisely why multiple strains from diverse genetic backgrounds need to be included as animal models. Given that the EDSP will be screening many chemicals with unknown mechanisms of endocrine disruption, the best way to ensure that all of the animal models are not genetically resistant is to conduct the screens in multiple, preferably isogenic, strains from diverse genetic backgrounds that have not been selected for traits associated with resistance to EDCs. It is also very important to include known sensitive strains.

Comment #3: Some EDMVS members noted that while testing in multiple inbred strains is an effective strategy for single-endpoint assays, the multiple endpoints in most of the *in vivo* assays being considered for the EDSP would make such a strategy nearly impossible to implement in EDSP.

Reviewer's Reply #3: It is no more difficult to measure the same endpoints on animals of multiple strains than it is on animals of the same strain. Reproductive endpoints, including reproductive organ weights, sperm counts, hormone levels, etc., are measured the same way across strains. While strains will differ in the timing of puberty, the measurement of reproductive endpoints such as uterine weights in 4-day uterotrophic assays would be no different. At each EDC dose level, for example, instead of having 12 to 15 animals in a single-strain experiment, in a multiple-strain experiment there might be 12 to 15 animals equally divided between three strains, e.g., 4 to 5 rats each of F344, DA and LEW or Wistar Furth inbred strains. The number of EDC dose levels and the total number of animals being compared in either experiment would be essentially the same. If a strain were shown to be much more sensitive or resistant to an EDC, experiments could be replicated with additional doses tested on identical isogenic strain genotypes to better define the NOAEL or LOAEL. The ability to replicate and extend such experiments is much better in isogenic strains due to the availability of identical genotypes within inbred strains, than in outbred strains where different genotypes are sampled with each replicate. The reviewer has conducted many reproductive endocrine and toxicology experiments using multiple strains and does not see the use of multiple strains as problematic, particularly when animal models are readily ordered from commercial suppliers. Major suppliers indicated to the reviewer that they would adjust their production of whatever strains are chosen to meet the demands of the EDSP (Mirley 2004; McNeelan 2004).

Overall, the same number of animals would be purchased, treated, and phenotyped in multiple-strain as in single-strain experiments. Depending on the age of the animal, the cost per inbred strain animal relative to SD or LE outbred strain rats is about 40 to 100% greater for F344 or LEW inbred strain rats. The costs will depend on the strains chosen but some suppliers have indicated that the cost of inbred strains might be substantially lower with higher volume use as anticipated in the EDSP (McNeelan 2004). Nevertheless, the costs of feed, bedding, animal care, and labor for conducting and analyzing the experiment are the same and are likely to be much greater than the cost of animals. Thus, relative to outbred animal models, the overall cost of using multiple isogenic strain animal models in EDC screening assays will be slightly greater but will provide much more reproducible and reliable data, which is less likely to underestimate effects on sensitive genotypes.

9. CONCLUSIONS

The objective of the EDSP is to identify and characterize endocrine-active chemicals that disrupt reproductive development and function. Since strains and genotypes differ markedly in susceptibility to several EDCs, the choice of strain(s) for EDSP assays is a fundamental issue that needs to be addressed to avoid underestimating EDC effects on susceptible individuals, populations, and species.

The EPA has not adequately considered how the breeding and long-term selection history of outbred strains has affected their susceptibility to EDCs. The EPA has validated the vast majority of EDSP assays in the SD-derived CD IGS rat. The SD rat strain has an exceedingly narrow genetic base, has undergone inbreeding and long-term selection for "high lactation, rapid growth, vigor, good temperament, and high resistance to arsenic trioxide" while at Sprague Dawley Inc. (Poiley 1953; Lindsey 1979). Following transfer to Charles River Laboratories (CRL), the SD-derived CD rat was then selected mainly for large litter size and vigor for another >80 generations. Crl:CD subpopulations were re-crossed and randomly selected to form the CD IGS population. Given this breeding and selection history, the SD rat and especially the highly prolific CD IGS strain cannot be considered representative of unselected outbred populations that have not undergone a major genetic bottleneck, much less the original rat population from which it was derived. Unfortunately, the implications of such long-term selection in this and other outbred strains on susceptibility to EDCs has not been adequately considered.

Reproductive traits are clearly under quantitative genetic control, e.g., they are controlled by several genes and by environmental factors, including exposure to EDCs. Selection for large litter size and increased prolificacy increases these traits and results in correlated responses in several mechanistically related reproductive and endocrine sensitivity traits (Bradford 1968; Bradford 1969; Land 1976; Eisen et al. 1981; Spearow 1985; Spearow et al. 1999). Although the physiological-genetic mechanisms involved are not well defined, the White Paper and this Appendix show that strains differ markedly in susceptibility to diverse EDCs. Given these findings, additional efforts are clearly needed to understand the physiological and toxicological mechanisms by which genetic variation and selection for high prolificacy in outbred strain animal models have increased the components of litter size and have altered susceptibility to diverse EDCs. Studies are also needed to understand if and how previous selection for high resistance to arsenic in SD rats has altered susceptibility to arsenic, toxic metals, and oxidative stress-inducing EDCs.

The White Paper contained several errors and omissions in reporting of strain sensitivity to EDCs. The Biological Factors and Study Design subpanel at the NTP/NIEHS Endocrine Disruptor Low Dose Peer Review (October 2000) recommended that animal model selection be based on responsiveness to known (positive control) endocrine active agents of concern, rather than on convenience and familiarity (NTP 2001; Melnick et al. 2002). Unfortunately, there were a number of errors in the reporting of strain differences in susceptibility to EDCs in the White Paper.

In several instances data on strain sensitivity to EDCs were reversed, erroneously reporting SD/CD rats to be more sensitive than F344 rats to EDCs such as estrogens and lead, when the original data showed the opposite. There were also several errors and omissions in the text, tables, and/or summaries, several of which resulted in underreporting strain differences in susceptibility to EDCs. For example, published data were not included in the text and/or tables of the White Paper

which showed that highly fecund SD or CD strain rats were more resistant at several endpoints than other strains examined to DES, Bisphenol A, and p,p'-DDE. Furthermore, the reviewer contends that it is inappropriate to describe a "less sensitive" strain as "sensitive" in the summaries. The reviewer contends that basing the strain selection on these flawed conclusions and other errors is ill-advised.

As discussed in sections 4 and 5 of this Appendix, the original data show that relative to F344 strain rats, SD/CD rats were less sensitive or insensitive to the disruption of reproductive development or function by many different compounds including estrogen, DES, BPA, and lead. Relative to F344 strain rats, SD/CD rats were also less sensitive or insensitive to the disruption of gestation by atrazine or the drinking water disinfection by product, bromodichloromethane (BDCM) and the disruption of estrous cyclicity by feed restriction. Relative to LE strain rats, SD and/or SD-derived CD rats are less sensitive or insensitive to the disruption of reproductive development or function by p,p'-DDE and dibutyl phthalate. In essence, SD rats are resistant to more EDCs than the Tables 2 and 3 and the summaries of the White Paper would lead the reader to believe. Given this selection history and relatively high resistance to many EDCs, the CD ISG rat strain is clearly inappropriate for use as the sole animal model in EDSP. If the CD IGS rat strain is used as the sole mammalian model, the EDSP will risk underestimating the effects of several classes of EDCs on sensitive genotypes.

Using the CD IGS and the LE IGS rat strain, is not an acceptable solution either. These are both Wistar-cross-derived strains and both have been selected for large litter size while at CRL. Thus they have overlapping genetic backgrounds and similar histories of selection for high prolificacy. Female CD and LE strain rats showed similar responses to Tamoxifen, methoxychlor, ethynyl estradiol, and atrazine in pubertal female assays (Gray 2003). Male CD and LE rats also showed similar responses to flutamide, p,p'DDE, and methyltestosterone in pubertal male assays (Gray 2003). However the SD strain was sensitive while the LE strain was insensitive to effects of Ketoaconazole on PPS (Gray 2003). In contrast, testes and seminal vesicle weights of CD IGS rats were less affected by dibutyl phthalate than that of LE rats (Gray 2003). These data suggest that CD and LE rats differ at a limited number of EDC susceptibility traits.

In contrast, the F344 strain rat is more sensitive than SD/CD strain rats to the disruption of reproductive function and development by estrogenic agents, BPA, and lead, as well as the disruption of gestation by atrazine and BDCM. Thus, isogenic F344 strain rats would provide a more sensitive and more appropriate animal model for testing the effects of these endocrine agents. However, the F344 is less sensitive or insensitive to partial estrogen agonists, tamoxifen, D4, atrazine, and the anti-androgen flutamide at some but not at other endpoints.

Highly fecund CD-1 strain mice were also less sensitive than C57Bl/6J (B6) strain mice to the disruption by estrogen of male gonadal and reproductive tract weights, sperm counts, as well as to the disruption by estrogen of gestation.

The magnitude of strain differences in susceptibility to EDCs reported in these studies range from a few percent to over 195-fold. However, in several studies a limited range of doses were used and on several occasions the resistant strains failed to respond. Thus, the magnitude of strain differences in sensitivity to several EDCs is unknown.

Other related points of disagreement in the conclusion

The reviewer partially disagrees with one of the conclusions of the White Paper, namely: "*a major question that cannot be answered is which animal model will provide the most appropriate data on the ability of the test chemical to interact with the endocrine system, in order to predict the effects of endocrine-active chemicals in humans, and/or other species of concern*." While men and

Wistar rats showed inhibition of spermatogenesis in response to blood levels of >30 to 40 µg/dl lead, SD/CD strain rats were not affected (Apostoli et al. 1998). Ethynylestradiol (EE) is clinically effective as a contraceptive in adult women at an oral dose of 0.5 to 1 µg/kg/day. While similar oral EE exposures were effective at increasing uterine weight in immature Wistar and CD IGS strain females (Kanno et al. 2001), oral exposure to 10 µg/kg/day EE did not alter any male or female reproductive endpoints of adult CD rats (Yamasaki et al. 2002). 50 to 200 µg/kg/day EE was required to alter estrous cycle features, reproductive tract weights, and histopathology of adult CD rats (Yamasaki et al. 2002). These findings suggest that a strain more sensitive to estrogenic agents and toxic metals than SD/CD rats would be more appropriate for predicting effects of EDCs on humans.

The conclusion of the White Paper argues against using the F344 strain, stating: "*the F344 has a small litter size, thus reducing the number of animals available for multiple evaluations,…*". Actually, F344 strain rats show a moderate litter size of about 7.5 to 8 pups (Harlan 2004a; McNeelan, 2004), which should be sufficient for the evaluation of most endpoints. The litter size of the F344 strain is among the highest of commercially available inbred strains, and its overall fecundity is in the middle of a larger set of inbred strains examined at NIH (Hansen 2004). Furthermore, the litter sizes of the outbred strains selected for large litter size are typically reduced/standardized soon after birth to avoid nutritional stunting, thereby diminishing some of the productivity advantages of outbreds. Also, is it appropriate to use an outbred strain that has been selected long-term mainly for high prolificacy and other traits associated with resistance to EDCs as an animal model representing "unselected" human populations?

The conclusion of the White Paper also states that: "*the F344 ... has a high incidence of spontaneous testicular tumors, which may confound potential effects on male reproductive organs*." The F344 does have a high incidence of spontaneous testicular cancer, but this occurs after 17 months of age (Weisburger et al. 2002), which is much later than animals will be examined in any planned EDSP procedures. The reviewer further contends that using a genetically sensitized animal model like the F344 rat is likely to improve chances of detecting EDCs with effects on testicular cancer, which seems to be increasingly common in human populations (Skakkebaek et al. 2001). For similar reasons, rather than using a strain that is resistant to prostate hypertrophy such as the SD rat, including a sensitive strain such as the Lewis or Wistar rat is likely to improve the detection of EDCs affecting this trait at the much earlier time points to be examined in the EDSP.

The studies reviewed by the White Paper show Strain x endocrine agent susceptibility interactions in many experiments and that no one strain was most sensitive to all EDCs. The data show that strains of rats and mice differ markedly in susceptibility to the disruption of reproductive development and function by many different EDCs at several endpoints. To better ensure that at least one of the strains is sensitive to the chemicals under evaluation, the EDSP needs to use multiple, preferably isogenic, strains from divergent genetic backgrounds as animal models. The EDSP also needs to include known sensitive strains and strains that have not been selected for traits associated with resistance to endocrine disrupting toxicants. Toxicological testing with multiple, genetically divergent isogenic strains rather than a single outbred strain increases the sensitivity of screening assays by increasing the likelihood that all of the strains tested are not resistant to the toxicant, as well as maximizing the precision, sensitivity, and reproducibility of endocrine disruption bioassays by using isogenic strains (Festing 1979; Festing 1987; Festing 1993; Festing 1995). The use of several isogenic strains with factorial statistical designs is an efficient means of toxicity testing and can be conducted with essentially the same total number of animals per experiment (Festing 1995; Festing et al. 2001; Festing et al. 2002).

The reviewer agrees with the statement in the conclusion of the White Paper that: "*These data support the case for performing reproductive toxicity assays in more than one strain to maximize the probability of detecting an effect at an endocrine endpoint." …"Obviously, it would be more thorough to conduct multi-strain assays to increase to chances of detecting endocrine effects"* … . The conclusion of the White Paper also accurately states that it is difficult to identify the strain that is most sensitive to all EDCs and endpoints. However, the White Paper then twists these issues. Rather than choosing a set of complementary strains so that at least one strain is most sensitive to all EDCs tested and the majority of endpoints, the White Paper then implies that the multiple-strain issue can be ignored, and the main question should be whether to use outbred or inbred strains. The reviewer does not agree with this twist in logic. Even though additional studies are needed to define the optimal set of strains, the use of strains already shown to be most sensitive for detecting certain classes of endocrine agents in multi-strain EDC screening assays would improve the sensitivity and reliability of screening assays over that of using the CD IGS rat in a single-strain assay. For example, the data show that including F344 strain rats in a multi-strain assay would improve the sensitivity and reliability of EDSP assays over that of using SD/CD rats alone for detecting pregnancy disrupting agents, toxic metals, and estrogenic xenobiotics at several endocrine, organ weight, and histopathological endpoints.

Need for additional screens of positive control EDCs to better define optimal multiple strain screening sets. Unfortunately, a standard set of diverse strains has not been tested and optimized to ensure sensitive detection of all mechanistic classes of EDCs. All we have is a patchwork of data from the testing of varying combinations of strains and substrains for sensitivity to different EDCs. We have no idea of the magnitude of variation in susceptibility between strains of rats to several different classes of EDCs, including environmental thyroid disruptors, such as perchlorate, or EDCs such as hydroxylated PCBs, which reportedly disrupt estrogen levels through inhibition of estrogen sulfotransferase (see Section 4K). Many of the multiple-strain studies that have been conducted have used closely related strains and several have not included known sensitive strains. Had the EPA screened divergent strains for their response to positive control EDCs in validation studies, the EPA would be in a much better position to pick a more optimal set of strains.

Instead, by focusing on validating EDSP assays in CD IGS outbred strain rats with a few validations in LE outbred strain rats, EPA has validated EDSP assays only in Wistar cross-derived, high fecundity selected outbred rat strains. Given their history of selection for high fecundity and resistance to arsenic trioxide, as well as the propensity for resistance to estrogenic xenobiotics, toxic metals, several other chemicals, and the disruption of cyclicity and gestation, CD/SD rats are clearly inappropriate for use as the sole animal model in the EDSP. Questions also need to be resolved regarding the susceptibility of the SD/CD rat to toxic metals and oxidative stress-inducing chemicals before it is considered as a member of a multiple strain screening set. SD/CD rats are nevertheless more sensitive than F344 strain rats to tamoxifen, D4, and atrazine at some endpoints but are less sensitive to atrazine at other endpoints. In addition, Hsd:SD and Crl:CD IGS rats have been separated for over 100 generations. CD rats were selected mainly for large litter size and vigor for about 80 of those generations, and are more extreme than SD rats at litter size, body weight, and insulin resistance/obesity-related endpoints. Thus, Hsd:SD and Crl:CD IGS strain rats need to be considered as separate substrains.

The F344 rat is a clear choice for an inbred isogenic strain that is sensitive to estrogenic agents, estrogenic xenobiotics, some toxic metals, and chemicals that disrupt gestation. But it seems

to be less sensitive or insensitive to partial estrogen antagonists such as tamoxifen and to the antiandrogen, flutamide. LE and Wistar rat strains also differ in sensitivity from SD or F344 or each other to a variety of EDCs. Unfortunately none of these other strains have been characterized for susceptibility to the full set of positive control EDCs.

Additional screens of strain Susceptibility to EDC. Ideally, in order to choose those most appropriate strains, a panel of diverse strains needs to be compared in EDSP assays with respect to susceptibility to known positive control EDCs in both sexes. Such validation and screening assays need to include known sensitive and isogenic strains from divergent genetic backgrounds that have not been selected for high fecundity, vigor, or resistance to arsenic. Otherwise, we will not know the role of genetic or strain variation in EDSP assays.

The reviewer realizes the need for controlling costs in the EDSP, and that the low fecundity of some inbred strains limits their use. The reproductive rate of a strain affects the purchase price, the number of mating pairs needed for gestational/lactational exposures and multigenerational experiments, as well as the precision of estimating effects of EDCs on breeding performance. Thus, moderate to high reproductive performance and high sensitivity to EDCs needs to be part of the criteria for choosing which inbred strains are included in additional validation screens and the EDSP. Due to economies of scale, the purchase price of isogenic strains for a large ongoing testing program such as the EDSP may be considerably lower than current list prices, especially with competition between suppliers.

The F344 strain is one of the more productive inbred strains available from CRL and Harlan, but intermediate amongst a larger set of inbreds strains examined at NIH. Other inbred strains with good overall reproduction include the DSS/2N, DA/PitN, MNS/N, INR/N, RHA/N, M520/N. In contrast, LEW/SsN, WKY/N, BN/SsN, and BUF/N showed successively lower productivities (Hansen 2004). For economic and EDSP throughput/accuracy reasons, strains with lower fecundity than LEW should be avoided in EDSP assays. While F344 and LEW are often considered as two of the most “standard” inbred strains for comparative general use (RatMap 2004), questions remain as to whether spontaneous autoimmune disease in some LEW substrains may limit their use in reproductive toxicology studies.

Ideally, a panel of: 1) Fisher344 and 2) an additional two to three diverse isogenic strains such as: Wistar Furth, Lewis, Lewis x BN F1, DA, Dahl Salt Sensitive (DSS) , RHA, MNS, Copenhagen, INR, and/or M520 would provide better genetic diversity for the EDSP. The process of choosing the rat strains for consideration in this screen could be optimized by including the input of individuals and institutions with extensive experience in strain selection issues with diverse inbred and outbred rat strains including: Dr. William Rall, NIH; Dr. Carl Hansen, NIH; Dr. Howard Jacob, Medical College of Wisconsin; Charlie Parady or Patricia Mirley, CRL; and Natalie McNeelan, Harlan. This could also facilitate integration of efforts by multiple federal agencies to utilize the same set of isogenic rat strains, which in the long run could enable substantial costs savings in developing and characterizing comparative rat strain models for understanding the effects of environmental agents on reproductive function and development.

However, with the exception of the fairly well characterized F344 strain, the response of the remaining inbred strains to most positive control EDCs is largely unknown. Thus, a set of isogenic strains would need to be screened in contemporary experiments to determine their relative strain sensitivity to diverse positive control EDCs. This should allow selection of a set of complementary strains to be used in all EDSP assays to better ensure that at least one strain is highly sensitive to each EDC at several reproductive endpoints. Alternatively, the inclusion of a sensitive mouse strain such as the C57BL/6J or B6C3F1 would add assurance that species differences were not missed.

Note that this list of strains does not include poor metabolizer strains other than the DA rat (CYP2D1/2 deficiency). However, the list does include the RHA strain, which would enable comparisons to RHA/jj strain rats, which lack UGT1A, thereby providing an animal model for poor UGT1A metabolizer humans. Nevertheless, additional studies beyond the EDSP will likely be required to determine the effects of many other common Phase I and II poor metabolizer and endocrine response mechanism mutations on susceptibility to EDCs.

Use of SD-derived CD IGS rats alone or in combination with Crl:LE rats as proposed in the White Paper is not suitable since both are Wistar-derived crosses, both have been selected for large litter size at CRL, and questions remain about the implications of using an animal model previously selected for high resistance to arsenic and high fecundity in toxicological screening assays. However, the reviewer realizes that fiscal considerations may mandate considering a less than optimal range of genetic diversity in EDSP screening assays. For example, inclusion of the LE outbred strain rather than one of the latter strains in the panel might retain sufficient diversity, but would be far less than optimal due to the segregating nature of this strain and previous selection for high fecundity. If an outbred strain had to be included, the use of an unselected Wistar outbred such as the Wistar Hannover which has a moderate litter size averaging 9.5 (Harlan 2004b), and moderate production index (McNeelan 2004) might be preferable, since it is likely to be more representative of humans that have not been selected long-term for high fecundity. Furthermore, Wistar rats are more sensitive than SD/CD rats to decreases in testes and seminal vesicle weights and testosterone levels by DES (Inano et al. 1996), delay of vaginal opening by BPA, (Tinwell et al. 2002); inhibition of sperm count and daily sperm production by BPA (Tinwell et al. 2002); induction of uterine weight and epithelial cell thickness by estradiol (Diel et al. 2004); and, inhibition of spermatogenesis by lead (Apostoli et al. 1998).

However, such inbred and outbred strains would still need to be included in the above prescreening panel to ensure that at least one strain most sensitive to each class of EDCs is included in the final screening panel. As discussed in Sections 3 and 4 of this Appendix, outbred strain models are not preferable due to the problems with variable genotypes within strain, genetic drift, decreased reproducibility, decreased precision of estimating EDC effects, and corresponding decreased sensitivity to EDC, as well as potential unrecognized selection for EDC resistance during the course of single and multigeneration experiments.

Only by using multiple, preferably isogenic, strains as animal models and including known sensitive strains, especially the F344 strain rat, will the EDSP be able to better ensure that effects of EDCs on susceptible genotypes are not underestimated. Such an approach will also provide the needed information on the nature of genetic variation in susceptibility to EDCs, and enhance future studies to identify susceptibility loci and protect human and other animal subpopulations at greater risk of environmentally induced disease. The role of genetic diversity in susceptibility to endocrine disruption needs to be considered in *in vitro* and *in vivo* assays in both Tier I and Tier II studies.

The White Paper did not address that using a soy-based diet high in antioxidants, combined with using an oxidative stress-resistant animal model, is likely to mask the endocrine disrupting effects of weak environmental estrogens and oxidative stress-inducing EDCs on humans and wildlife eating less than optimal diets.

The reviewer is also troubled by the statement in the White Paper that a benefit of including an additional strain in validation studies is for "*providing more flexibility to laboratories in selecting strains for performance of the assays.*" If a testing laboratory is allowed flexibility in choosing which strain it wants to use to test a given chemical, what prevents the laboratory from using only a strain known to be resistant to a mechanistic class of chemicals? To avoid the potential for the abuse of genetics, multiple strain assays in the EDSP need to involve a defined set of strains,

and always need to include strains known to be most sensitive to a given mechanistic class of EDC. Since relatively few strains/genotypes have been screened to date, the EDSP also needs to be responsive to protecting more sensitive genotypes or populations identified in future studies.

Addressing Human Susceptibility to Endocrine Disruption: There are many polymorphisms in genes coding for proteins involved in steroid/xenobiotic biosynthesis, metabolism, and signal transduction mechanisms that can affect susceptibility to environmental endocrine agents. It is also clear that individual humans respond differently to several endocrine and toxicological compounds. Our understanding of the role of genetic variation in reproduction and susceptibility to disease will increase dramatically by utilizing human, mouse, and rat genomic sequence information. Sequence analysis of DNA repair genes of a sample of "normal" humans found a large number of functional mutations, many of which are not neutral (Xi et al. In press). There is no reason to anticipate that the role of genetic variation in susceptibility to environmentally induced disease will be different.

Relatively little is known about genetic variation in susceptibility to the thousands of chemicals to be screened in the EDSP. The SDWA and FQPA instruct the EPA Administrator to implement a plan for studies of subpopulations at greater risk of adverse effects from environmentally induced disease. The EDSP could and should play a critical role in this process. Using outbred strains like the SD and/or LE rat, and especially using a single outbred strain like the SD rat, will not provide this information. Variability within outbred strains often further masks the detection of EDCs and the detection of susceptible individuals/genotypes if they occur at a low frequency in an outbred strain. Given the marked insensitivity of SD outbreds rats to several EDCs, the use of this strain as a sole animal model will clearly underestimate risk for several EDCs. Screening EDCs with different laboratories using different strains/substrains, feed, bedding and testing procedures further confounds all these factors, allowing no direct comparisons (Kanno et al. 2001; Kanno et al. 2003), and therefore is an unsuitable experimental design. Only by using multiple, preferably isogenic, strains as animal models in <u>contemporary</u> comparisons, will the EDSP be able to provide the needed information to determine which chemicals are EDCs and the nature of genetic variation in susceptibility to EDCs. It is important to understand that the studies reviewed by the White Paper show that the genetic susceptibility of an animal model to EDCs and whether a chemical is an EDC are not independent considerations.

Identification of differences in susceptibility to EDCs between highly reproducible inbred strains will also enable future studies to genetically map, identify, and characterize genes controlling susceptibility to endocrine disruption. Such multiple strain screens will define animal models suitable for determining the physiological, biochemical, endocrine, and genetic mechanisms that mediate strain differences in susceptibility to endocrine disruption. Such information will enable the rapid identification of genetic markers and therapeutic treatments and/or preventive measures to better ensure that susceptible individuals are not harmed by reproductive toxicants. The reviewer realizes that it is well beyond the scope of the EDSP to determine all of the mechanisms of genetic susceptibility to EDCs. Nevertheless, only by using multiple, genetically divergent, preferably isogenic strain animal models will the EDSP be able to lay the foundation for future studies to identify susceptible individuals and protect human, wildlife, and domestic animal subpopulations at greater risk of environmentally induced disease.

Given the biodiversity in vertebrate species including humans, efforts are needed to protect not only the average and most resistant individuals, but also the more sensitive individuals and populations, from endocrine disruption. If the EDSP is conducted in a single outbred strain or even two closely related outbred strains, the nature and magnitude of genetic diversity in susceptibility to

endocrine disruption will remain a mystery. Failure to adequately consider the issues of genetic susceptibility of animal models used in endocrine disruption screening assays will put sensitive/susceptible individuals, populations, and species at risk of not only infertility and reproductive disease but also population declines.

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