Work Assignment 3-7

White Paper on Species/Strain/Stock in Endocrine Disruptor Assays

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PREPARED BY:

Sherry P. Parker, Ph.D.
Rochelle W. Tyl, Ph.D., DABT

REVIEWED BY BUT NOT IN COMPLETE CONCURRENCE:

Jimmy L. Spearow, Ph.D.

FOR:

James Kariya
WORK ASSIGNMENT MANAGER
U.S. ENVIRONMENTAL PROTECTION AGENCY
ENDOCRINE DISRUPTOR SCREENING PROGRAM
WASHINGTON, DC

BATTELLE
505 KING AVENUE
COLUMBUS, OH 43201

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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1.0 Executive Summary

This white paper is a review of the interspecies and intraspecies similarities and differences in endocrine endpoints in the absence and presence of test chemicals, in order to determine whether specific species/strains should be preferred or avoided when screening for endocrine activity. There is much evidence that different species and strains within species exhibit differing sensitivities to endocrine-active compounds, specific for chemicals and endpoints evaluated. Thus selection of appropriate species and strain(s), or at least understanding the differential responsivity of them is crucial to detecting effects in animal models which are extrapolable to human risk. This white paper is limited to the species being considered for inclusion in the Endocrine Disruptor Screening Program (EDSP) and also limited in scope to the endocrine endpoints under consideration. Currently, the reproductive and developmental toxicology Environmental Protection Agency (EPA) guideline studies recommend using the rat and not strains with low fecundity. The most commonly used rat strain for these Guideline studies is the CD Sprague-Dawley (SD) rat (the CD-1 Swiss mouse is also frequently used). Though the majority of historical data exists in this species/strain, there is evidence that endocrine-active chemicals may have very different dose-response curves for certain endocrine-related reproductive endpoints, which may, in part, be due to a differential sensitivity of different species/strains and endpoints in these species/strains to these chemicals. Since confounding factors make interlaboratory comparisons of data problematic, multi-strain studies conducted under the same experimental conditions and same laboratory were primarily used in the species/strain/stock comparisons.

Comparisons revealed a lack of consistency in effects produced by endocrine-disrupting chemicals on endocrine endpoints from strain to strain. Endocrine effects were chemical specific, strain specific, endpoint specific, and, in some cases, laboratory specific. There were more sensitive and less sensitive strains to endocrine-active compounds among outbred and inbred strains, depending on the chemical used and the endpoints evaluated. Clearly, strain (genotype) by environmental agent by endpoint interactions need to be considered in selecting the appropriate species/strains for EDSP assays.

2.0 Introduction and Background

In 1996, the Food Quality Protection Act was enacted by Congress. It directs the United States Environmental Protection Agency (EPA) to screen pesticides for endocrine activity. Thus, the EPA is implementing an Endocrine Disruptor Screening Program (EDSP). In this program, comprehensive toxicological and ecotoxicological screens and assays are being developed to identify and characterize the endocrine effects of various environmental contaminants, industrial chemicals, and pesticides.

The program's aim is to develop a two-tiered approach, i.e., a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) to identify substances with the potential to interact with the endocrine system, and a set of definitive apical *in vivo* assays (Tier 2) to determine whether the substances identified in Tier 1 cause adverse effects, identify the adverse effects, and determine the quantitative relationship between dose and adverse effects. The EDSP is required to use "validated" test systems. The Endocrine Disruptor Methods Validation Subcommittee (EDMVS) provides technical advice on the validation of most of the assays.

In order to determine necessary modifications to standard reproductive and developmental toxicology guidelines, to detect and characterize the effects of endocrine disruptors, the National Toxicology Program/National Institute of Environmental Health Sciences (NTP/NIEHS), at the request of the EPA, organized a peer review panel which convened in October 2000. The panel consisted of scientists from academia, industry, and the government. One of the subpanels of the NTP's Endocrine Disruptor Low Dose Peer Review Panel investigated the concern that animal models used in assays to detect endocrine disruption have been chosen on the basis of convenience and familiarity, and species/strains/stocks which are more frequently used are those which are bred specifically for robust fecundity and likely reduced sensitivity to endocrine perturbations (NTP's Report of the Endocrine Disruptors Low Dose Peer Review, 2000). The subpanel addressed this issue with respect to the mammalian two-generation assay and had the following remarks:

On *animal model selection*: The subpanel recommended that the selection of species or strain for future studies should be the result of a more deliberate thought process, rather than based on availability, convenience, or familiarity. Development of a core of historical data across mouse and rat strains (inbred and outbred), with known endocrine-disrupting chemicals and characterization of the reproductive endpoints of interest, was also recommended. These data could be used to modify current protocols with respect to the number and doses/concentrations of dose groups, group size, and endpoint selection.

On *species/strain selection*: The subpanel asserted that although there is an abundance of historical control data in CD-1 mice and SD rats collected in reproductive and developmental toxicology studies, inbred F1 strains such as the B6C3F1 mouse may provide less variable responses to endpoints assessed. In addition, the advantage of historical data may be compromised by genetic drift and/or selective breeding.

In the December 2001 meeting of the EDMVS, committee members discussed strains and stocks and concluded that the EPA should prepare a white paper summarizing what is known about intraspecies strain/stock similarities and differences in the neuroendocrine control of reproduction/development and in responses to endocrine-active chemicals, and provide the rationale for strain/stock selection. This review is the "white paper" requested by the EDMVS, designed to assess the interspecies and intraspecies variability of endocrine endpoints in *in vivo* assays under consideration by the EDSP. Please note that the uterotrophic assay and the

Hershberger assay are under consideration by the EDSP, for inclusion in testing guidelines but are being standardized and validated in a cooperative effort between US EPA and OECD and participating laboratories on three continents; therefore, these assays will not be standardized or validated in this project, and will not be discussed in this white paper.

2.1 Purpose

The purpose of this review is to summarize the interspecies and intraspecies similarities and differences in response to endocrine endpoints, in order to determine whether specific species/strains should be preferred or avoided when screening for endocrine activity. Currently, the recommended species for reproductive and developmental toxicology EPA guideline studies is the rat. Though the majority of historical data exists in the SD rat and CD-1 mouse strains, there is evidence that endocrine-active chemicals may have very different dose-response curves for responses to certain endocrine-related reproductive endpoints, and this may in part be due to a differential sensitivity of different species/strains to these chemicals. Whether or not non-monotonic dose-response curves are eventually shown to be a general phenomenon of endocrine disruptors (or a class of them), it is appropriate to ask whether screening for endocrine activity is being carried out in appropriately sensitive test systems.

A literature review was performed to identify key references on the following two general topics:

1) Influence of rat strain/stock on endocrine endpoints measured in the mammalian *in vivo* assays being considered for the EDSP

2) Interspecies similarities and differences in neuroendocrine control of reproduction/development and in responses to endocrine-active chemicals (reported since 1986)

2.2 Literature Search Strategy

Literature databases, accessible through the RTI Information Technology Services, were searched for published peer-reviewed and nonpeer-reviewed articles using on-line electronic literature databases, followed by a focused literature screening process. Full citations, including abstracts (if available) were retrieved for review.

2.2.1 Databases Searched

MedLine, PubMed, Biological Abstracts, Chemical Abstracts, Toxline including DART (Developmental and Reproductive Toxicology)

2.2.2 Database Search Strategies

- *English language articles*. The literature search was performed to include all applicable English language articles.
- *Foreign language articles with English abstracts*. The literature search was performed to exclude foreign language articles with foreign language abstracts. However, the literature search was performed in a manner that accepted foreign language articles that also have English language abstracts. This strategy was used to allow authors to review some literature published in any number of foreign languages.

2.2.3 Keywords and Phrases Used

Articles were identified through the use of keywords in the literature search. Individual sets of keywords were selected for each of the topics listed in "Objectives" above. Combinations of keywords in Column A were combined with keywords in Column B for Task 2 in order to identify key articles addressing the influence of rat strain and stock on endocrine endpoints from pubertal male and female, *in utero* lactational, adult male, and two-generation reproductive toxicity studies. Initially, "rat strain" and "rat stock" were combined with keywords in Column B. When more than 100 hits were found per combination of keywords, additional terms from Column A were added to limit the search. Since the rabbit is not a species under consideration for use in the EDSP, it has been deliberately omitted from the white paper.

A.	Rat strain	Fisher (F344)
	Rat stock (supplier)	Wistar
	Rat genetic variation	Lewis
	Sprague-Dawley (CD, SD)	Noble
	Long-Evans (hooded, LE)	Holtzman (HTZ)
	Alderly Park (ALK or ALP)	Outbred rats
	Dark agouti	Inbred rats
	Norway	
B.[1]	Reproductive toxicity	cells)
	Anogenital distance	Lactational exposure
	Nipple retention	Thyroid development
	Ovarian corpora lutea	Thyroid development and pregnancy
	Precoital interval	Lactation
	Sperm, sperm production	Blood testis barrier
	Estrous cyclicity	Spermatogenesis
	Areolar retention	Mammary glands
	Vaginal patency	17β-Estradiol (E2)
	Preputial separation	Estrus
	Uterotrophic	Litter size
	Embryo loss	Gestation, gestational loss
	Testes (Leydig, and Sertoli	Pregnancy

INSL3
Reproductive tract development
Müllerian ducts, Müllerian Inhibitory Substance
Wolffian ducts
Reproductive and accessory organs
Thyroid hormones
Hypothalamic, pituitary, and gonadal hormones
Puberty
Implantation
Fetal survival, mortality

[1]keywords were obtained from approved EDSP protocols and preliminary search results.

In addition to searches performed with combinations of both columns of keywords, papers by the following authors were searched for relevant articles:

vom Saal, FS; Ashby, J; Odum, J; Gray, LE; Ostby, J; Cooper, RL; Spearow, JL; Michna, H; Diel, P; Festing, M.

The focus of the search was on "rat strain." When there was a paucity of references pertaining to a general endocrine endpoint, "mouse strain" was added to the search.

For interspecies comparisons of reproductive and developmental endpoints, combinations of keywords in Column C were combined with keywords in Column D, and the search was limited to articles published since 1986, in order to identify key articles addressing interspecies differences in neuroendocrine control of reproduction/development, with emphasis on parameters addressed by the NTP's Endocrine Disruptor Low Dose Peer Review Subpanel on Biological Factors and Study Design.

C. Reproductive toxicity, endocrine
Developmental toxicity, endocrine
Mouse/mice
Rat
Interspecies

D.[2]Intrauterine position (IUP)
Estradiol
LH
LHRH (GnRH)
FSH
ACTH
Hypothalamic-pituitary-gonadal (HPG) axis
Puberty
Prolactin
Testosterone (T)
Thyroid hormone (T_3, T_4)
TSH
Oxytocin
Growth hormone
Diet
Caging
Bedding
Genetic variability
Gene expression
Strain differences
Genotype

Oogenesis
Meiosis
Mitosis
Endocrine-mediated nondisjunction
Hypospadias
Steroidogenesis
Reproductive tract malformation
Cryptorchidism
Spermatogenesis
Nipple retention
Anogenital distance
Uterus
Progesterone
Dihydrotestosterone (DHT)
Gestation
Pregnancy
Gestational loss
Embryo loss
Implantation
Fetal survival, mortality
Androsterone
Androstenedione
Prostate
Hypothalamic-pituitary-thyroid (HPT) axis
Areolar retention
Vaginal patency
Preputial separation (balanopreputial
Epispadia
Ovary, ovaries
Testis, testes
Gonad
Aromatase

[2]Keywords were obtained from the NTP's Report of the Endocrine Disruptors Low Dose Peer Review (Subpanel on Biological Factors and Study Design), October 2000, and preliminary search results.

The focus of the search was reproductive and developmental toxicity and endocrine effects. When a search of combined terms from Columns C and D yielded more than 100 hits, specific animal species names (in Column C) were added to limit the search.

2.2.4 Summary of the Review Process

After key articles were identified, individual references were retrieved and further searched to identify additional key articles (i.e., tree search). References of interest were electronically downloaded to Reference Manager, retrieved, evaluated, organized by topic, and retained for use in preparing the white paper.

2.3 Definitions

The Institute for Laboratory Animal Resources (ILAR) defines a strain as "inbred" when it has been mated brother x sister for 20 or more consecutive generations (ILAR Journal, 1992). "To ensure isogenicity, as well as homozygosity, a single brother x sister pair must be selected in the twentieth or a subsequent generation to perpetuate the strain. Parent x offspring matings may be substituted for brother x sister matings, provided that in the case of consecutive parent x offspring matings, each mating is to the younger of the two parents; this will prevent repeated backcrossing to a single individual" (ILAR Journal, 1992) . A strain is defined as "outbred" when it has been maintained as a closed colony for at least four generations. "To minimize changes caused by inbreeding and genetic drift, the population should be maintained in such

numbers as to give less than 1 percent inbreeding per generation. Under these conditions, a heterozygous breeding population is expected to reach equilibrium and to produce a stock of stable genetic composition. Formerly inbred strains may be included after four generations of closed outbreeding, provided that continued outbreeding is intended. Outbred stocks are not necessarily highly variable genetically. The degree of genetic variability of any individual stock can only be determined by studying the appropriate genetic markers" (ILAR Journal, 1992). "Wild type" refers to the genotype or phenotype that is found most commonly in nature or in the standard laboratory stock for a given organism, before mutations are introduced.

2.3.1 Inbred and Outbred Strains

The term "strain" refers to a closed population of organisms of the same species, with distinctive hereditary characteristics that distinguish them from other groups within the species. "Strains" are artificially maintained to promote certain characteristics by manipulation of population size, mating system, as well as the intensity and direction of artificial selection (Lynch and Walsh 1998). The terms "strain," "stock," and "line" are used somewhat interchangeably on inbred and outbred strains. The most commonly used outbred strains of rats include Wistar, Long Evans, SD, and CD (caesarean-derived CR® SD). Outbred mice include ICR, SENCAR, NMRI, CFW, CF1, CD-1, and "Swiss" mice. The most commonly used inbred rat strains include Fischer (F-344), Brown Norway (BN), ACI, Lewis (LEW), Noble, DA, Copenhagen, Dahl Salt Sensitive (SS), spontaneously hypertensive rat (SHR), and WKY. Commonly used inbred mouse strains include A, AKR, BALB/c, CBA/Ca, CBA, C3H/He, C57BL/6, C57BL/10, DBA/2, FVB, NOD, NZB, SJL, and SWR. Commonly used isogenic rat F1 crosses include F-344 x BNF1 (FBNF1) and LEW x BN F1 (LBNF1). Commonly used isogenic mouse F1 crosses include B6D2F1, B6C3F1, B6AF1, CAF1, CB6F1, and NZBWF1.

Strain differences, in response to xenobiotics and hormonally active compounds, are an extremely common finding in the few studies that have compared several strains of mice or rats (Festing 1979; Festing 1987; Festing 1995; Steinmetz et al. 1998; Spearow et al. 1999; Long et al. 2000). Since most toxicologists and physiologists only use a single strain of mice or rats, the amount of genetic variation between strains is not usually apparent. Many assume that finding a fairly uniform response to a given hormonally active toxicant in a commercially available outbred strain indicates a lack of genetic variation in susceptibility in the species. As will be discussed in detail, such assumptions are often invalidated by the extremely narrow genetic base, history of long-term selection for large litter size, and correlated changes in reproductive development and function traits characteristic of many commercially available outbred strains of mice and rats. This section will consider the features and selection history that have defined available inbred and outbred mouse and rat strains, in particular, factors relating to genetic variation in susceptibility to endocrine disruption.

One criticism against utilizing the most commonly used outbred strains is based on the fact that these animals have been bred specifically for robust fecundity and

relative insensitivity to endocrine perturbations. Selective breeding can alter reproductive traits, including natural and hormone-induced ovulation in the rat, litter size, testis weight, and sperm production (Bradford 1969; Johnson et al., 1994; Okwun et al., 1996a; Okwun et al., 1996b; Spearow and Barkley 2001). Selection for large litter size can affect the hypothalamic-pituitary-gonadal axis, resulting in differential sensitivity of the ovaries to gonadotropins and changes in follicular steroidogenesis in female mice, increased testis weight in males, and altered sensitivity of testis weight to estrogen in males (Spearow 1985; Spearow et al., 1987; Spearow et al., 2001).

Inbred strains have several features that make them valuable for biomedical research (Festing, 1979). They are produced by at least 20 generations of brother x sister matings, with all individuals of a strain in the 20th or subsequent generation tracing back to a single common ancestral breeding pair (Festing 1987). While a small number of genes may continue to segregate as residual heterozygosity, especially in the 20th to 30th generation of inbreeding, practically speaking, all members of an inbred strain are isogenic, i.e., essentially genetically identical individuals (Festing 1987). Thus, each isogenic inbred strain represents an infinitely repeatable, genetically defined set of identical twins which are homozygous at essentially all loci. A complete mouse genomic map and mouse genomic DNA sequence are available for the C57BL/6J inbred mouse strain (www.informatics.jax.org; www.ensembl.org/Mus_ musculus/). In addition, a rat genomic map, and a rat genomic DNA sequence are in development for the Brown Norway BN/SsNHsd/MCW (BN) rat strain (Rattus norvegicus) (www.ensembl.org/Rattus_norvegicus/; also see http://bacpac.chori.org/rat230.htm).

Genetically defined, isogenic inbred strains are an important resource for determining the toxicological effects of chemicals on biodiversity within such species. Because of their very high level of homozygosity, inbred strains stay genetically uniform and constant over many generations, with only a slight amount of genetic drift due to new mutations (Festing 1987). Their utility in toxicological research is due to the highly consistent and reproducible genotype, ideal for testing many different chemicals for toxicity over time (Festing 1979; Festing 1987; Festing 1995). Inbred strains are genetically monitored to confirm genetic integrity of each strain stain, and to ensure they have not been accidentally outcrossed by using coat color, biochemical, immunogenetic, as well as microsatellite and Single Nucleotide Polymorphism (SNP) molecular genetic markers.

F1 crosses between inbred strains are also commonly used in toxicological research, including B6C3F1 mice by the NTP. Isogenic F1 crosses are uniformly heterozygous at all loci, differing between parental inbred strains. As parental inbred strain genotypes and genetic sequence become increasingly defined, the genotype of F1 crosses of genetically defined inbred strains can be accurately predicted *in silico* (Manly et al. 2001; Marshall et al., 2002). This enables the production of an almost infinite number of genetically defined isogenic F1 cross animals with identically defined homozygous and/or heterozygous genotypes at all known loci. While the F1 crosses of inbred strains are isogenic, their F1 + F1 crosses to generate F2 animals and/or

backcrosses produce offspring which are segregating at many loci. The increased genetic and phenotypic variability in such segregating crosses confounds the description of treatment-related effects and thereby decreases the sensitivity of detecting treatment effects, and therefore limits their use in toxicological studies with offspring of F1 parents.

A wide variety of specialized, genetically defined, inbred strain genetic resources are also available, and are especially useful for characterizing, mapping, and identifying genes controlling strain differences in a wide variety of traits (Silver 1995). Two strains are referred to as consomic when they differ by one complete chromosome pair, and a congenic strain when it carries a small genetic region (ideally a single gene) from another strain, but which is otherwise identical to the original inbred strain. Several congenic inbred strains of mice and rats are available, each with a single chromosomal region from one strain backcrossed onto the genetic background of another strain. Several consomic (chromosome substitution) inbred strains are also available, each with a single chromosome from one strain introgressed by backcrossing onto the genetic background of another strain. Available rat consomic strains include several SS.BN chromosome-specific consomic strains with individual BN chromosomes on the Dahl Salt-Sensitive (SS) genetic background. Available mouse consomic strains include a full set of C57BL/6J-A chromosome substitution strains, each with a single A/J chromosome substituted on the C57BL/6J (B6) genetic background (Nadeau et al., 2000). Several mouse and rat recombinant inbred (RI) strain sets are also available. These RI strains are formed by crossing two inbred parental strains, intermating the F1 to produce F2s, and then producing a set of inbred lines by inbreeding *ad infinitum* from each of several F2 mating pairs. Available mouse RI strain sets include BXD RI, AXB,BXA RI, AKXD RI, and AKXL RI (http://jaxmice.jax.org/jaxmicedb/html/rcbinbred.shtml). Available rat RI strain sets include the SHR x BN (HXB/BXH) RI and the LEW x BN (LXB) RI.

Outbred strains are also commonly used in biomedical research and are intended to be genetically more diverse by maintenance of large, heterogenous, random mating populations and avoiding inbreeding. Yet, the diversity of many outbred mouse strains is limited, in part, by the narrow genetic base of the founders and, in some cases, early inbreeding programs to select against deleterious recessive genes. For example, laboratory stocks of Norway rats (*Rattus norvegicus*) were developed from albino mutants that had been bred by fanciers (Gray 1977). Around 1900, H.H. Donaldson obtained rats from fanciers for a breeding colony at the University of Chicago. This stock was transferred to the Wistar Institute in 1906 and maintained as a closed colony. This Wistar stock contributed to, and is therefore related to, several other outbred strains. In 1915, a few albino Wistar Institute females were crossed with a single wild gray male and then used to develop the Long-Evans rat stock (http:/www.criver.com/03CAT/rm/rats/longevansRats.html).

In 1925, Robert Dawley crossed a single hybrid hooded male of unknown origin with an albino female of the Douredoure strain (probably from Wistar). This single hooded foundation male was subsequently backcrossed to his albino female offspring

for seven successive generations. Multiple daughter lines, developed by inbreeding, were then crossed to form the stable heterogeneous SD stock, which was then maintained as a closed outbred population (http://www.harlan.com/). Thus, while the SD rat strain can be traced back to a single (most likely a Wild x Wistar) hooded hybrid male and a Wistar female, due to the repeated backcrossing of successive generations of daughters to the hooded hybrid foundation male, the vast majority of the genes in this bred strain originated from the single hooded hybrid male. Furthermore, the process of inbreeding in each of multiple lines enables enhanced selection against deleterious recessive genes present in the initial population, and enables increased litter size means and improved selection responses in litter size following crossing (Falconer 1971; Eklund and Bradford 1977; Falconer 1989). In other words, by inbreeding with selection in several lines followed by crossing these lines, Robert Dawley was very likely to have eliminated deleterious recessive genes commonly found in outbred populations. By then selecting for increased fecundity and docility, he was able to develop the highly productive SD strain. Charles River Laboratories obtained SD breeders in 1950, Cesarean derived them in 1955, and selected long-term for large litter size and vigor to develop the CD® (SD) rat strain (http:/www.criver.com/03CAT/rm/rats/longevansRats.html). Given this breeding and selection history, the SD strain, and the SD-derived CD strain have less genetic diversity and less deleterious recessive genes than what is found in wild type populations. As discussed below, long-term selection mainly for increased prolificacy may have resulted in even greater genetic divergence for reproductive and correlated traits.

A stock of Swiss mice was also developed from two male and seven female non-inbred albino mice by Dr. de Coulon, Lausanne, Switzerland. This stock was imported into the U.S. by Dr. Carla Lynch at Rockefeller Institute in 1926, and transferred to the Institute for Cancer Research in Philadelphia in 1948, where it was selected for high production and growth rate (http://www.taconic.com/addinfo/icrorigin.htm; http://www.criver.com/03CAT/rm/mice/cd1Mice.html). This closed ICR Swiss stock was used to establish several ICR strains. ICR was Caesarean derived in 1959 by Charles River Laboratories (CRL) and used to bred the CD-1 strain which was also selected long-term for large litter size and vigor.

Other outbred strains of mice are also from a very narrow genetic base. For example, the CRL CF-1® strain, which likely originated from non-Swiss mice at Rockefeller Institute, was intensively inbred for over 20 generations by Carworth and then outbred from a single mating pair (http://www.criver.com/03CAT/rm/mice/cf1Mice.html). This outbred stock, like all the other outbred strains at CRL, was long-term, selected primarily for high prolificacy.

Population Genetics:

Mutations, inbreeding, genetic drift, and differential selection may account for genetic differences in the same outbred strains provided by different suppliers or even different colonies of the same supplier. In outbred strains, spontaneous mutations and

recombinations between alleles supplement each other in generating and maintaining a multiple allelic system, which provides even more genetic variation on which selection can act. The amount of genetic variability in a randomly selected and randomly mated strain or population depends, in part, on the initial heterozygosity, the rate of inbreeding, and the number of generations of inbreeding (Pirchner 1969; Falconer 1989). Inbreeding is the change in genotype frequencies resulting from the mating of related individuals. The rate of inbreeding is dependent on the effective population size (Pirchner 1969; Falconer 1989). For pair-mated species, the rate of inbreeding per generation = 1/(2N), where N= total number of unrelated individuals in a population (Pirchner 1969). For populations in which males are mated to several females, the rate of inbreeding per generation = (1/(8Nm)) + (1/(8Mf)), where Nm= number of males and Nf=number of females (Pirchner 1969).

Genetic drift is the change of allele frequencies as a result of sampling populations of limited size. A colony of limited population size also undergoes genetic drift and therefore differs from other colonies of the same origin (Kacew and Festing, 1996). Genetic drift occurs when the frequency of alleles in a population change due to chance, i.e. sampling, rather than by natural or artificial selection. The "founder effect" is an extreme form of genetic drift, where gene frequency in a small founding subset of a population is different than the larger population from which it was derived. For commercially available outbred strains, genetic drift and inbreeding are likely greatest in the generations with small effective population size, at which Caesarean derivations were performed to establish each outbred strain and each outbred substrain. In contrast, a large number of breeders (typically several hundred to a thousand) are selected for continuing the line in most generations of breeding in most outbred mice and rat strains. During these generations (with large effective population sizes), if mating is random, the theoretical rate of inbreeding is likely to be quite low in the 0.05% to 0.5% range per generation. Nevertheless, genetic quality control data comparing outbred strain subpopulations showed evidence for considerable genetic drift between colonies or subpopulations. For example, Major Histocompatibility Complex RT1 haplotypes showed considerable genetic drift between colonies of Crl: CD® (SD) BR rats (Rodent Genetics and Genetic Quality Control for Inbred and F1 Hybrid Strains, Part II, Winter 1992, Table 8: http://www.criver.com/techdocs/rodent2.html).

DNA fingerprinting analysis showed that the diversity within outbred rat strains was much less than that found among ten commonly available inbred rat strains (Festing 1995). Genetic and biochemical marker typings in 63 rat inbred laboratory strains and 214 substrains showed an average polymorphism of 53% between strains, with wild-derived Brown Norway (BN) strain rats showing the greatest genetic divergence (Canzian 1997). While much more diverse than within any inbred strain, the genetic diversity within outbred rat strains is far lower than that found between commonly available inbred strains and more closely represents the level of diversity found in island populations (Festing 1995). For many traits, these commercial outbred strains show much lower variability than that found in genetically heterogeneous populations, such as an F2 cross between inbred strains. The limited genetic diversity of common commercial outbreds, such as the CD rat, should not be surprising, given its

extremely narrow genetic base, early inbreeding to purge deleterious recessive genes, and subsequent history of selection mainly for large litter size and vigor.

Migration/Outbreeding:

The amount of genetic variability in a strain or population also depends on the frequency of migration or outbreeding from other populations and the difference in gene frequencies between such populations (Pirchner 1969; Falconer 1989).

Genetic Monitoring:

Genetic monitoring of outbred strains can be used to confirm a strain's genetic integrity. The main purpose of genetic quality control is to preserve isogenicity (http://www.criver.com/techdocs/ rodent1.html). The loss of isogenicity, i.e., substrain or subline divergence, has three main causes: mutation, drift in residual heterozygosity, and genetic contamination caused by an unintended outcross. Genetic drift resulting from mutation or residual heterozygosity is difficult to detect and control and has minimal impact on most research. The greatest cause of subline divergence affecting the usefulness of inbred strains is genetic contamination. Genetic quality control procedures are aimed at preventing and detecting genetic contamination by strict colony management and routine genetic monitoring (http://www.criver.com/techdocs/rodent1.html).

Genetic and Environmental Sources of Variation:

Selection has defined the available outbred strains of mice and rats. While some of this genetic divergence between outbred line substrains is likely due to genetic drift, some of the divergence between strains and substrains is also very likely to be due to selection. Early in their development, outbred strains (including Wistar, SD, and Long Evans rats) were selected, at least in part, for docility and increased reproductive performance (Gray 1977). For economic and productivity reasons, most outbred strains continued to be selected by commercial breeders for large litter size and vigor. For example, following caesarean derivation from the SD line, CRL selected the CD rat strain from 1950 until 1991, i.e., for approximately 80-100 generations, mainly for large litter size in their 2nd to 5th litters and to a lesser degree for increased vigor (Charlie Parady and Patricia Mirley, CRL, personal communication). Following selection of large litters at birth, litters were reduced to 13 pups per litter, and vigorous pups from larger litters were selected at weaning as breeders for the next generation of the line. While pedigrees were not maintained, selected individuals with different birth dates were randomly mated to avoid inbreeding. CRL used this selection criteria and mating system within each subpopulation of outbred mice and rats. While much of the selection pressure was for large litter size, the limited selection for vigor following rearing in large litters (mice only have ten teats) may have also resulted in some selection for increased lactational yield and body weight.

ICR Swiss outbred mice were also rigidly selected for high productivity and

growth rate by Dr. TS. Hauschka at the Institute for Cancer Research (www.taconic.com/addinfo/icrorigin.htm). Following Caesarean derivation from ICR outbreds in 1959, the CD-1 mouse was again selected mainly for large litter size, with some selection for vigor through 1991 (Charlie Parady and Patricia Mirley, CRL, personal communication). The result of these selective breeding and random mating programs has been large, vigorous, highly prolific, high lactating outbred mouse strains that have been widely used as animal models in biomedical research.

Results of Controlled Selection Experiments:

Unfortunately, unselected controls were not maintained during the many generations of selection that defined these commercial outbred laboratory animal strains. Nevertheless, several selection experiments have been conducted by academic researchers that did maintain appropriate, randomly-selected control lines or divergently-selected control lines. These selection experiments in outbred stocks showed dramatic responses to selection for large litter size, growth rate, and litter weight gain (Bradford 1968; Bradford 1969; Land and Falconer 1969; Eisen et al. 1970; Land 1970; Eisen 1972; Land et al. 1974; Eisen 1975; Bradford 1979; Eisen and Durrant 1980). Long-term selection for large litter size results in increased litter size, with limited effects on body weight (Bradford 1968). In contrast, selection for increased growth rate increases growth rate and mature weight but decreases longevity (Eklund and Bradford 1977). Many of the reproductive endpoints of interest, including puberty, litter size, and cyclicity, are threshold traits, invoking need to consider threshold and scale effects (Falconer 1989).

Selection for large litter size increases ovulation rate and embryo-fetal survival (Bradford 1968; Bradford et al. 1979; Durrant et al. 1980; Eisen and Durrant 1980; Spearow and Bradford 1983; Spearow 1985; Pomp et al. 1988). Selection for growth rate also increases ovulation rate, but through different physiological mechanisms than selection for large litter size (Durrant et al. 1980; Eisen and Durrant 1980; Spearow and Bradford 1983; Spearow 1985; Pomp et al. 1988). While the physiological genetic mechanisms by which genetic selection for increased prolificacy in rodents are not fully understood, they involve changes in the regulation of the hypothalamic-pituitary-gonadal axis, serum gonadotropin levels, and follicle populations, as well as changes in sensitivity to gonadotropins, estrogens and estrogen negative feedback, the induction of gonadal LH receptors, the induction of gonadal steroidogenesis, the induction of follicular growth, and follicular atresia (Bradford et al. 1979; Durrant et al. 1980; Spearow and Bradford 1983; Spearow 1985; Spearow 1986; Pomp et al. 1988; Lubritz et al., 1991). In essence, selection for increased litter size has dramatic effects on the endocrine mechanisms regulating reproductive endocrine function and development traits.

Comparison of reproductive endocrine traits between inbred strains, congenic substrains, and recombinant inbred lines clearly show major genetic differences in these traits between strains of mice, further confirming that these reproductive endocrine traits have a genetic basis. This includes evidence for significant to highly

significant differences between strains of mice in: hormone-induced ovulation rate (Spearow 1988a; Spearow 1988b; Spearow and Barkley 1999), the hormonal induction of follicle maturation, hormonal control of follicular atresia and follicle number (Spearow et al., 1991), ovarian steroidogenesis and aromatase activity, estrogen-induced immature uterotrophic weight (Griffith et al. 1997; Roper et al. 1999), estrogen-induced uterine eosinophil and macrophage numbers (Griffith et al. 1997; Roper et al. 1999), and estrogen-induced susceptibility to vaginal candida infection (Parmar et al. 2003). Strains of inbred mice and rats also differ in normative testes and seminal vesicle weights (Zidek et al., 1998; Zidek et al., 1999).

SD and ACI strain rats also differ dramatically in the incidence of mammary cancers in response to DES (Shellabarger et al. 1978), as reviewed by Festing (1987). Whereas DES failed to cause mammary cancer in any SD rat (0/33), DES increased the incidence of mammary cancers from 0% to 53% in ACI strain rats. In contrast, in response to atrazine, female SD rats developed mammary cancer while F-344 rats did not, apparently due to a disruption of ovarian function leading to persistent estrus and increased E2 in SD but not in F-344 rats (Wetzel et al. 1994; Stevens et al. 1999).

Additional evidence for genetic variation in reproductive endocrine function has also been demonstrated in Quantitative Trait Loci (QTL) linkage studies that have mapped genes or QTL controlling reproductive endocrine traits to specific chromosomal regions. Genes controlling the increased natural ovulation rate of large-litter size selected Quackenbush-Swiss strain mice over that of C57BL/6J strain mice map to regions of chromosome (Chr) 2 and 4 (Kirkpatrick et al., 1998). These regions of Chr 2 and 4 overlap with loci controling major strain differences in hormone-induced ovulation rate and ovarian steroidogenesis. Markers on Chr 13 are significantly associated with strain differences in testes weight in the mouse BXD recombinant inbred strain set (Zidek et al., 1998).

Several genes controling E2-induced uterine hypertrophy and/or E2-induced uterine leukocyte responses have also been mapped to specific chromosomal regions (Roper et al. 1999). An interacting genetic factor on Chr 10 controls E2-induced uterine weight as well as E2-induced leukocyte responses (Roper et al. 1999). These and other studies in genetically-defined inbred strains of mice clearly confirm that differences in reproductive endocrine traits and sensitivity/susceptibility to estrogenic agents found among selected strains have a genetic basis.

Due to genetic variation in reproductive development and function, selection can also have a major effect on reproductive function in other mammalian species. Lines of sheep selected for large litter size increased the frequency of alleles with major effects on reproductive development, function, and ovulation rate (Bindon 1984; McNatty et al. 1985; McNatty et al., 1995). QTL linkage mapping and positional cloning studies showed that a Chr 6 mutation (FecB) in the intracellular kinase signaling domain of bone morphogenetic protein IB (BMP-IB) receptor (also known as ALK-6), which binds members of the transforming growth factor-beta (TGF-beta) superfamily, has a major

effect on reproductive function, development, ovulation rate, and litter size (Wilson et al. 2001).

Sources of Variation in Traits:

The phenotype of an individual can be considered as the sum of its genotypic value (G), the environmental effects (E), and the genotype x environment interaction, i.e., phenotype = G + E + GxE interaction (Falconer 1989; Lynch and Walsh 1998). Geneticists normally consider each of these factors as variance components in analyzing trait phenotypes. Potential endocrine-disrupting chemicals represent environmental or nongenetic sources of variation affecting a given trait. The purpose of the EDSP is to determine if a given chemical, i.e., environmental factor, has detrimental effects on reproductive development and functional phenotypes. However, it is not just the occurrence of genetic differences in reproductive phenotypes, but also the potential for genotypes to interact in a nonparallel manner with environmental factors that is of concern in designing EDSP assays. If G and GxE interactions are not important, any strain of animals could be used for testing chemicals for endocrine disruptor activity. However, if the genetic variance and especially if the genetic x environmental variance are significant, care must be taken to avoid screening chemicals with resistant strains, since the effects on sensitive animal strains would be underestimated (Narotsky et al. 2001; Spearow and Barkley 2001). The ultimate question is, of course, the effects, if any, on humans and other species of concern. The best approach would be the use of the most relevant animal model, if known; the default approach is the use of the most sensitive animal model.

The genetic variance in a trait can be further considered broken down into its components, including the additive genetic variance, dominance genetic variance, and the epistasis genetic variance, e.g., Total Genetic = Additive + Dominance + Epistasis (Falconer 1989; Lynch and Walsh 1998). In biological terms, the additive genetic variance is the variation in the average additive effects of alleles. Alleles at additive-acting loci behave in an step-wise or additive manner, with each + allele increasing the phenotype in an additive manner. Conventional mass selection for a trait acts mainly on additive genetic variance to increase the frequency of alleles, which on average have a beneficial effect on the trait(s) under selection. An animal's breeding value is the sum of the additive effects of its genes. Dominance is defined as a nonadditive interaction between alleles at a given locus. While loci that show dominance for a trait can also have an additive genetic component, they show a deviation, e.g. dominance deviation, from the regression line between the phenotypic means of the low homozygote and the high homozygote. For example, consider a trait showing complete dominance where the phenotype of individuals that are aa = 0, Aa= 2 and AA=2. While the average of the two homozygotes =1, the heterozygote (Aa) has a mean of 2, and thus shows a dominance deviation. Epistasis describes the nonadditivity of effects between loci.

The effects of selection, inbreeding, and crossing are very different on traits controlled by additive versus dominance genetic variation. Selection can utilize additive

genetic variance but not the dominance genetic variance in a population to improve a trait. Traits controlled by additively acting loci do not show inbreeding depression, since the number of loci proportional to the initial gene frequency will fix for the - versus the + acting alleles. In contrast, loci showing dominance generally decline with inbreeding due to the fixation of less desirable recessive alleles. Finally, F1 crosses generally show heterosis or hybrid vigor (e.g., increased phenotypic variance) in traits showing dominant gene action but not in traits controlled by additive gene action unless there is also complementarity among component traits.

Relative Importance of Historic Inbreeding Versus Selection for High Prolificacy in Defining Currently Available Strains:

In full sib (brother-sister) mating programs during the development of inbred strains, the largest evolutionary force is genetic drift and random fixation of alleles. This is especially true of reproductive traits with medium to low heritability. There will be an inbreeding depression for phenotypes controlled by dominantly acting loci and perhaps for some loci controlled by dominant epistatic interactions (Lynch and Walsh, 1998). In contrast, on average, inbreeding without selection will not change the phenotypes controlled by additively acting genes since as many + as - acting alleles are likely to fix in a given inbred strain. This is particularly true for traits controlled by a large number of additive loci. Nevertheless, for traits controlled by a very small number of additive loci, there is likely substantial genetic drift in the trait, depending on whether inbreeding fixes a given inbred strain for a + or - acting allele. Without selection, the expectation is for no net change in the number of + or - acting alleles affecting a trait.

In contrast, long-term selection mainly for a single trait will dramatically increase the frequency of + alleles, even for medium to low heritability traits like litter size (Bradford 1968; Eklund and Bradford 1977). Furthermore, since commercial outbred populations were selected for large litter size in large populations, it is likely that any beneficial mutations which occurred in the population would also be utilized to increase prolificacy even further.

Correlated Trait Responses:

Correlated traits are generally due to the action of genes, with pleiotropic effects on several traits or physiological processes (Falconer 1989). Comparison of strains selected for high fecundity with randomly selected control strains revealed correlated trait responses in several male characters for reproductive development function. In addition to changing reproductive function in females, selection for large litter size also increases the weights of testes, epididymides, and seminal vesicles (Eisen and Johnson 1981; Spearow et al. 1999; Spearow et al. 2001). Strains of mice and rats selected for large litter size are more resistant than unselected strains to disruption of testes weight, accessory gland weights, spermatogenesis, and steroidogenesis by estradiol or DES (Spearow et al. 1987; Inano et al. 1996; Spearow et al. 1999; Spearow et al. 2001). These observations suggest that one of the correlated responses to

selection for large litter size is resistance to endocrine disruption by estrogenic agents (Spearow et al. 1999; Spearow and Barkley 2001; Spearow et al. 2001).

Some of the genes with pleiotropic effects on related reproductive traits have been mapped, suggesting potential genetic mechanisms mediating correlated trait responses. Markers on Chr 8 showed a significant association with seminal vesicle mass and a suggestive association with litter size in HXB and BXH recombinant inbred strain sets derived from SHR and Brown Norway (BN) rat strains (Zidek et al. 1999). Since litter size was significantly associated with seminal vesicle mass, these data suggest that both of these traits are under the control of the same QTL or tightly linked QTL on rat Chr 8 (Zidek et al., 1999). Thus, this rat Chr 8 QTL may have pleiotropic effects on litter size and seminal vesicle mass, explaining, at least in part, how selection for large litter size also affects male reproductive developmental traits.

Considerable alarm was raised by the toxicology community when it was noted that certain outbred stocks, including CD rats, were showing excessive litter size, increased mature body weights, and decreased longevity (Pettersen et al. 1996) (CRL reference paper Vol 11, #1, 1999). When raised in the same environment, CRL: CD males were found to be significantly heavier than Taconic: SD males which were heavier than Hsd: SD males (Klinger et al., 1996). While it is unknown whether the increased body weights of CD rats are a correlated response to selection for vigor or unintended selection for increased body weight, the study of Klinger et al. (1996) suggests the substrain differences are genetic. Increased body weight is correlated with decreased longevity (Eklund and Bradford 1977), and a higher proportion of CD rats failed to survive to the age required in two-year carcinogenicity studies (Petterson et al., 1996). Thus, large commercial suppliers such as CRL have reconsidered and changed their long-term selection criteria and mating system (CRL reference paper Vol 11, #1, 1999).

Genetic Standardization of Genetic Variability in Outbred Strains:

Due to the observed increased litter size and body weight, as well as decreased longevity, CRL has recently initiated an effort to "standardize" certain outbred strains such as the CD® (SD) IGS BR rat, Wistar Han IGS rat, and CD-1 (CD-1®(ICR)BR) mouse. For example, in the early 1990s, 100 pairs of CD rat breeders were selected from each of eight diverse CRL CD rat colonies world wide and rederived in isolators to form the CD IGS reference foundation colony in Wilmington, MA. Selection criteria were relaxed, and this CD IGS (international genetic standard) rat reference population was then managed with procedures to minimize genetic drift to establish each CD IGS colony world wide. CRL plans to further minimize genetic drift by migrating additional breeders in both directions between the IGS reference population and isolated colonies over time (CRL reference paper Vol 11, #1, 1999). By also using a pedigreed mating system designed to minimize inbreeding and by improving genetic quality control monitoring, CRL is anticipated to dramatically reduce genetic drift and variation between outbred IGS colonies.

Genetic variation within “narrow genetic base outbred strains”: While CRL refers to this as the International Genetic Standard (IGS) program and has the aim of minimizing genetic drift and producing a "standardized" outbred rat, it is the variability of the population that is standardized, not the individual Crl:CD®(SD)IGSBR rat (abbreviated as CD IGS). While much less diverse than outbreds of essentially any other mammalian species that have not undergone an extreme genetic bottleneck followed by long-term selection mainly for increased prolificacy, the SD strain and the SD derived CD IGS rat strain are segregating at many loci. Even with a narrow genetic base, it is impossible to predict or repeat the genotype of any given CD IGS strain, or for that matter any other outbred strain. Selection for increased prolificacy is likely to have also increased the frequency, or fixed alleles conferring resistance to endocrine disruption by some hormonally active agents, but the genes conferring genetic susceptibility to diverse hormonally-active compounds have not been identified. Thus, the use of such outbred animals makes it impossible to replicate the susceptibility genotypes and therefore the conditions used for testing any toxicant x dose combination in the EDSP. This makes replication of experiments involving outbred animal models problematic, regardless of whether replicates are conducted by the same or different laboratories. Such unpredictable genetic variation within even narrow genetic base outbred strains will greatly complicate and limit efforts to use conventional reproductive toxicological, genomic bioinformatic, microarray, and proteomic approaches to identify chemicals with endocrine disrupting activities, as well as future efforts to characterize their mechanisms of action and loci controlling genetic susceptibility. Furthermore, the within strain genetic variation common to outbred strains is also likely to be a major component of "between litter" effects.

While the extremely narrow genetic background, early inbreeding, and long-term history of selection, mainly for high prolificacy and vigor, has resulted in highly robust and productive CD IGS rats, it is highly doubtful these animals are representative of any natural mammalian outbred population. The fact that Robert Dawley backcrossed daughters of a hybrid male back to the same male for seven consecutive generations indicates that well over 90% of the gene pool (an estimated 99.6 % of the genes minus deleterious alleles purged by selection) of the SD strain came from the single foundation male rat. Thus, the SD strain and any substrains derived from this closed population represent an exceedingly narrow genetic base. The seven consecutive generations of backcrossing daughters to the same foundation male rat is also likely to have purged most deleterious recessive genes, including those affecting reproductive development and function. While crossing inbred sire-daughter lines generated from the single foundation male is likely to have provided some heterozygosity at certain loci in the SD population, most of the genetic diversity in this strain had to come from the single hybrid foundation male that was backcrossed for seven consecutive generations to his daughters.

Just as important, the IGS program does very little to eliminate the changes in gene frequency affecting reproductive and correlated traits brought about by over 80 generations of selection for increased litter size. Since selection of the CD rat was conducted in large, narrow genetic base outbred populations, any additional mutations

improving litter size would also be selected for. It has been argued that outbred laboratory animal populations show phenotypes more representative of wild outbred populations and more phenotypic diversity than inbred strains. This may be true for unselected traits in a fully outbred laboratory animal strain, but it is clearly not true regarding traits (and their correlated traits) for which outbreds have undergone long-term selection (Eklund and Bradford 1977). Many long-term selected outbred strains show phenotypic means and distribution for traits under selection and their correlated traits, which are well beyond the normal distribution of values from an unselected control population. While long-term selection for large litter size increases the mean litter size, it also decreases the additive genetic variance for this trait (Eklund and Bradford 1977) and is thus likely to decrease the additive genetic variance in traits correlated with high fecundity. 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 pleitropically-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.

Data from Spearow's laboratory show dramatic differences in susceptibility to endocrine disruption by estrogenic agents between strains of mice, and that CD-1 strain mice selected for high fecundity are highly resistant to E2. This includes approximately 16- to 100-fold differences in sensitivity between strains of mice in susceptibility to the disruption of testes weight, spermatogenesis, epididymal sperm counts, testicular sulfotransferase activity, and gestational fetal losses (Spearow et al. 1999; Spearow et al. 2001). Data from other laboratories also show that the SD rat and the SD-derived CD rat are less sensitive than other strains in susceptibility to estrogenic agents, including estrogen and the xenoestrogen Bisphenol A (BPA) (Steinmetz et al. 1997; Steinmetz et al. 1998; Long et al. 2000). SD rats are also much more resistant than Wistar/MS or Fisher 344 rats to the reduction in testis and seminal vesicle weights by DES (Inano et al. 1996), and are much more resistant than several other strains, including F344, to estrogen- and DES-induced pituitary tumors (Gregg et al. 1996; Wendell et al. 2000). Thus, the fact that outbred strains such as the SD-derived CD rat and the Swiss and ICR derived CD-1 mouse have been previously long-term selected for increased fecundity and vigor is of special concern in EDSP assays due to the correlated trait response of increased resistance to endocrine disruption by estrogenic agents.

However, resistance or sensitivity to endocrine disruptors is not uniform across test chemicals and endpoints. For example, SD rats are more sensitive than F344 rats to the uterotrophic effects, including increased uterine weight and epithelial cell height effects of endocrine-active compounds such as tamoxifen (Bailey and Nephew 2002). There are many other examples of strain differences in endpoint- and test substance-

specific responses to endocrine-active compounds (see Tables 2 and 3 for a range of responses in different rat strains).

Even though the “outbred” strains come from a relatively narrow genetic base and may not represent all sensitive rats of the world, there is a wider range of responses in outbred strains, due to genetic heterogeneity, than in inbred strains. The selection of an outbred versus an inbred strain for use in these assays depends on whether one can select the most sensitive inbred strain for an assay with all the confounders discussed above and below, or whether the broader response of an outbred strain provides a significant advantage at the sensitive end of the curve.

Genetic Variability in Toxicological Assays:

Since all members of a given isogenic strain (inbred strains and F1 hybrids) are essentially genetically identical, the dramatically reduced genetic variability in isogenic strains enhances the reproducibility and comparability of data generated in these stocks (Festing 1979; Festing 1993). The crucial characteristic common to both inbred and F1 hybrid strains is isogenicity, i.e., the fact that all individuals of an authentic strain are genotypically the same and therefore phenotypically more uniform than individuals of outbred stocks (http://www.criver.com/techdocs/rodent1.html). Isogenicity of such inbred stains and F1 crosses leads not only to a much greater genetic and phenotypic uniformity but also a high, long-term genetic stability. Thus, it has been suggested (Festing 1995) that toxicologists should treat genetics like every other variable and control it by utilizing isogenic strains (F1 hybrids heterozygous at every locus). 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.

The view is held by some researchers (Spearow et al. 1999; Spearow et al. 2001) that commercial outbred strains are resistant to endocrine disruption by estrogenic agents at some endpoints, most likely as a correlated response to long-term selection for high prolificacy. However, toxicity testing in outbreds represents testing the effects of toxicants on a sampling of outbred strain genotypes. One argument is that the segregation of any genes in outbred strains controlling traits that have not been fixed by early inbreeding or long-term selection will result in genetic noise and increased phenotypic variability. Since toxicity testing usually involves the calculation of dose-response curves, the use of phenotypically variable nonisogenic stocks reduces the precision with which such curves can be estimated and therefore the sensitivity of the assay (Festing, 1979). The counterargument is 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.

Multiple Strain Assays:

There is a risk in using a single strain in toxicological safety testing, particularly when that strain is known to be highly resistant to one or more classes of chemicals and/or endpoints to be tested in the EDSP. Any time there is considerable genetic variation in a susceptibility trait, a single isogenic or outbred strain may be resistant to the compound being tested or the endpoint being assessed. If so, a chemical that is toxic to other genotypes may be judged to be relatively safe (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." This is particularly true for traits showing major strain differences in susceptibility to endocrine disruption by estrogenic agents. As an alternative, Michael Festing has pointed out for two decades the benefits of toxicological testing with multiple divergent isogenic strains, rather than a single strain, to better ensure that all the test animals are not resistant (Festing 1979; Festing 1987; Festing 1993; Festing 1995), by the use of several isogenic strains from diverse genetic backgrounds in a factorial experimental design to overcome the problem of testing animals, all of which are genetically resistant to the compound to be tested (Festing 1995; Festing et al. 2001; Festing and Altman 2002).

An advantage of testing with multiple strains is that identification of strain differences enables an additional resource to determine the mechanisms of toxicity (Narotsky et al. 2001). Once parental strains are shown to differ, congenic inbred, consomic inbred, and recombinant inbred strains can be compared and strain distribution profile phenotypes used to map and characterize genes controling susceptibility. Congenic inbred, consomic inbred, and recombinant inbred strains are highly reproducible strains with defined genotypes (Silver 1995). Once a strain set has been genotyped at molecular markers along each chromosome, genes controling traits differing between parental strains can be mapped to specific chromosomal regions, following scoring biochemical or physiological phenotypes of the set of strains (Matin et al. 1999; Cowley et al. 2001; Liang et al. 2002) with appropriate consomic strains, if available. Such congenic inbred, consomic inbred, and recombinant inbred strains resources, along with available gene mapping software, also enable simple as well as complex multigenetic physiology, disease, and toxic susceptibility traits to be broken down to allow the identification of individual susceptibility genes (Manly et al. 2001; Williams et al. 2001). Thus, the use of several highly divergent, genetically-defined inbred parental strains in endocrine disruptor assays could greatly enhance the possibility of identifying genes controlling susceptibility to endocrine disruption.

2.3.2 Species Selection for Endocrine Disruption Assays and Genetic Variability

Variability across strains, in both rats and mice in reproductive parameters, must be considered in the selection of the appropriate strains/species. Historically, the most common strains/species selected for assays of endocrine disruption have been the SD rat and CD-1 mouse. Since the objective of the EDSP study is to examine the effects of a multitude of chemicals on reproductive developmental structural and functional toxicity, strain variation in developmental rates, as well as other biochemical endocrine and signal transduction mechanisms, may be important in considering the range of

susceptibilities to reproductive and developmental toxicity, as are evidenced by effects on endocrine endpoints.

In selecting the appropriate species for EDSP assays, the rat has an advantage over the mouse due to larger size, which allows easier analysis of serum hormone concentrations and certain other physiological endpoints. While considerably more genetic resources are available in the mouse, progress is currently being made in sequencing the genome of Brown Norway rat strain. If recombinant inbred, congenic, and/or chromosome-specific consomic strains derived from the parental inbred strains to be used in the EDSP were available, the utility of the rat for such studies would be further improved.

At present, one of the biggest problems with species or strain selection is that only a small number of studies have examined genetic differences in susceptibility to endocrine disruption. Since most reproductive toxicology studies involve only a single stock of laboratory animals, we do not know whether the response to a given xenobiotic is under genetic control (Festing, 1987). Furthermore, an even smaller number of studies have compared genetically-defined isogenic strains.

The fecundity of a strain limits the number of offspring available following gestational or lacational exposures. Strains with low fecundity are not recommended in OPPTS reproductive and developmental test guidelines. Since SD rats have been bred for high fecundity and have the largest historical database, they have been used the most frequently for regulatory reproductive toxicity studies. Nevertheless, provided the supplier maintains enough breeders to provide the animal needs of the EDSP, strains with moderate fecundity might not limit the number of animals available from the supplier for conducting pubertal or adult exposures. Furthermore, even with gestational and lactational exposures, a strain with moderate fecundity would not limit an EDSP assay under current guidelines of retaining and examining one individual of each sex per litter at adulthood.

Another consideration of species and strain selection relates to the maintenance of early pregnancy in many species, which is dependent on the gonadotropins maintaining corpus luteum (CL) progesterone production. In humans, the maintenance of pregnancy is dependent on LH modulation of CL progesterone production prior to implantation, and hCG modulating CL progesterone production following implantation (see discussion of Narotsky et al 2001 Section 2.5.2). Human epidemiological evidence indicates that exposures to low-dose bromodichloromethane (BDCM, a common drinking water disinfection by-product) are associated with an increased incidence of pregnancy loss in humans (Waller et al. 1998). Following exposure to 75 mg/kg BDCM on days 6-10 of gestation, 62% of Hsd:F344 litters showed full litter resorption (Bielmeier et al. 2001). In contrast, 0% of the Hsd:SD litters showed full litter resorption in response to 75 or 100 mg/Kg BDCM (Bielmeier et al. 2001). Thus, sensitive strains such as the F344 might be more appropriate to estimate human risk of BDCM-induced abortion. While utilizing strains with low fecundity may reduce the number of animals available for the multitude of assessments required in reproductive toxicity testing

guidelines, use of a potentially resistant strain of animals may underestimate the risk to sensitive genotypes.

A discussion of assays under consideration for testing guidelines, a detailed comparison of strain differences in endocrine endpoints, and sensitivity of these endpoints to endocrine-disrupting chemicals which follow provides a summary of potential problems with species/strain selection in reproductive toxicity studies. Specific examples of differential sensitivity of endocrine endpoints in different species and strains to many endocrine-disrupting chemicals will be discussed in Section 3.0.

2.3.3 Confounders Affecting Comparisons of Reproductive Toxicity Data

The focus in this White paper is on intra-species and inter-species comparisons of responses to EACs. The critical papers are those in which the same laboratory evaluates two or more different strains/stocks (intra-species) or the two or more different species at the same time under the same laboratory conditions. Comparisons of intra- and inter-species differences in response to EACs performed at different times, in different laboratories under different laboratory conditions, are more difficult to interpret due to the following likely confounders to the determination that the strains/stocks/species, per se, differ in their responses:

Same laboratory, different times. Source of the animals, genetic drift, age of animals, status of the plastic cages and/or water bottles (new versus damaged), change in feed lot, bedding lot numbers, water supply, change in technical staff.

Different laboratories

A. Animals

- Source/supplier (local closed colony, national/international commercial supplier, change in location from commercial supplier, etc.; the same strain from different suppliers, will most likely be genetically different)
- Age, weight, and health status

B. Husbandry

- Housing: Group housing versus single housing impacts on many endpoints in both sexes for rats and mice. When male mice are group housed, a “dominance hierarchy” is established, with one dominant, aggressive male and the remaining males as subordinates (Haemisch et al. 1994; McKinney and Pasley, 1973; Van Loo et al. 2001). The subordinates exhibit reduced circulating testosterone levels, testes weight, epididymides, accessory sex organs, epididymal sperm numbers and motility (Koyama and Kamimura, 1999; 2000), and testicular spermatid head counts. The subordinates also exhibit signs of increased stress such as increased circulating stress hormones and adrenal gland weights,

altered nervous system and neuroendocrine functions (D'Arbe et al. 2002; Karolewicz and Paul, 2001), altered immune competence, and increased incidence of tumors (Bartolomucci et al. 2001; Grimm et al. 1996; Haseman et al. 1994). In many cases (incidence varies by mouse strain), the dominant male "barbers" the subordinates by removing their whiskers and large patches of fur (Strozik and Festing, 1981; Reinhardt and Militzer, 1979; Sarna et al. 2000; Long, 1972). Even phenotypic variance is affected by housing in C57BL/6J mice (Nagy et al. 2002). Therefore, a major source of differences among study results in different laboratories may be due to the major confounder of housing conditions, if they vary from laboratory to laboratory. Group housing will also result in inter- and intra-cage variability and therefore intra-group variability, confounding detection of any treatment-related inter-group differences. Singly-housed mouse males provide a more uniform population in terms of the endpoints under consideration in this White paper, which are affected by group housing. Dominance hierarchy is also established in group-housed male and female rats (Becker and Flaherty, 1968; Westenbroek et al.,2003; Sharp et al. 2003). The group-housed subordinate rats exhibit effects on delta-opioid receptor function (Pohorecky et al. 1999), immune status (Stefanski 1998; 2000), endocrine status (Taylor and Costanzo, 1975; Popova and Naumenko, 1972), circadian rhythms (Greco et al., 1989), and behavioral and neuroendocrine parameters (Blanchard et al., 1993). The dominant rats display aggressive behavior (Taylor and Moore, 1975) and "barber" the subordinate rats (Bresnahan et al., 1983). The subordinate female rats also exhibit stress-like responses in group housing situations (Westenbroek et al. 2003; Sharp et al. 2003). Therefore, the same confounders may exist in studies in rats across laboratories if housing situations vary.

- Caging: Polycarbonate caging is transparent, autoclavable, and in common use. Recent evidence (Hunt et al. 2003; Koehler et al. 2003) indicates that crazed, cracked, damaged polycarbonate caging from inappropriate and inadvertent use of a harsh detergent releases BPA (a weak xenoestrogen), which can cause adverse reproductive effects (meiotic aneuploidy) in certain sensitive mouse strains (Hunt et al. 2003). Meiotic aneuploidies are associated with embryo and fetal mortality as well as Down's Syndrome (Trisomy 21) in humans. If such damaged cages are in use in a laboratory, its ability to detect effects of a potentially estrogenic chemical (intentionally introduced) may be compromised by the inadvertent presence of BPA (Hunt et al. 2003). A laboratory can carefully check their polycarbonate cages and discard damaged ones or switch to caging which is not made of polycarbonate (e.g., polypropylene). However, polypropylene caging is translucent but not transparent.

- Water bottles: Damaged polycarbonate water bottles can also release BPA (Hunt et al. 2003). A laboratory can carefully check and discard damaged water bottles or switch to glass water bottles.

- Feed: Laboratory animal feed can be categorized into semipurified, purified, certified, and standard open or closed formulas. Different lots of the same feed from the same vendor, as well as different feeds from different vendors, may differ in their relative content of nutrients, pesticides, other contaminants, and phytoestrogens: genistein, diadzein, formononetin, and biochanin A (the isoflavones), as well as coumestrol (the coumestans) from soybeans, flax, wheat, barley, corn, alfalfa, and oats commonly used in laboratory animal diets (Thigpen et al., 1999). Phytoestrogens and estrogenic mycotoxins from contaminating molds and fungi can bind to the estrogen receptor (although they are much weaker than the endogenous steroidal estrogens of humans and animals) and induce estrogen-like effects in animals, humans, and cells in culture. Phytoestrogens can also affect sex-specific behavior, gonadotropin function (Whitten et al. 1995), and postnatal development (Lewis et al. 2003). The predominant phytoestrogens in feed are genistein and daidzein from soybeans. The concentrations of these phytoestrogens vary significantly across rodent diets and within rodent diets by batch (Thigpen et al. 1989; 1999). The content is influenced by the use of different plant species, portion of the plant used, geographic location, time of harvest, and method of processing into pellets or meal (Eldridge and Kwolek, 1983).

- Water: Different laboratories use tap water, deionized water, deionized/distilled water, distilled water, reverse osmosis (R0) water, etc. Separate from palatability issues, different salts, ions, organic contaminants, pesticides, disinfection by-products (DBPs), etc., in different concentrations are present in each type of water and will vary by season, by geographic location, by different water disinfectants, by where the disinfectant is added in the water purification process, and by where the water is sampled in the supply lines. Certain DBPs have been shown to affect reproduction, especially those which are brominated; e.g., bromochloroacetic acid (Klinefelter et al. 2003), dibromoacetic acid, bromochloromethane, bromodichloromethane (Narotsky et al. 1993; Bielmeier et al. 2001), etc., as well as perchlorate which inhibits iodide symporters in the thyroid, reducing import of organic iodide into thyroid cells, and thereby reducing synthesis, export, and circulating and local T_3 and T_4 levels, causing hypothyroidism in adults and children (Lamm and Doemland, 1999).

- Temperature and relative humidity: Are these parameters continuously recorded, monitored, and adjusted? Elevated temperatures can affect

spermatogenesis in males. Reduced temperatures can cause stress responses. Reduced relative humidity can affect pup survival.

- Light cycle: 12:12 or 14:10 can make a difference in circulating hormone levels for those hormones keyed to light/dark cycles (i.e., circadian rhythms; Greco et al. 1989).

- Technician skills and experience: The better trained and more experienced the technical staff, the better the dissections and examination of animal data, and the more uniform the evaluations (low inter-technician variability).

- Source of the test material: The source and purity of the test material, amount, and identification of impurities, storage stability, etc., will affect the results if the performing laboratory does not know the status of these parameters and does not adjust for them, if necessary.

C. Study Design

- Number of animals/group; number of dose groups; source, species/strain/stock, age and weight of the animals

- Route of administration, identity of vehicle, doses (in mg/kg/day), dosing volume (in ml/kg), chemical concentration (in mg/ml); adjustment of dosing volume, based on most recent body weight: the same dose (in mg/kg), administered at a different concentration and dosing volume, can result in different results

- Frequency of body weights to adjust dose and detect effects, clinical observations, feed consumption

- Duration of dosing period (age of animals at end of dosing)

- Technician experience and expertise in dosing, observation of clinical signs; are the technicians “blind for dose?”

- Time of necropsy (age of animals)
  - Anesthesia/euthanasia method
  - Endpoints evaluated
  - Experience of technicians in necropsy, trimming and weighing organs (fresh/fixed weight, especially for small organs such as pituitary, adrenal glands, thyroid gland, ovaries; risk of organs drying out)
  - Choice of fixative (testes in Bouin’s, other organs in buffered 10% formalin)

- — Trimming in tissues, fixation, embedment (GMA plastic for testes, paraffin for other organs), section location and thickness, microscope slide preparation, staining (PAS/H for testes, hematoxylin and eosin for other organs), coverslipping
- — Experience of pathologist reading slides
- — Blood collection (location, volume, speed [hemolysis?])
- — Analysis of hormones, validation method, intra- and inter-assay variability, intertechnician variablity (single terminal blood sample versus longitudinal evaluation by serial tail vein sampling or from cannulated animals)

• Summarization and statistical analyses of study data
- — Are the correct statistical tests used for the correct parameters?
- — Does the laboratory maximize sensitivity and power (number of independent entities, “n” per group)?
- — Does the laboratory have and use historical control data (to interpret concurrent control values in the context of historical control values, and to track the control values over time and between studies)?

2.4 Assays Under Consideration of the EDSP and Associated Endocrine Endpoints

The EPA is in the process of implementing the EDSP. To support this program, the EPA has contracted with Battelle (as prime and RTI International as subcontractor) to provide comprehensive toxicological and ecotoxicological screening, including chemical, analytical, statistical, and quality assurance/quality control support to assist the EPA in developing, standardizing, and validating a suite of *in vitro*, mammalian, and ecotoxicological screens and assays for identifying and characterizing endocrine effects through exposure to pesticides, industrial chemicals, and environmental contaminants. The studies conducted will be used to develop, standardize, and validate methods; prepare appropriate guidance documents for peer review of the methods; and develop technical guidance and test guidelines in support of the Office of Prevention, Pesticides and Toxic Substances regulatory programs.

In vivo mammalian assays under consideration by the EDSP include: (1) pubertal male and female, (2) *in utero*/lactational, (3) adult male 15-day study, all for Tier I,and (4) two-generation mammalian reproductive assay (Tier 2). In addition, as an alternative to the mammalian two-generation assay, a one-generation assay has been described in the EDSTAC Final Report (1998). Table 1 summarizes the assays under consideration and their associated endpoints. A discussion of each proposed study follows Table 1.

Table 1. Assays Under Consideration by the EDSP and Associated Endocrine Endpoints

Endocrine Endpoints	One-Generation*	Pubertal Male and Female	*In Utero* Through Lactation	Adult Male	Two-Generation
Period of exposure/ testing	F0: 2 wks of prebreed exposure, 2 wks of mating; 3 weeks of gestation,3 wks of lactation, then selected F1 animals exposed for minimum of 10 wks postwean	F1 offspring dosing from pnd 23-52/53 (males), pnd 22-42/43 (females)	F0 maternal dosing from gd 6 to pnd 21 F1 cohorts: 1) F1 females, 1/litter for utero-trophic assay, sc injection pnd 21-24 2) F2 females, 2/litter for pubertal assessments, oral dosing pnd 21-42 3) F1 males, 2/litter, for pubertal assessments, oral dosing pnd 21-70	Dosing for 15 consecutive days in adult males	F0: 10 wks of prebreed exposure, 2 wks of mating, 3 wks of gestation, 3 wks of lactation, then selected F1 animals exposed for minimum of 10 wks prebreed, mating, gestation, and lactation, with termination at weaning (pnd 21) of F2 offspring
Litter size/gest-ational indices	yes	no	yes	no	yes
Pup viability	yes	no	yes	no	yes
Anogenital distance	yes, pnd 0, 21 and 95 (males)	no	yes, pnd 0, 21 and at necropsy (pnd 42 females, pnd 70 males)	no	yes (triggered in F2 in 1998 OPPTS Testing Guidelines)
Nipple/areolar retention	yes, pnd 11-13 (males and females), 21 and 95 (males)	no	yes, pnd 11-13	no	yes (not in 1998 OPPTS Testing Guidelines)
Vaginal patency	yes	yes	yes	no	yes
Preputial separation	yes	yes	yes	no	yes

Table 1. (continued)

Endocrine Endpoints	One-Generation*	Pubertal Male and Female	*In Utero* Through Lactation	Adult Male	Two-Generation
Hormone levels	yes, T_3/T_4 (TSH triggered)	TSH, and T_4	TSH, T_3, T_4 and E2	Serum T, E2, DHT, follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PL), thyroid stimulating hormone (TSH), thyroxine (T_4), and triiodothy-ronine (T_3)	not in 1998 OPPTS Testing Guidelines; likely to include TSH, and T_4 for the EDSP (personal communication with J. Kariya)
Urethral vaginal distance	no	no	yes	no	no
Estrous cyclicity	yes	yes	yes	no	yes
Uterine Weight	no	no	yes	no	no
Reproductive tract develop-ment	yes	yes	yes	no	yes
Testes descent	yes	no	no	no	yes
Behavioral evaluations (clinical observations)	yes	yes	yes	yes	yes
Andrology	yes	no	yes	yes	yes
Reproductive organ weights and histopath-ology	yes	yes (reproduct-ive organs and thyroid)	yes	yes	yes

*as described in the EDSTAC (1998) Final Report as an alternative to the mammalian two-generation reproductive toxicity study.

2.4.1 One-Generation Assay

Two assays were proposed by the EDSTAC (FR, 1998) as an alternative to the mammalian two-generation reproductive toxicity study, a one-generation assay and an "Alternative Mammalian Reproductive Test" (AMRT). The proposed one-generation assay is a shortened, scaled down version of the OPPTS guideline for reproductive toxicity testing, and was designed to assess the reproductive effects and developmental effects after an *in utero* lactational and post-lactational exposure. During continuous exposure, the assay is designed to assess onset of puberty (VO and PPS), estrous cyclicity, and andrological parameters. The period of exposure begins two weeks prior to mating and continues through weaning of the F1 offspring, followed by a ten-week postwean exposure in the F1 animals. The AMRT differs from the one-generation assay in that is doesn't include prebreed exposure, but includes mating of the F1 offspring and evaluations of the F2 offspring, with no direct exposure in the F1 and F2 animals.Though the exposure periods and durations of the two alternative assays differ, the endpoints evaluated are essentially the same (and are similar to the endpoints evaluated in the two-generation assay).

2.4.2 Pubertal Male and Female Assays

The EDSTAC also recommended the use of an intact female 20-day pubertal assay to evaluate test materials for effects on the thyroid, hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-thyroid (HPT) axes, aromatase and estrogens (and/or other test materials) that are only effective orally or after a dosing duration longer than that used in the uterotrophic assay (EDSTAC Report, 1998, Vol. 1, Chapter 5, p. 5-26). EDSTAC also recommended, as an alternate assay to be evaluated, the intact male 20-day thyroid/pubertal assay in rodents (EDSTAC, 1998, Vol. 1, Chapter 5, p. 5-30).

The EDSTAC discussion on the usefulness of the female pubertal assay and its endpoints included the following:

> "The determination of the ages at "puberty" in the female rat is an endpoint that has already gained acceptance in the toxicology community. Vaginal opening (VO) in the female is a required endpoint measured in the new EPA two-generation reproductive toxicity test guideline. In this regard, this assay would be easy to implement because these endpoints have been standardized and validated and VO data are currently being collected under GLP conditions in most toxicology laboratories. In addition, VO data are reported in many recently published developmental and reproductive toxicity studies (i.e., see studies from Drs. R.E. Peterson's, R. Chapin's and L.E. Gray's laboratories on dioxins, antiandrogens, and xenoestrogens).
>
> In the pubertal female assay, oral dosing is initiated in weanling rats at 21 days of age (10 per group, selected for uniform body weights at weaning to reduce variance). The animals are dosed daily, 7 days a week, and examined daily for vaginal opening (one could also check for age at first estrous and onset of estrous cyclicity). Dosing continues until VO is attained in all females (typically two weeks after weaning, unless delayed). Age at VO is also determined in the female rat. Rats are dosed by gavage with xenobiotic and examined daily for VO. The advantage over the uterotrophic assay is that one test

detects both agonists and antagonists, it detects xenoestrogens like methoxychlor that are almost inactive via sc injection, it detects aromatase inhibitors, altered HPG function, and unusual chemicals like betasitosterol. In addition, at necropsy one should weigh the ovary (increased in size with aromatase inhibitors, but reduced with betasitosterol), save the thyroid for histopathology, take serum for T4, and measure TSH.

Exposure of weanling female rats to environmental estrogens can result in alterations of pubertal development (Ramirez and Sawyer, 1964). Exposure to a weakly estrogenic pesticide after weaning and through puberty induces pseudoprecocious puberty (accelerated vaginal opening without an effect on the onset of estrous cyclicity) after only a few days of exposure (Gray et al., 1989), or precocious pubery with both accelerated VP and onset of estrous cyclicity. Pubertal alterations also result in girls exposed to estrogen-containing creams or drugs which induce pseudoprecocious puberty and alterations of bone development (Hannon et al., 1978).

Several examples of estrogenic chemicals affecting vaginal opening in rodents are known and include methoxychlor (Gray et al., 1989), nonylphenol, and octylphenol (Gray and Ostby, 1998). This endpoint appears to be almost as sensitive as the uterine weight bioassay, but the evaluation is easier to conduct and does not require that the animals be euthanized, so they can be used for additional evaluations. For example, treatment with methoxychlor at weaning (6 mg/kg/day or higher) caused pseudoprecocious puberty in female rats. Vaginal opening occurs from two to seven days earlier in treated animals than controls, in a dose-related fashion, but methoxychlor did not alter estrous cyclicity at the low dosage levels, indicating a direct estrogenic effect of methoxychlor on vaginal epithelial cell function without an effect on hypothalamic-pituitary maturation. Similar effects have been achieved with chlordecone, another weakly estrogenic pesticide, and octylphenol. Chlordecone also induces neurotoxic effects (hyperactivity to handling and tremors). In addition to estrogens, the age at vaginal opening and uterine growth can be affected by alteration of several other endocrine mechanisms, including alterations of the hypothalamic-pituitary-gonadal axis (Shaban and Terranova, 1986; Gonzalez et al., 1983). In rats, this event can also be induced by androgens (Salamon, 1938) and EGF (Nelson et al., 1991). In the last 20 years, there have been over 200 publications which demonstrate the broad utility of this assay to identify altered estrogen synthesis, ER action, growth hormone, prolactin, FSH or LH secretion, or CNS." (EDSTAC Report, 1998, Vol. 1, Chapter 5, pp. 5-26 - 5-27)

Based on the EDSTAC's recommendations, one of the assays that the EPA has proposed to include in an EDSP is a female pubertal assay (see FR Vol. 63, No. 248, pp. 71541-71568, December 28, 1998). This assay is the most comprehensive female-specific assay in the proposed Tier 1 battery of assays, as it is capable of detecting substances that alter thyroid function, that are aromatase inhibitors, estrogenic, antiestrogenic, or are agents which interfere with the HPG and HPT axes. Results from other shorter assays and/or with the use of ip injection as the route of administration have also been reported (O'Connor et al. 1996, 1999).

The EPA is also pursuing the validation of a male pubertal assay as a potential alternative to other assays in the Tier 1 battery. The EDSTAC discussion on the usefulness of the male pubertal assay and its endpoints included the following:

"This assay detects androgens and antiandrogens *in vivo* in a single stage apical test. "Puberty" is measured in male rats by determining age at PPS (preputial separation). Animals are dosed by gavage beginning one week before puberty (which occurs at about 40 days of age) and PPS is measured. Androgens will accelerate and antiandrogens and

estrogens will delay PPS. The assay takes about 3 weeks, and allows for comprehensive assessment of the entire endocrine system in one study (10 per group, selected for uniform body weights to reduce variance). The animals are dosed daily, seven days a week, and examined daily for PPS. Dosing continues until 53 days of age; the males are then necropsied. The body, heart (thyroid), adrenal, testis, seminal vesicle plus coagulating glands (with fluid), ventral prostate, and levator ani plus bulbocavernosus muscles (as a unit) are weighed. The thyroid is retained for histopathology and serum is taken for T4, T3, and TSH. Testosterone, LH, prolactin, and dihydrotestosterone analyses are optional. These endpoints take several weeks to evaluate and are affected not only by estrogens but by environmental antiandrogens, drugs that affect the hypothalamic-pituitary axis (Hostetter and Piacsek, 1977; Ramaley and Phares, 1983), and by prenatal exposure to TCDD (Gray et al., 1995a; Bjerke and Peterson, 1994) or dioxin-like PCBs (Gray et al., 1995b). In contrast to these other mechanisms, only peripubertal estrogen administration accelerates this process in the female and delays it in the male. Preputial separation in the male rodent is easy to measure and this is not a terminal measure (Korenbrot et al., 1977).

Age and weight at puberty, reproductive organ weights, and serum hormone levels can also be measured. Delays in male puberty results form exposure to both estrogenic and antiandrogenic chemicals including methoxychlor (Gray et al., 1989), vinclozolin (Anderson et al., 1995a), and p,p'DDE (Kelce et al., 1995). Exposing weanling male rats to the antiandrogenic pesticides p,p'DDE or vinclozolin delays pubertal development in weanling male rats as indicated by delayed preputial separation and increased body weight (because they are older and larger) at puberty. In contrast to the delays associated with exposure to estrogenic substances, antiandrogens do not inhibit food consumption or retard growth (Anderson et al., 1995b). Antiandrogens cause a delay in preputial separation and affect a number of endocrine and morphological parameters including reduced seminal vesicle, ventral prostate, and epididymal weights. It is apparent that PPS is more sensitive than are organ weights in this assay. In addition, responses of the HPG are variable. In studies of vinclozolin, increases in serum LH were a sensitive response to this antiandrogen, whereas serum LH is not increased in males exposed to p,p'DDE during puberty (Kelce et al., 1997). Furthermore, a systematic review of the literature indicates that the sex accessory glands of the immature intact male rat are consistently more affected than in the adult intact male rat.

In summary, preputial separation and sex accessory gland weights are sensitive endpoints. However, a delay in preputial separation is not pathognomonic for antiandrogens. Pubertal alterations result from chemicals that disrupt hypothalamic-pituitary function (Huhtaniemi et al., 1986) and, for this reason, additional *in vivo* and *in vitro* tests are needed to identify the mechanism of action responsible for the pubertal alterations. For example, alterations of prolactin, growth hormone, gonadotrophin (LH and FSH) secretion, or hypothalamic lesions alter the rate of pubertal maturation in weanling rats.

As indicated above, the determination of the age at "puberty" in the male rat are endpoints that already have gained acceptance in the toxicology community. Preputial separation in the male is a required endpoint in the new EPA two-generation reproductive toxicity test guideline. In this regard, this assay would be easy to implement because these endpoints have been standardized and validated and PPS data are currently being collected under GLP conditions in most toxicology laboratories. In addition, PPS data are reported in many recently published developmental and reproductive toxicity studies (i.e., see studies from R.E. Peterson's, J. Ashby's, R. Chapin's and L.E. Gray's laboratories on dioxins, PCBs, antiandrogens, and xenoestrogens).

Sex accessory gland weights in intact adult male rats also can be affected directly or indirectly by toxicant exposure. The HPG axis in an intact animal is able to compensate for the action of antiandrogens by increasing hormone production, which counteracts the effect of the antiandrogen on the tract (Raynaud et al., 1984; Edgren, 1984; Hershberger, 1953)." (EDSTAC, 1998, Vol. 1, Chapter 5, pp. 5-30 through 5-32).

Based on the EDSTAC's recommendations, one of the assays that the EPA has also proposed to validate as a potential alternative for other assays in the Tier 1 battery in an endocrine disruptor screening program is a male pubertal assay (see FR Vol. 63, No. 248, pp. 71541-71568, December 28, 1998). This assay is the most comprehensive male-specific assay in the proposed Tier 1 battery of assays, as it is capable of detecting substances that alter thyroid function, that are aromatase inhibitors, androgenic, anti-androgenic, or that are agents which interfere with the HPG and HPT axes. Results from other shorter assays, and/or with the use of ip injection as the route of administration, have also been reported (O'Connor et al., 1996, 1999).

2.4.3 *In Utero* Through Lactation Assay

The proposed protocol has been identified by the EPA as the "*In Utero*/Lactational Exposure Testing Protocol" and has been assigned for development under the EDSP. The objective of this bioassay is to detect effects mediated by alterations in the estrogen, androgen, and thyroid-signaling pathways as a consequence of exposure during pre- and postnatal development in the laboratory rat. The treatment paradigm allows for an evaluation of effects on organogenesis, sexual differentiation, and puberty. In using a developing system as the basis for the assay, it is understood that modes of action, other than those of the estrogen, androgen, and thyroid-signaling pathways, may be involved in the induction of toxicity. As such, any observed effects will have to be interpreted in light of the overall weight of the evidence that they are endocrine dependent.

2.4.4 Adult Male Assay

One of the assays considered by EDSTAC as an alternate assay was a short-term screen in an intact adult male with assessment of levels of various circulating hormones at necropsy (see Table 1). The adult male assay was developed to detect effects on male reproductive organs that are sensitive to antiandrogens and agents that inhibit testosterone synthesis or inhibit 5-alpha-reductase (see EDSTAC FR Vol. I, p. 5-30, August, 1998). Results from this assay and/or with the use of ip injection as the route of administration, and other assays with a similar purpose, have been reported (O'Connor et al. 1996, 1999, 2002a,b).

Based on the EDSTAC's recommendations, one of the assays that the EPA has proposed to validate as a potential alternative for other assays in the Tier 1 battery, in an endocrine disruptor screening program, is an adult male *in vivo* assay (see FR Vol. 63, No. 248, pp. 71541-71568, December 28, 1998). The utility of this battery for screening unknown compounds for endocrine activity will be evaluated. Endocrine endpoints for this study are listed in Table 1.

2.4.5 Two-generation Assay

For the Tier 2 battery, EDSTAC recommended a mammalian two-generation reproductive toxicity study. The two-generation reproductive toxicity study in rats is

designed to evaluate the health effects of chemicals on reproduction and viability through two generations as performed in accordance with EPA Guideline OPPTS 870.3800 (1998), and OECD Guideline 416 (2001). Endocrine endpoints for this study are listed in Table 1. In the two-generation reproductive toxicity assay, potential endocrine-disrupting effects can be detected through behavior, fertility, gestational duration, litter size, sex ratio, viability of the offspring, developmental landmarks, and reproductive development (histopathology of reproductive organs, onset of puberty [acquisition of preputial separation and vaginal opening], and estrous cyclicity) in the F1 offspring exposed initially as gametes (from exposed F0 parents), their gestation (in exposed F0 females), lactation (nursed by exposed F0 females), and directly through adulthood and reproduction to produce the F2 generation. This study is intended to evaluate the effects of chemicals on sensitive life stages of reproduction and development in a transgenerational design. Proposed endocrine endpoints for this study are listed in Table 1. Table 1 indicates what is tested and what is proposed with no separation.

2.5 Endocrine Endpoints Under Consideration for EDSP Assays and Intraspecies Variability

Study designs for use in risk assessment require endpoints that have been shown to be robust, reproducible, appropriately sensitive, biologically plausible, and relevant to the adverse outcomes of concern. Definitions of the attributes of such endpoints are as follows:

Reproducible: These endpoints must be reliable; i.e. the same findings occur under the same conditions within the initial reporting laboratory (intra-laboratory) and among other laboratories (inter-laboratory). If the results from endpoints are not reproducible, they cannot form the basis for future research and are most likely not useful for risk assessment.

Robust: These endpoints must significant enough to be present after comparable routes of exposure, (e.g., dosed feed or dosed water), at the same doses over time. Different effects, both quantitative and even qualitative, may be observed when different routes of administration are used. The use of oral gavage, a bolus dose once/day, may result in exacerbation of the endpoint if the parent material is the proximate toxicant and is metabolized to a nontoxic metabolite, if bolus dosing overwhelms the metabolic capacity of the organism or preparation, or it may result in diminution or loss of the endpoint if the parent compound must be metabolized to the active form. The use of nonoral routes, such as inhalation, topical application, injection, etc., will also likely result in different effects, since these routes bypass “first-pass” metabolism by the liver. The findings from routes unrelated to human or environmental exposures may not be as useful for risk assessment.

Sensitive: These endpoints should not be so sensitive that they are dependent on unique conditions (e.g., intrauterine position [IUP], etc.), especially those which are not relevant to the species at risk. Sensitivity is the ability of an endpoint to detect small differences reliably. These endpoints should not exhibit high variability (insensitive) or be greatly affected by confounders (too sensitive).

Relevant: These endpoints must be biologically plausible and related to adverse effects of interest/concern. If there are no adverse consequences at the dose/duration/route evaluated, these endpoints should be predictive of other adverse effects at higher doses, after longer exposure duration, and/or by different routes, etc.

Consistent: These endpoints should occur in the presence of effects in other related, relevant endpoints, if possible, at the same dose, timing, duration, routes of exposure, etc. (i.e., characteristic syndrome of effects).

Individual endocrine endpoints are discussed below.

2.5.1 Fertility and Gestational Indices

Fertility and gestational indices and litter size parameters are used as measures of reproductive performance. Examples of these indices and parameters follow.

***Gestational parameters*:**
No. of mating pairs
No. (%) females sperm/plug positive

Mating index = $\left(\frac{no.\,sperm\,/\,plug\,positive}{no.\,paired}\right)\times 100$

Precoital interval (time in days from pairing to evidence of copulation)

No. (%) females pregnant

Pregnancy index = $\left(\frac{No.\,pregnant}{No.\,sperm\,/\,plug\,positive}\right)\times 100$

Gestational length in days

No. (%) females with live litters

Gestational index = $\left(\frac{No.\,females\,with\,live\,litters}{No.\,females\,pregnant}\right)\times 100$

***Litter size parameters*:**

No. ovarian corpora lutea/dam

No. uterine implants/dam

No. (%) preimplantation loss = $\left(\frac{No.corpora\ lutea - no.uterine\ implants}{No.corpora\ lutea}\right) \times 100$

No. resorbed implants/litter

No. dead fetuses/litter

No. nonlive (resorbed and dead) implants/litter

No. (%) litters with $\geq$ 1: resorptions, dead fetuses, and nonlive

No. (%) postimplantation loss = $\left(\frac{No.uterine\ implants - no.live\ fetuses\ or\ pups}{No.uterine\ implants}\right) \times 100$

No. malformed implants/litter

No. affected implants (nonlive plus malformed)

No. live pups/fetuses/litter

2.5.2 Survival and Growth Indices

Survival and growth indices are used as measures of pup viability. Pups are evaluated on pnd 0 for the following:

Live birth index = $\left(\frac{No.live\ pups\ at\ birth}{Total\ no.\ pups\ born}\right) \times 100$

Stillbirth index = $\left(\frac{No.dead\ pups\ at\ birth}{Total\ no.\ pups\ born}\right) \times 100$

No. live pups/litter: total and by sex

No. dead pups/litter total and by sex

Mean pup body weight/litter: total and by sex

Anogenital distance (absolute in mm, relative to body weight, or adjusted with body weight as the covariate) by sex/litter

Pups are evaluated during lactation for the following:

Sex ratio (% male offspring/litter)

4-Day survival index = $\left(\frac{No.\ pups\ surviving\ 4\ days\ (precull\ *)}{Total\ no.\ live\ pups\ at\ birth}\right) \times 100$

7-Day survival index = $\left(\frac{No.\ pups\ surviving\ 7\ days}{Total\ no.\ live\ pups\ at\ 4\ days\ (postcull\ *)}\right) \times 100$

14-Day survival index = $\left(\frac{No.\ pups\ surviving\ 14\ days}{Total\ no.\ live\ pups\ at\ 7\ days}\right) \times 100$

21-Day survival index = $\left(\frac{No.\ pups\ surviving\ 21\ days}{Total\ no.\ live\ pups\ at\ 14\ days}\right) \times 100$

Lactation index = $\left(\frac{No.\ pups\ surviving\ 21\ days}{Total\ no.\ live\ pups\ at\ 4\ days\ (postcull\ *)}\right) \times 100$

*If the litters are standardized to a fixed number (normally eight or ten) on pnd 4.

Several studies have investigated the effects of strain differences on implantation and pregnancy outcomes. One study by Cummings et al. (2000) compared the effects of atrazine on implantation and early pregnancy in four strains of rats: Holtzman, SD, LE, and F344. Since atrazine has been known to affect prolactin surge, which is important in the initiation of pregnancy (two surges, one diurnal and one nocturnal, occur daily in the first ten days of pregnancy), the rats were dosed on the first eight gestational days (gd) either diurnally or nocturnally with 0, 50, 100, or 200 mg/kg/day with atrazine. In F344 rats, atrazine (at the top two doses) increased the percent preimplantation loss only after nocturnal dosing. Holtzman rats showed a trend toward increasing preimplantation loss, while SD and LE rats were not significantly affected at this endpoint. Percent postimplantation loss was significantly higher in Holtzman rats only. Clearly, the F344 and Holtzman strains were most sensitive to the effects of atrazine on early pregnancy.

The effects of atrazine on full litter resorption and pregancy outcome were investigated in three rat strains: F344, SD, and LE (Narotsky et al., 2001). The dams were dosed from gd 6-10 with 0, 50, 100, and 200 mg/kg/day with atrazine and then

allowed to deliver their pups, thus allowing for an assessment of pup viability. At the highest dose (200 mg/kg/day), atrazine caused similar rates of full litter resorption in all three strains. Prenatal loss was significantly increased in F344 dams, resulting in reduced litter sizes for dams with live litters. At lower doses (e.g. 50 and 100 mg/kg/day), atrazine caused pregnancy loss in only F344 rats, while SD and LE litters were unaffected. The period of sensitivity to atrazine-induced pregnancy loss coincided with the period of LH/prolactin dependency on the maintenance of pregnancy. While gestational loss was induced in the sensitive F-344 strain by 50 mg/kg atrazine administered on gd 6-10, even the highest dose of atrazine (200 mg/kg) was without effect when administered after the LH dependent period on gd 11-15. The authors concluded that F344 rats were more sensitive than SD and LE rats to the reproductive effects of atrazine, and that maternal toxicity, which occurred in all three strains at higher doses, was not predictive of full litter resorption.

In another study, the effects of a drinking water disinfection-by-product, BDCM, on pregnancy loss were studied in two rat strains, F344 (dosed with 0 and 75 mg/kg/day based on previous studies), and SD (dosed with 0, 75 and 100 mg/kg/day) (Bielmeier et al. 2001). Daily dosing with BDCM (75 mg/kg) from gd 6 to 10 produced a 62% incidence of full litter resorption (pregnancy loss) in F344 rats and no effect on pregnancy in SD rats. Since body weights were significantly reduced in both strains, it is possible that toxicokinetic differences in the strains may not be responsible for the differential sensitivities of the strains to BDCM-induced pregancy loss. Thus, the data of Bielmeier et al. (2001) suggest that F344 rats are genetically more sensitive than SD rats to BDCM-induced diminishment of luteal cell responsiveness to LH (or perhaps BDCM induced luteolysis).

Though these studies were not performed in the same laboratory, Liberati et al. (2002) compared reproductive and litter parameters in Wistar Hannover rats with historical CD rat data from the same laboratory and from CD rat data provided by CRL. Pregnant female Wistar Hannover rats were dosed daily with distilled water from gd 6 to 15. Wistar Hannover rats were found to have lower pregnancy rates and smaller litter sizes. In addition, they were found to have higher percentages of preimplantation loss, postimplantation loss, and resorptions versus CD rats (14.1, 7.4 and 7.2% versus 5.9, 5.6, and 5.1 %, respectively). Thus, it appears likely that fundamental differences in reproductive parameters occur between outbred stocks of rats.

2.5.3 Reproductive Tract Development

Reproductive development involves both morphological and hormonal aspects, which operate together to result in correctly formed, functional, and responsive reproductive systems in both males and females. In mammals, gonadal origins begin early in embryonic development, prior to sexual differentiation (Schardein, 1999). Initial stages are the same for both male and female. Sexual differentiation and maturation are under hormonal control. Thus, both physical and hormonal indicators of

reproductive development can be monitored to detect the presence of endocrine-disrupting activity.

— **Wolffian duct (male development).** Initially, the gonads appear as a pair of longitudinal undifferentiated genital ridges in the dorsal abdominal cavity of the embryo. The primordial germ cells migrate into the genital ridges from the extra-embryonic yolk sac at about gd 10-12 in the rat. Concomitantly, the genital ridges form primitive sex cords, which are indistinguishable, male from female. These small primitive indifferent gonads are held in place in the abdominal cavity by cranial suspensory ligaments (from gonad cephala to diaphragm) and by gubernacular cords (from gonad caudally to the base of the abdominal cavity). In the male, under the initiation of the sry gene on the Y-chromosome, the primitive sex cords continue to proliferate and form the testis, including the interstitial Leydig and the intratubular Sertoli cells. The Leydig cells begin to produce T. The epididymides, vas deferens, ventral prostate, and seminal vesicles are formed from the embryonic structures known as the Wolffian ducts in the presence of fetal T. The secondary sex cords characteristic of female development (Müllerian ducts) regress in the presence of testosterone and of Müllerian Inhibitory Substance (MIS; inhibin) produced by fetal Sertoli cells, as male sexual differentiation proceeds. DHT (produced locally by the Leydig cells from conversion of testosterone by the enzyme 5-alpha-reductase), directs the differentiation of the male genital tubercle into the external genitalia and the urogenital sinus into the prostate at Cowper's (bulbourethral) glands; DHT also causes regression of nipple anlagen in the fetal male rodent. In the presence of T, the cranial suspensory ligaments regress, as the gubernacular cords thicken (also under the control of the INSL3 gene) to cause the testes to descend to the inguinal ring *in utero* (in rodents) and then into the scrotal sacs, i.e. testes descent during late lactation (in rodents).

— **Müllerian duct (female development).** After formation of the primitive sex cords, genetically female embryos undergo differentiation as the primitive sex cords proliferate to form the ovaries, whereas the secondary sex cords (Müllerian ducts) form the uterus, oviducts, and upper end of the vagina. The lower end of the vagina and external genitalia are formed from the female genital tubercle. The Wolffian ducts regress in female fetuses in the absence of T. Also in the absence of T, the gubernacular cords regress, while the cranial suspensory ligaments are retained to keep the ovaries held abdominally just below the kidneys.

— **Pre- and postnatal development.** Visual examination of the reproductive tracts of both males and females at birth and during the postnatal period provides a measure of both pre- and postnatal development as described above and below.

— **Puberty.** Acquisition of puberty, identified by the age (in days) of acquisition of vaginal patency (VP) in offspring females and the age (in days) of acquisition of balanopreputial separation (preputial separation, PPS) in males, can be used to compare the relative effects of a compound on male and female reproductive development. In the authors' laboratory, the age at acquisition of these indicators of puberty is consistent, with very tight variances intra- and inter-studies. Acquisition of puberty is a critical endpoint in endocrine disruptor assays. Section 2.5.7 contains a more detailed discussion of pubertal endpoints.

2.5.4 Anogenital Distance

The sex differences in anogenital distance (AGD) at birth and beyond (male AGD is approximately twice as long as female AGD in rats, mice, and newborn humans) are under androgen control, specifically dihydrotestosterone (DHT) (Gray et al. 1998; Gray and Ostby, 1998) and do not appear to be affected by estrogens (Biegel et al. 1998a) but are affected by pup body weights (Ashby et al. 1997). Data (Gallavan et al. 1998) from 1501 control CD® (SD) rat pups indicated that a 1 gm increase in body weight results in a 0.19 mm increase in AGD. Very small (but statistically significant) increases in female AGD (with no effects on males) on pnd 0 have been reported for dietary *p*-tert-octylphenol (OP; Tyl et al. 1999) and BPA (Tyl et al. 2002) in a number of dose groups, not dietary dose related, with no developmental or reproductive sequelae. If AGD values are shorter in either sex (especially if in both sexes) in a treatment group with reduced pup body weights, it is highly likely that the AGD effect is due to the body weight effect, and can be teased out by analyses of covariance (ANCOVA) with body weight as the covariate. The precision with which laboratories measure AGD on newborns ranges from use of a dissecting microscope with an ocular micrometer and eyepiece grid or a vernier caliper (and the pup flat on the microscopic platform) to hand-held pups and a ruler. Obviously, the accuracy and variance of the values will differ, depending on the method. Precise methods result in very tight values, which may result in very similar statistically significantly differences in group means, for which the biological significance and relevance, if any, are unknown. AGD has also been shown to be significantly reduced in CD® (SD) newborn rats whose dams were on 50% feed restriction from gd 7 (Holsapple et al. 1998; Carney et al. 1998).

AGD is DHT-mediated, and the endocrine-mediated effects persist into adulthood. However, since it is confounded by body weight, the current practice is to present the data as mm, mm/cube root of the body weight, and/or to analyze the data by ANCOVA (analysis of covariance), with the body weight at measurement (birth, weaning, etc.) as the covariate. These procedures help to account for differences in body weight (especially in groups where there is systemic toxicity, expressed as reduced parental and offspring body weights).

2.5.5 Urethral Vaginal Distance (UVD)

The measurement of UVD in female rodents has been proposed as an endpoint, possibly sensitive to levels of E2; analogous to AGD under DHT control. It is currently under evaluation in the authors' laboratory.

In a study which investigated the effects of gestational exposure to TCDD on reproductive development of female rat offspring in two strains of rats, LE and Holtzmann (Gray and Ostby, 1995), administration of TCDD on gd 15 (1 µg/kg) produced malformations of the external female genitalia and vaginal orifice, a delay in puberty, and significantly increased UVD in both rat strains.

2.5.6 Retention of Nipples in Preweanling Males

This is evaluated usually in male rats on pnd 11-13 (and in male mice on pnd 9-11) and is DHT-mediated. Effects may persist into adulthood. In the authors' laboratory, retained nipples have never been observed in control preweanling CD® (SD) males, although areolae have been observed in our laboratory in 0-7.5% of control males on pnd 11-13 (based on examination of over 5000 males *in toto*). This is a sensitive indicator of altered testosterone and/or DHT levels (effects on synthesis, degradation, receptor binding, transcriptional activation, etc.). Male pups with retained nipples (especially as weanlings and/or adults) are more likely to exhibit reproductive system malformations, but the correlation is not perfect (i.e., some males with nipples exhibit no malformations, some males with no nipples do exhibit malformations). Retention of nipples is also a reasonable, but not infallible, predictor of male reproductive malformations caused by perinatal exposures at similar or higher doses (McIntyre et al. 2001; McIntyre et al. 2002).

One study by You et al. (1998) was designed to compare male sexual development in two strains of rats after gestational exposure to an antiandrogenic compound. In LE and SD rats exposed *in utero* on gd 14-18 to *p,p′*-DDE (a metabolite of DDT) at 0, 10, or 100 mg/kg/day, the high dose produced a significant 14% decrease in AGD in male LE, and a 7.8% decrease (not statistically significant) in SD rats on pnd 2**,** with no effect on AGD in females of either strain. On pnd 13, males from both the low and high-dose groups in the SD rats and males from the high-dose group only in the LE rats had retained nipples. Preputial separation occurred in control SD and LE rats at about the same time, but vaginal opening occurred earlier in control LE versus SD rats. Regardless, neither preputial separation nor vaginal opening in either strain were affected by developmental exposure to *p,p′*-DDE**,** and growth of male reproductive organs was also not affected. Flutamide (an androgen receptor antagonist), which was used as a positive control in this study, produced decreases in AGD, nipple retention, and changes in male reproductive organ growth (decrease in testis, ventral prostate and epididymis weight in SD rats, and a decrease in seminal vesicle weight in LE rats). Both strains of rat had a differential sensitivity to the effects of *p,p′*-DDE. In LE but not SD rats, AGD was decreased by 100 mg/kg/day of *p,p′*-DDE and in SD rats, nipple retention was produced at a lower dose (10 mg/kg/day) than in LE rats. In response to the 100

mg/kg dose, LE rats showed 6- to 8-fold higher serum concentrations of *p,p'*- DDE than SD rats. One explanation for the differential effects with *p,p'*-DDE may be due to different tissue levels of *p,p'*-DDE from potentially different pharmacokinetic characteristics in the two strains.

In a study comparing the effects of developmental exposure to VIN (an antiandrogen which has metabolites that bind to the androgen receptor) in Wistar and LE rats, similarities and differences were reported (Hellwig et al. 2000). Exposure to 200 mg/kg/day from gd 14 to pnd 3 produced similar effects on male offspring of both strains, including reduced AGD, nipple and areolae retention lasting into adulthood, hypospadias, penile hypoplasia or development of vaginal pouch, transient paraphimosis (penile edema), and reduced function and chronic inflammation of the epididymides, prostate, seminal vesicles, and coagulating glands. In adults, LE had testis atrophy and chronic inflammation of the urinary bladder, which were not observed in Wistar offspring. Exposure to 12 mg/kg/day produced only transient nipple/areolae retention in male offspring of both strains, but produced nipple/areolae retention persisting into adulthood in a few LE but no Wistar males. In addition, adult LE but not Wistar exposed to 12 mg/kg/day had slightly reduced prostate, seminal vesicle and coagulating gland weights. Overall there were more similarities than differences in the effects of VIN in both strains, and the NOAEL was 12 and 6 mg/kg in Wistar and LE rats, respectively.

2.5.7 Puberty

Acquisition of puberty can be determined in both females and males by a number of physical changes. For females, vaginal patency and age of first estrus are most often used, whereas in males, preputial separation is most often monitored. In both sexes, acquisition of puberty is affected by body weight, so the current approach is to covary the age at acquisition by the body weight at acquisition, at an arbitrary age during the time of acquisition, or by some measure of weight gain during the postlactational or prepubertal period (the selection of the end date for weight gain is problematic). Small changes in acquisition ($\leq$3 days) may indicate body weight-related delays in development; large changes (accelerations or delays of $\geq$ 4 days) most likely indicate effects from endocrine disruption, especially in the absence of body weight effects. Minor delays/accelerations in puberty in the BPA rat study (Tyl et al. 2002) were presented and analyzed as absolute values, and as values covaried by body weight at acquisition and at an arbitrary age.

Statistically significant differences in age at acquisition of puberty may indicate endocrine-mediated effects, especially if the effects are different for the sexes (e.g., VP is delayed and PPS is accelerated or unchanged, VP is accelerated and PPS is delayed or unchanged, etc.) and if the effects are profound (acceleration or delay of many days versus only a few days). However, acquisition of developmental landmarks is dependent on both age and weight (i.e., heavier animals acquire the landmark earlier, while lighter animals acquire the landmark later), but lighter animals do acquire the landmark (unless there is another cause for the delay) and in many cases acquire the

landmark at a lighter weight than the heavier animals. This observation is consistent with the recognition by the EPA (1996, p. 56295) that "body weight at puberty may provide a means to separate specific delays in puberty from those that are related to general delays in development." The significance (i.e., the consequence, if any) and "the biologic relevance of a change in these measures of a day or two is unknown" (EPA, 1996, p. 56295).

The recognition that body weight is important in analyzing and understanding acquisition of puberty is strengthened by the work of Kennedy and Mitra (1963) and Carney et al. (1998) who showed that body weight and food intake are factors in the initiation of puberty in the rat, and by the work of Holsapple et al. (1998) who put groups of 26 timed-mated SD rats on standard diets at 100% (control), 70%, or 50% of historical control feed intake levels from gd 7 through weaning on pnd 21. Selected weanlings were continued on feed restriction until ten weeks (with 100% feed from ten to 20 weeks of recovery) or until 20 weeks of age, with necropsy of all offspring at 20 weeks of age. Feed restriction resulted in reduced weight gains for dams and pups related to the degree of restriction. In both the 50% and 70% feed restriction groups, gestation length was significantly increased, and age at VP and PPS was also delayed (by one day at 70% restriction and by six days at 50% restriction for both parameters). AGD at birth was significantly reduced in both sexes in the 50% restriction group, but AGD:body weight ratios were essentially identical across groups, indicating that smaller (low body weight) pups had shorter AGDs and that the effects were proportional. The authors conclude that "these results show that certain reproductive and developmental endpoints are altered by feed restriction in the range relevant to common testing scenarios" (Holsapple et al. 1998).

2.5.7.1 Vaginal Patency in Females

In females, acquisition of puberty is indicated by vaginal opening or patency (VP), dependent on E2 and resulting from E2-dependent cornification of the vaginal seam. In control CD® (SD) rats in the authors' laboratory, the grand mean age at VP is 31.1 days (based on 16 studies from 1996 to 2000). VP may be observed first as the appearance of a small "pin hole(s)" or perforations but is recorded as acquired when vaginal opening is complete. Vaginal threads across the vaginal opening may be temporary or persistent (Wolf et al. 1999; Flaws et al. 1997).

Vaginal opening may be advanced by estrogenic compounds, and estrogen receptor modulators either advanced or delayed with various environmental chemicals (see Review by Goldman et al. 2000). In a study of the effects of BPA on sexual development in two strains of rats, SD and Alderley Park (AP), Tinwell et al. (2002) found strain-related differences in VP. Vaginal opening was significantly delayed in AP rats and not SD rats exposed to BPA. There was no effect on age of first estrus (an explanation of this endpoint follows).

2.5.7.2 Age of First Estrus in Females

On or within a few days of VP, the female exhibits her first estrus, so age at first estrus (absolute age and/or interval from VP to first estrus) is also useful. Late follicular growth of the first ovulatory cells is stimulated about the time of vaginal opening, although there is some variation in the initial release of oocytes. Following vaginal opening, daily vaginal smears are monitored to determine the age of first estrus and/or first vaginal cycle. Irregular estrous cycles are often seen in the immediate postpubertal period (Goldman et al. 2000).

2.5.7.3 Preputial Separation in Males

Acquisition of puberty in males is indicated by preputial separation (PPS; balanopreputial separation) or separation of the foreskin of the penis from the glans. PPS is dependent on androgens. PPS is a process that leads to the cleavage of the epithelium through cornification, forming the squamous lining of the prepuce of the penis (Goldman et al. 2000). As a sign of puberty and an essential prerequisite for further development of the ejaculatory process, PPS has been used as a reliable, noninvasive endpoint by which to monitor rodent pubertal development and perturbations of this process. This landmark of acquisition generally occurs during the peripubertal period (pnd 36-55 or 60; Stoker et al. 2000). In control CD® (SD) rats in the authors' laboratory, the grand mean age at PPS is 41.9 days (based on 16 studies from 1996 to 2000).

Estrogenic and anti-androgenic compounds have been shown to delay PPS, while androgen receptor agonists accelerate PPS (see review by Stoker et al. 2000). Tinwell et al. (2002) found that BPA had no effect on PPS in two strains of rats (AP and SD) at a dose that delayed vaginal opening in female AP rats only. In AP rats, vaginal opening was at 33.8 ± 0.8 days in control animals, compared to 35.4 ± 0.6 days in rats exposed to 50 mg/kg/day BPA. Sensitivity to BPA was found to be not only strain related but sex related.

2.5.8 Estrous Cyclicity and Ovulation Rate in Postpubertal Females

After the initial release of ova, female rats begin to exhibit four- to five-day estrous cycles, with accompanying changes in vaginal cytology and circulating hormones. The acquisition of estrous cyclicity results from shifts in the hypothalamic-pituitary-ovarian endocrine axis and is the culmination of the maturation of reproductive processes that began prenatally. As indicated above, irregular estrous cycles are more common in the first weeks after acquisition of puberty.

Ovulation rate is affected by the dose and ratio of FSH to LH, stage of the cycle, and age of the female. Large genetic differences in ovulation rate exist between strains of mice and in response to exogenous gonadotropins (Spearow et al. 1999; Spearow and Barkley, 1999).

Ovulation rate (the number of eggs ovulated per female) is not included in the endpoints discussed in this White Paper because the protocols of the proposed EDSP assays preclude measurement of ovulation rate in order to measure other relevant endpoints. Ovulation rate is based on the number of corpora lutea counted on the ovaries. These are postovulation ruptured follicles (one per ovulated ovum) producing large amounts of P4 and lesser amounts of E2, to prepare the uterus for implantation of the conceptuses. However, maternal ovarian corpora lutea involute beginning at delivery of offspring (with involution completed on or about pnd 4) to become corpora albicans, indistinguishable from corpora albicans from previous ovulation cycles. All of the studies in Tiers I and II that involve production of offspring require that the dams remain with their pups through lactation to weaning. The parental females are necropsied at the weaning of their litters on pnd 21, when the corpora lutea are no longer present on the ovaries. The inability to collect ovarian corpora lutea counts in these studies also precludes calculation of percent preimplantation loss:

$$\left(\frac{No.corpora\,lutea - no.uterine\,implants}{No.corpora\,lutea}\right)\times 100$$

What can be ascertained, and is therefore included in the list of endpoints to be discussed, is percent postimplantation loss, which is based on the number of uterine implantation sites (i.e., the number of conceptuses implanted; these “nidation scars” persist at least 40 days after delivery) and the number of total pups delivered. Both of these parameters are present and recorded in the Tier I and II studies involving generation of offspring. The calculation for percent postimplantation loss is:

$$\left(\frac{No.uterine\,implants - no.live\ fetuses\,or\,pups}{No.uterine\,implants}\right)\times 100$$

In studies comparing estrous cycles across rat strains, there were strain-related differences in estrous cyclicity in response to food deprivation (Tropp and Markus, 2001). Prior to food restriction, Brown Norway rats had irregular estrous cycle patterns while SD, LE, and F344 rats had regular estrous cycle patterns. By day 5 of food deprivation, 75% of F344 rats and 100% of Brown Norway rats stopped cycling and SD and LE rats were unaffected (the animals’ weights were reduced to 85% of *ad libitum* body weight). It is possible that SD and LE rats, which have generally larger body masses may have more energy store and therefore be less sensitive to changes in body weight. Another possibility is that sensitivity to food deprivation is higher in inbred versus outbred strains. However, given the fact that food deprivation schedules were adjusted to account for differences in initial body weight, it would seem unlikely that simple strain differences in body weight account for the results. These data suggest outbred strains selected for larger litter size are relatively resistant to the disruption of estrous cyclicity by dietary restriction.

In a study comparing estrous cycles in Lewis and F344 rats, by obtaining vaginal smears and quantitating E2, P, FSH, and LH levels at different phases of the cycle, Smith et al. (1994) reported that metestrus was significantly longer, while diestrus and estrus were significantly shorter in Lewis rats compared to F344 rats. Proestrus was similar in both strains. During estrus, E2 levels were significantly higher in Lewis compared to F344 rats, and P levels were significantly higher in all stages of the estrous cycle in Lewis compared to F344 rats. LH and FSH levels did not differ between strains at any stage of the estrous cycle. The authors suggest that elevated E2 and P levels may affect corticosterone levels which could affect hypothalamic-pituitary-adrenal axis responsiveness.

In response to endocrine-disrupting chemicals, strain differences in the ovarian cycle have been reported. Cooper et al. (2000) reported that LE rats were more sensitive than SD rats to atrazine-induced disruption of the ovarian cycle. In addition, Ando-Lu et al. (1998) found that in aging Donryu rats, estrous cycle abnormalities (e.g. persistent estrus) were more common than in F344 rats. Finally, in a study by Eldridge et al. (1994), 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, decreased P, and increased incidence of mammary tumors in SD rats only, with no significant treatment-related effects in F344 rats.

BPA, an environmental estrogen, has been found to stimulate Prl secretion in F344 but not SD rats (Steinmetz et al. 1997). More recently, Long et al. (2000) found that BPA increased DNA synthesis and cell proliferation in the vaginal epithelium of F344 rats but not SD rats. Thus, the rat vagina, an estrogen target tissue, is more sensitive to the effect of BPA in a strain-specific manner. Long et al. (2000) also showed that F344 and SD rats showed no difference in clearance of 3H-BPA from the blood, concentration or affinity of estradiol receptor, or induction of early gene c-fos in response to BPA. Since BPA increased vaginal cell proliferation and DNA synthesis in F344 but not in SD, these data show that strains differ in the intermediate effects of these xenoestrogens downstream of the ER.

Differences in estrous cyclicity have been reported in outbred strains of mice which have been selected for large litter size, high embryo survival, or small litter size (Barkley and Bradford, 1981; DeLeon and Barkely, 1987). Selection for large litter size (Line S1) and high embryo survival (Line E) increased the regularity of estrous cycles, and selection for small litter size (CN) dramatically decreased the regularity of estrous cycles. Therefore, the BN rat, F344 rat, or the CN mouse may provide better animal models than strains that have been bred for large litter size.

2.5.9 Andrology

Depending on the age of the male rodent when sampled, the cauda epididymis ($\geq$ 80 days old) or the entire epididymis (65-80 days old) is evaluated for total number of sperm per cauda or per gram cauda, motility and progressive motility (as percent of total sperm examined. This evaluation must be done within two minutes of animal's demise

with microscope slide and buffer kept at 37°C). Percent malformed sperm should be examined usually manually by microscopic examination of 200 fixed and stained (Eosin Y) sperm per male. In addition, one testis at necropsy should be frozen and subsequently homogenized in buffer and evaluated for homogenization-resistant spermatid head counts (SHC) to calculate daily sperm production (DSP) and efficiency of DSP.

For andrological studies, epididymal sperm counts and testicular homogenization-resistant spermatid head counts are found to be good markers for altered spermatogenesis. Wilkinson et al. (2000) compared the outbred strains of Wistar and SD with the inbred strain Dark Agouti (DA). While a small number of SD were used in this study, the DA rat has lower absolute and relative (% body weight) testes weight, and more variability in sperm counts but there was no significant difference in testicular histology, sperm count per gram of testis, or epididymal sperm count. There were also no differences in weights (relative to body weight) of the epididymis, seminal vesicles, or ventral prostate or of testosterone values for whole blood. DA rats are deficient in CYP2D1 activity, and several P450 cytochromes may also be absent.

In the Tinwell et al.(2002) study that found that the weak xenoestrogen BPA had no effect on PPS in two strains of rats (AP and SD), they reported that 50 mg/kg BPA decreased total sperm count and daily sperm count in AP rats but not in SD rats. Thus, there were strain-related differences in the effects of BPA in rats.

Apostoli et al. (1998), in a review article on the toxicology of lead**,** stated that SD rats appeared to be relatively resistant to the toxicological effects of lead. However, in general, lead impaired spermatogenesis and decreased androgens in other rat strains (e.g. Wistar and Charles Foster rats). Concentrations of blood lead > 40 µl/dl were associated with decreased sperm counts, volume, motility, morphology and endocrine effects.

In mice, strain differences in andrological parameters have been observed. For example, CD-1 mice have been shown to be much greater than 16-fold more resistant than C57Bl/6J (B6) or C17/Jls strain mice to the inhibition of spermatogenesis by pubertal exposure to estradiol (Spearow et al. 1999). Additional studies in Spearow's laboratory have confirmed these observations and have also shown CD-1 mice to be much more resistant to the inhibition of testes weight, elongated spermatids per seminiferous tubule crosssection and epididymal sperm counts than outbred wild-derived Mus spretus/RP/Jls mice.

2.5.10 Organ Weights and Histopathology

- **Reproductive (including accessory sex organ weights).** Reproductive organ weights should be obtained at adulthood and should include: (a) ovaries and uterus for females and (b) testes, epididymides (total and separated into caput, corpus and cauda), prostate (whole, and dorsolateral and ventral lobes separately; dissection may be postfixation), seminal

vesicles, coagulating glands, preputial glands, bulbourethral (Cowper's) glands, and levator ani/bulbocavernosus (LABC) complex for males.

- **Thyroid.** Thyroid hormones (T_3 and T_4) are necessary for normal growth, development, differentiation, and regulation of most organ systems (Goldman et al. 2000; Stoker et al. 2000). Disruption of the feedback control of thyroid function may result in either a hypertrophic (goiter) or hypotrophic thyroid, depending on the mechanism of disruption. These changes would be evident in the weight of the thyroid gland. Since the thyroid gland surrounds the trachea, the thyroid plus embedded trachea is fixed and the trachea dissected away post fixation. The thyroid can then be weighed with little or no damage to the organ for subsequent histopathology.

- **Systemic (liver, kidneys, brain, etc.).** Systemic organ weights should be obtained at adulthood in both sexes and should include liver, kidneys, adrenal glands, pituitary, brain (regions), etc. Comparison of the effect of the test compound on these organ weights (absolute and relative) to effects on reproductive organ weights will provide a more complete characterization of toxicity and suggest whether observed toxicity is more or less targeted to the endocrine system.

- **Absolute and relative to body weight (and brain weight).** Organ weights (both reproductive and systemic) should be presented as absolute and relative to terminal body weight and brain weight. Relative organ weights will correct for effects on body weights (i.e., systemic toxicity). Brain weight is generally considered more stable than body weight after exposure to exogenous compounds and provides a basis for determination if changes in organ weights are primary or secondary to altered body weights.

In a study by Putz et al. (2001), the estrogenic effects of neonatal exposure to ß-estradiol-3-benzoate (EB) were studied in two rat strains, SD and F344. Neonatal rats were injected with EB (over a 7-log range of doses from 0.015 µg/kg/day to 15 mg/kg/day in SD and a 5-log range of doses from 0.15 µg/kg/day to 1.5 mg/kg/day in F344) on pnd 1, 3, and 5. While F344 were not examined on pnd 35, SD male rats on pnd 35, exhibited significant increases in absolute and relative testis and epididymis weights at the low dose, 0.015 µg/kg/day, and significant reductions at higher doses (1.5 and 15 mg/kg/day). Since hepatic testosterone hydroxylase activity was increased in the low-dose animals, it may have advanced puberty, therefore resulting in increased organ weights. On pnd 90, in SD males exposed neonatally to the highest dose used in both strains (1.5 mg/kg/day), there were significant reductions in absolute and relative seminal vesicle, and coagulating gland weights, but not in testis or epididymis weights. SD rats on pnd 90 also showed an increase in testis and epididymis weights at the lowest dose (at one order of magnitude lower than the increase observed on pnd 35). This dose was not tested in F344 rats. In F344 males, the reduction in male reproductive organ weights (absolute and relative to body weight) at pnd 90 was greater at the highest dose (1.5 mg/kg/day) than in SD rats. Whereas relative testicular weights

were 44% of controls in F344 rats, they were 67% of controls in SD rats. Similarly, 1.5 mg/kg EB reduced epididymal weights to 36% of controls in F344 versus 87% of controls in SD rats. Pnd 90 testis and epididymal weights were reduced much more by 1.5 mg/kg EB in F344 than at a 10-fold higher dose (15 mg/kg) in SD rats. Thus, SD rats were greater than 10-fold more resistant than F344 to the inhibition of testes weight by EB.

Strain-related differences in absolute pituitary weights have been reported in ovariectomized rats exposed to E2 (silastic implants) for 10 or 20 days (Schechter et al. 1987). Pituitary weights were dramatically increased in F344 rats, with comparatively minimal effects in SD rats, and Prl levels were dramatically increased in F344 rats ($\geq$ 1000 fold), while only moderately increased in SD rats (100 fold). In addition, E2 implants in F344 strain rats produced a dramatic hyperplasia of anterior pituitary lactotropes, activation of phagocytic folliculo-stellate cells (FS), increase of cells positive for basic fibroblast growth factor, and reorganization of the blood supply from vessels in the adjacent meninges. Estradiol-treated SD rats did not show comparable responses (Schechter and Weiner 1991). Pituitary weights were also different across strains in ovariectomized rats exposed to E2 (10 mg s.c. pellet) for four weeks (Yin et al. 2001). Control pituitary gland weights were the lowest in Brown-Norway rats (4.4 ± 0.2 mg), and more than two-fold higher in Wistar rats (13.0 ± 2.1 mg); F344 control pituitary weights were 7.5 ± 0.1 mg, and Donryu 10.7 ± 1.0 mg. After exposure for four weeks to E2, there was a significant > 3 fold increase in pituitary weights in F344 rats, a significant >0.5 fold increase in pituiary weights in Brown-Norway rats, and no difference in pituitary weights of Wistar and Donryu rats. The F344 strain was the most susceptible to estrogen induction of pituitary tumorigenesis, followed by Wistar and Brown-Norway. The work of Schechter et al. and Yin et al. demonstrates that the pituitary gland of F344 rats is more sensitive to the effects of E2.

Differential effects of DES in particular rat strains have been demonstrated in studies by Gorski et al., who showed strain differences in estrogen dependent pituitary mass (Edpm) and pituitary tumor growth (Wendell et al. 1996; Wendell and Gorski 1997; Chun et al. 1998; Wendell et al. 2000). While F344 are highly susceptible to DES-induced pituitary growth/tumors, Brown Norway (BN) and SD rats are highly resistant. Following DES treatment, F344 strain rats and F344 x BN F2 rats with largest pituitary tumors showed a reduction in retinoblastoma susceptibility gene product (pRb) (Chun et al. 1998). QTL linkage analysis in a F344 x BN F2 mapped several additive and epistatic loci controlling Edpm, including susceptibility alleles from F344 and from BN (Wendell and Gorski 1997). Through QTL mapping in a (F344 x BN)x F344 backcross Wendell et. al. (2000) showed that several QTL including Edpm2-1, Edpm3, Edpm5, and Edpm9-2 all had significant effects on pituitary mass. While Edpm2-1 and Edpm9-2 primarily affected DNA content, Edpm5 primarily affected hemoglobin/DNA ratio, and Edpm3 affected all of these traits equally (Daun et al. 2000). These data defining genes controlling susceptibility to estrogenic agent-induced tumors among genetically defined strains provide a powerful tool for understanding genetic differences in susceptibility to endocrine disruption by estrogenic agents. These data also have value as historic controls. Since these studies used genetically-defined isogenic parental strains, they

can easily be repeated and enhance the identification of genes controling susceptibility to environmentally-induced disease in humans as well.

In a review by Kacew et al. (1995), strain-related differences in mammary tumorigenesis were summarized. SD rats are more susceptible to mammary tumorigenesis after exposures to 2-acetylaminofluorene, 1,4-bis(4-fluorophenyl)-2-propynyl-N-cyclooctyl carbamate, and atrazine than were F344 rats. In addition, Wistar and SD rats were more susceptible than Copenhagen or LE rats to the effects of DMBA (7,12-dimethylbenz (a)anthracene), while Wistar were more sensitive than LE to the effects of 2-acetylaminofluorene. Therefore, there is an inherent difference in mammary tissue sensitivity among rat strains. In males, the sensitivity of the tumorigenic response in the prostate of F344, ACI, Lewis, CD and Wistar rats to 3,2'-dimethyl-4-aminobiphenyl (DMAB) was ordered as follows: F344>ACI>Lewis>CD; the Wistar rats were insensitive (Shirai et al. 1990).

Strain differences in susceptibility to the effect of chemicals on testis weight in mice have been reported. Oishi (1993) found that administration of di-2-ethylhexyl phthalate (DEHP) (0, 0.1, 0.2, 0.4, and 0.8% in feed, for two weeks) to two strains of mice (Jcl:ICR and CD-1) caused significant increases in absolute and relative liver weights in both strains at the highest doses and reduced testicular weights in CD-1 mice only (at a dose as low at 0.2%). DEHP was associated with testicular atrophy in CD-1 mice only, at the doses administered.

In another report demonstrating strain differences in mice, Nagao et al. (2002) exposed male C57BL/6N and ICR mice to BPA at 0, 2, 20 or 200 µg/kg/day for various periods encompassing adulthood, the juvenile period (just after weaning), and the embryo/fetal period. Though BPA did not affect male reproductive organ weights during any dose/exposure period, E2 (10 µg/kg from pnd 27 to 48, as a positive control) produced significant decreases in absolute and relative testes, epididymides, and seminal vesicle weights (as low as 55% of control values) compared to controls in C57BL/6N mice, while ICR mice were unaffected. Histopathology showed that 10 µg/kg E2 was without effect on ICR males, while B6 males showed slight to severe effects on elongated spermatids, decreased epididymal sperm, and seminal vesicle atrophy. Thus, C57BL/6N mice were more sensitive than ICR mice to the effects of E2. These data are consistent with data presented by Spearow et al. (1999).

In male mice, strain-related differences in susceptibility to endocrine disruption by endocrine-active chemicals have been reported (Spearow et al. 1999; Spearow et al. 2001). Mouse strains included B6 (an inbred strain), CD-1 (outbred, with larger litter size), C17/Jls (bred randomly, then inbred), and S15 (bred for large litters, then inbred). In control mice, testicular weight (absolute and relative to body weight) was higher in CD-1 and S15 strains selected for larger litter sizes. In juvenile male mice exposed for three weeks to E2 (at 0, 2.5, 10, 20 or 40 µg in silastic implants), B6 and C17/Jls were sensitive to E2, showing a maximal suppression of testis weight and spermatogenesis even at the lowest dose of E2 (2.5 µg), with no effect on testis weight or spermatogenesis in CD-1 or S15 at any dose up to 10 µg E2. Thus, Spearow et al.

(2001) demonstrated genetic differences in sensitivity to estrogen that may be related to breeding animals for high fecundity.

Additional studies exposing juvenile male mice from 3 to 7 weeks of age to 0, 0.625, 2.5, 10, 40 and 160 µg E2 in silastic implants showed a dramatic strain difference in susceptibility to endocrine disruption (Spearow et al. 2002; Spearow et al. 2003). CD-1 mice were greater than 195-fold more resistant than B6 mice to the disruption by E2 of testes weight, number of elongated spermatids per tubule and Spermatogenic Index (SI). CD-1 strain mice were also >41 times more resistant than B6 strain mice to the inhibition by E2 of epididymal sperm counts, and were more resistant than outbred wild-derived Mus spretus mice to the disruption of testes weight and spermatogenesis by E2.

In a separate experiment, immature B6 males, outbred CD-1, CD-1 derived inbred strains CD10 and CD3, and F1 crosses were implanted subcutaneously at 3 weeks of age with silastic implants containing 0, 2.5, or 40 µg E2 (Spearow et al. 2003). Susceptibility to endocrine disruption by estrogenic agents (SEDE) was evaluated 4 weeks later by determining testicular weight, histology and epididymal sperm counts. The effects of Strain, Dose of E2 and the Strain x E2 Dose interaction were all highly significant on testes weight (TW), seminiferous tubule diameter, elongated spermatids per tubule, spermatogenic index and epididymal sperm counts ($P<0.0003$) (Spearow et al. 2003). Resistance of mouse strains to disruption of testes weight by E2 ranked: B6 << CD3 < CD10 < CD-1. While CD10 x B6 F1 mice showed limited hybrid vigor or heterosis for resistance to the disruption of testes weight by E2, the CD10 x CD3 F1 showed a large amount of heterosis in this trait. The data suggest that susceptibility to the disruption of testes weight by estrogen is controlled by additively and non-additively acting genes. Thus the observed > 16-fold to > 195-fold strain differences in susceptibility to the disruption of spermatogenesis and testes weight between strains questions the adequacy of the standard 10-fold within-species safety factor if only genetically resistant strain(s) are used for toxicological safety testing.

2.5.11 Behavioral Assessments/Clinical Observations

Courtship and mating behaviors in both sexes, and maternal and neonatal behaviors involving nesting, pup retrieval, and nursing are also under the control of the endocrine system. Qualitative evaluation of these behaviors, as they affect viability and ability to thrive, provides another measure of possible endocrine-disrupting activity of a test compound. Strain-related differences in lordotic behavior have been reported. In LE rats exposed gestationally to 1,4,6-androstatriene-3,17-dione, high levels of lordotic behavior are observed in male adult offspring treated with estrogen and progesterone, while SD rats only showed slight effects (Whalen et al. 1986). In an earlier study by Emery and Larsson (1979), Wistar males retained copulatory behavior longer than SD males following castration and systemic para-chlorophenylalanine treatment (which facilitates copulatory behavior). The castrated Wistar males also were more responsive to androgen replacement than SD males. In ovariectomized females, Wistar females were behaviorally more sensitive to estrogen than SD females.

2.5.12 Hormonal Controls

The endocrine system (also referred to as the hormone system) is made up of glands located throughout the body, hormones that are synthesized and secreted by the glands into the bloodstream, hormone carrier proteins (e.g. steroid hormone binding proteins, globulin and albumin, α-fetoprotein), receptors in the cell membranes, cytosol and nucleus of the cells of various target organs, and tissues that recognize and respond to the hormones. The function of the system is to regulate a wide range of biological processes, including control of blood sugar (through the hormone insulin from the pancreas), growth and function of reproductive systems (through the hormones T and estrogen and related components from the testes and ovaries), regulation of metabolism (through the hormones cortisol from the adrenal glands and thyroxin from the thyroid gland), development of the brain and the rest of the nervous system (estrogen and thyroid hormones), and development of an organism from conception through adulthood and old age. Normal functioning of the endocrine system, therefore, contributes to homeostasis (the body's ability to maintain itself in the presence of external and internal changes) and to the body's ability to control and regulate reproduction, development, and/or behavior. An endocrine system is found in nearly all animals, including mammals, nonmammalian vertebrates (e.g., fish, amphibians, reptiles, and birds), and invertebrates (e.g., snails, lobsters, insects, and other species). In humans, the system comprises more than 50 different hormones, and the complexity in other species appears to be comparable.

Puberty, the period in which sexual maturation occurs, begins in the hypothalamic-pituitary-gonadal (HPG) axis and leads to the development of secondary sex characteristics and fertility in both males and females (Stoker et al., 2000; Goldman et al., 2000). Within the hypothalamus, gonadotropin-releasing hormone (GnRH) from neurosecretory neurons act as the primary controller, whereas in the anterior lobe of the pituitary, gonadotropes, which secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and lactrotropes, which secrete prolactin (Prl), serve the controller function. The primary gonadotropin-responsive elements in males are the Leydig and Sertoli cells in the testes, whereas in the female, the thecal and granulosa cells in the ovarian follicle respond.

- **Hypothalamus (GnRH).** It is generally believed that the CNS is the trigger point for initiation of sexual maturation in the male and female rat (Goldman et al., 2000; Stoker et al., 2000). GnRH is present in the fetal brain and slowly increases until the second postnatal week in females and the third postnatal week in males. At that point, GnRH increases steeply and remains elevated until puberty. At puberty, the GnRH neurons undergo a morphological change, developing spiny-like processes that may be related to an increase in synapses on the cells. It has been shown that at puberty, the GnRH neurons become more responsive to neurotransmitter (norepinephrine and dopamine) stimulation. GnRH is released in a pulsatile manner in both male and female animals, which induces a similar pattern of LH and FSH secretion from the

anterior pituitary. GnRH levels can be viewed as an indicator of initiation of sexual maturation.

- **Pituitary (FSH, LH, Prl, TSH).** The gonadotropins FSH, LH, and Prl, secreted by the anterior pituitary, are essential in the process of sexual maturation. In the male, LH stimulates T secretion by direct action on the Leydig cells in the testis, and FSH binds to the Sertoli cells within the seminiferous tubules to aid spermatogenesis. FSH also increases the number of LH receptors in the testis, which in turn increases T production and testis growth. Increased prolactin is associated with growth of the prostate and seminal vesicle glands. In the female, FSH and LH act on the ovarian follicular granulosa and thecal cells, respectively, to stimulate production of E2, follicular/oocyte maturation and ovulation. An increase in prolactin levels is essential in the acquisition of vaginal opening and the transition to sexual maturity. Thyroid stimulating hormone (TSH) also from the anterior pituitary, is the trigger for the release of T_3 and T_4 from the thyroid gland (though T_3 is also produced locally in target organs) , and is essential in the regulation of thyroid activity (see below).

Endocrine-disrupting chemicals have been shown to alter levels of pituitary hormones, such as prolactin and LH. Cummings et al. (2000) dosed four strains of rats, either diurnally or nocturnally with atrazine (0, 50, 100, or 200 mg/kg/day on the first eight days of pregnancy). There were reductions in LH levels in Holtzman rats and LE rats but not in SD or F344 rats after diurnal dosing, and reductions in LH levels in LE and F344 at the highest dose (200 mg/kg) after nocturnal dosing, with no effect on SD or Holtzman rats. There were strain-related differences in control levels of LH. For example, serum LH levels were low in Holtzman and F344 rats and significantly higher in SD and LE rats. Control progesterone levels tended to be higher in F344 but the ranking of other strains differed according to time of collection. Thus basal levels of pituitary hormones may contribute to the sensitivity of certain strains to endocrine-disrupting chemicals.

In a study by Cooper et al. (2000), 50-300 mg/kg/day of atrazine was administered to ovariectomized SD and LE rats for 1, 3, or 21 days, and surges of LH and Prl induced by estrogen were examined. After one or three doses of atrazine (300 mg/kg), LH and Prl were suppressed in ovariectomized LE but not SD rats. After 21 doses, LH and Prl were suppressed in both rat strains in a dose-dependent manner. Therefore, although a longer exposure resulted in similar effects in both strains, LE rats were more sensitive to shorter exposures of atrazine.

The differential effects of an environmental estrogen, bisphenol A (BPA) were studied in female F344 and SD rats by Steinmetz et al. (1997). Basal levels of Prl were 40 and 25 ng/ml in F344 and SD rats respectively. Within three days, E2 (in silastic capsules inserted s.c.) significantly increased Prl levels 10-fold in F344 rats and only 3-fold in SD rats; while BPA significantly increased Prl levels 7-8-fold in F344 rats, with no effect on SD rats. Interestingly, E2 increased anterior pituitary weight in F344 rats, but not in SD rats, while BPA had no significant effect on pituitary weight in either strain.

While the authors speculated that genetic differences in estrogen receptors may be involved in strain-related sensitivities, subsequent studies in uteri and vagina showed that F344 and SD rats differ in the intermediate effects of xenoestrogens downstream of the estrogen receptor (Long et al. 2000).

- **Gonads (T, DHT, E2, P).** Androgens are essential in the development of the male reproductive tract, as well as for feedback regulation of the hypothalamic-pituitary axis, sex accessory organ development and maintenance, and spermatogenesis (Goldman et al., 2000; Stoker et al., 2000). T and DHT are the two most active androgens. Testes descent and development; maturation of the epididymides, vas deferens, seminal vesicles, coagulating glands, prostate, Cowper's glands, levator ani/bulbocavernosus, and other aspects of the male reproductive tract are dependent upon T, whereas DHT is responsible for male AGD, and external genitalia. DHT is key in the development and maintenance of the external genitalia, prostate, and urethra. E2 and progesterone (P) serve similar developmental and maintenance functions in the female.

In four strains of rats, Holtzman, LE, SD and F344, dosed with atrazine (0, 50, 100 or 200 mg/kg/day, for the first eight days of pregnancy) one group diurnally and the other nocturnally, Holtzman rats were the only strain to show a significant postimplantation loss and decreased P levels (Cummings et al., 2000). In the same study, serum E2 was increased only in SD rats dosed diurnally with the high dose of atrazine (200 mg/kg). In control animals, there were strain-related differences in both P and E2 levels. For example, serum P levels on day 9 controls were significantly higher in F344 and Holtzman rats than in SD or LE rats. At the same time, E2 levels in F344 rats were significantly lower than those of the other three strains. Thus, there are strain differences in control gonadal hormone levels, as well as in response to atrazine.

In 19.5 day old male rat fetuses, gestational exposure to TCDD (0, 0.5, 0.1, 0.5 or 1.0 µg/kg) was associated with increases in prenatal T and pituitary LH production in Han/Wistar but not LE rats (Haavisto et. al., 2001). The lowered sensitivity of fetal LE rats may be associated with prenatal T levels which are only 15% of those in Han/Wistar rats. Conversely, adult LE rats are 1000 times more sensitive to TCDD compared to Han/Wistar rats (Pohjanvirta et al., 1988). Interestingly, strain-related differences in sensitivity to TCDD, are also age-dependent. In the same report, gestational exposure to DES (100 µg/kg in Han/Wistar rats; 100, 200 or 300 µg/kg in SD rats) significantly decreased prenatal T production in SD and Han/Wistar male rats, thus the authors concluded that both TCDD and DES exposure in utero may interfere with the timing of the prenatal T surge.

- **Thyroid (T_3, T_4).** TSH released from the pituitary gland stimulates the thyroid to secrete triiodothyronine, T_3, and thyroxine, T_4. T_4 is more prevalent in the blood (98%) than is T_3 (2%). T_3 is predominantly produced locally in target tissues. Prenatally, maternal T_4 is essential for normal offspring development. Thyroid hormones are well known to play essential roles in vertebrate

development (Dussault and Ruel, 1981; Myant, 1971; Porterfield and Hendrich, 1993; Porterfield and Stein, 1994; Timiras and Nzekwe, 1989). Experimental work focused on the effects of thyroid hormone on brain development in the neonatal rat supports the concept of a "critical period" during which thyroid hormone must be present to avoid irreversible damage (Timiras and Nzekwe, 1989). Though the duration of this critical period may be different for different thyroid hormone effects, the general view has developed that the period of maximal developmental sensitivity to thyroid hormone occurs during the lactational period in the rat (Oppenheimer et al., 1994; Timiras and Nzekwe, 1989). Although thyroid hormone receptors are expressed in fetal rat brains (Bradley et al., 1989; Strait et al., 1990) and thyroid hormone can exert effects on the fetal brain (Escobar et al., 1990; Escobar et al., 1987; Escobar et al., 1988; Porterfield, 1994; Porterfield and Hendrich, 1992, 1993; Porterfield and Stein, 1994), the lactational period represents a stage of rapid expansion of the thyroid hormone receptors (Perez-Castillo et al., 1985) and an increase in the number of demonstrated effects of changed levels of thyroid hormone on brain development.

In a study of the effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) on two strains of adult rats, LE, and Han/Wistar, there were strain-related differences in control and treated rats (Pohjanvirta et al., 1989). Control T_3 values in both strains were not significantly different, but T_4 levels were about 1.2 times higher and TSH levels twice as high in Han/Wistar compared to LE rats. Rats were injected once i.p. with 0, 5, 50, or 500 (Han/Wistar only) µg/kg of TCDD, and tissues and hormones were collected at 1, 4, 8 and 16 days post treatment. TCDD decreased T_4 levels slightly more in LE (59%) compared to Han/Wistar rats (43%) after 4 days. After 16 days, T_4 levels had returned to basal levels in the two highest dose groups in the Han/Wistar rats, but in LE rats, T_4 levels remained more than two fold lower than controls with no sign of recovery. By day 16, TCDD increased T_3 levels in the two highest dose groups in the Han/Wistar rats and had decreased T_3 levels by one-half in LE rats. Thus, LE rats exhibited a greater sensitivity to TCDD with respect to thyroid hormone levels. The greater sensitivity of LE rats to TCDD has been confirmed in more recent work by Pohjanvirta et al. (1999) who showed that LE rats are 1000 times more sensitive to the acute lethal effects of TCDD than are Han/Wistar rats.

Another study compared the effects on levels of TSH, T_3 and T_4 after an endocrine challenge test [with thyrotropin-releasing hormone (TRH), and TSH], on two strains of male rats, SD and F344 (Fail et al., 1999). Both strains of rats responded to the challenge with increases in TSH levels. In F344 rats, there were significant increases in levels of T_3 and T_4, while in SD rats, there were only increases in T_4.

Strain-related differences in various hormone levels and organ weights were reported after exposure to the weak antiandrogen, *p,p*'-DDE, by O'Connor et al. (1999). After exposure of adult male LE and CD rats for 15 days to *p,p*'-DDE (0, 100, 200, 300 or 350 mg/kg/day in CD rats; 0, 200 or 300 mg/kg/day in LE rats), the following effects were reported.

CD	LE
↑ relative liver weight, ↓absolute epididymis weight	↑relative liver weight, ↑absolute epididymis and relative accessory organ weight
↑E2 (≥200 mg/kg/day), no change in T, DHT	↑E2 (300 mg/kg/day), T, DHT(≥200 mg/kg/day)
↓FSH (≥200 mg/kg/day) , no change in Prl, LH	no change in FSH, Prl or LH
↓T_4 (≥100 mg/kg/day), no change in TSH	↑TSH and ↓T_4 (≥200 mg/kg/day)

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.

There is also a report by Dhaher et. al, (2000) of intraspecies differences in estrogen receptor number and binding affinity between Balb/c strain mice and a strain used as a systemic lupus erthematosus (SLE) model, MRL/MP-lpr/lpr (Dhaher, Greenstein et al. 2000). The MRL mice showed significantly higher affinity for E2 binding (using 3H-moxestrol as ligand) than did the Balb/c mice, which may be related to the exacerbation by E2 of SLE in the mouse model.

2.5.13 Uterine Weight

Uterine weight is sensitive to estrogenic compounds. The increase in uterine weight after estrogenic compound exposure may be due to increased fluid uptake (imbibition), increased cell size (hypertrophy), and/or increased cell number (hyperplasia). Although the uterotrophic assay is being standardized and validated by an OECD/EPA initiative, the measurement of uterine weight is a sensitive parameter for inclusion in many of the *in vivo* screens involving females. The uterotrophic assay was designed to identify chemicals that act as estrogen receptor agonists or antagonists, directly on the uterus in ovariectomized females (since the HPG axis is not intact). An alternative is to use prepubertal intact females as the animal model. In this case, the test chemical may affect any point along the HPG axis, and the end organs such as the uterus, and the chemical may not be an ER agonist or antagonist since it does not require ER binding in the uterus. A description of processes and endpoints to evaluate chemical effects on uterine weight follows.

1. Ovariectomized adult females are exposed to test material for 3-5 days (po, sc, etc.) and are evaluated for the following:

   A. Increased uterine wet/blotted weight, 6 to 24 (hypertrophy and hyperplasia, respectively) hours after test dose; must be due to a uterine

estrogen receptor-mediated response (since gonad is missing, HPG axis is not intact); an estrogen receptor agonist will be detected.

B. Administration of authentic E2, the potent endogenous estrogen plus the test material; if uterine wet and blotted weights increased with E2, but to lesser extent from E2 plus test chemical, then test material is an estrogen antagonist.

2. Intact prepubertal females are exposed to test material for 3 days (po, sc, etc.) prior to normal onset of puberty and are evaluated for the following:

A. Increased uterine wet/blotted weight (can occur through HPG axis since it is intact; if uterine weight is increased, the test material is an estrogen-mimic, or estrogen-like (not necessarily an estrogen receptor agonist).

B. Adminstration of E2 and test material for detection of anti-estrogens (need not be an estrogen receptor antagonist).

A three-day uterotrophic assay for detecting the estrogenic activity of octylphenol, nonylphenol, methoxychlor, and bisphenol A in prepubertal LE rats was found to be the most accurate method of detecting estrogenic activity when compared to age of VO and estrous cyclicity (Laws et al., 2000), and may provide a sensitive endpoint for detection of endocrine disrupting chemicals that act via estrogen receptor binding (ovariectomized female), or via interaction with the HPG axis (intact prepubertal female).

In studies performed in ovariectomized Wistar, Da/Han, and SD rats, dosed two weeks after surgery with increasing doses of genistein (25, 50 and 100 mg/kg/day), p-tert-octylphenol (5, 50 and 200 mg/kg/day), bisphenol A (0, 5, 50 and 200 mg/kg/day), and as a positive control 100 µg/kg EE, there was a strong stimulation by EE in uterine weight in the Wistar and Da/Han rats and less pronounced response in the SD rats (Diel et al., 2001). All 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. No doses of bisphenol A applied stimulated uterine wet weight in Wistar or Sprague Dawley rats, whereas in the Da/Han rats a slight stimulation was detected in the highest dose (200 mg/kg BW). These studies demonstrated a strain- and chemical-specific sensitivity in the uterotrophic assay with SD rats less sensitive than DA/Han rats to EE and BPA. While Wistar rats were more sensitive than SD to EE, both Wistar and SD rats were resistant to BPA.

Similarly, EE (1,3,10 or 30 µg/kg/day), DES (0.5, 1.5, 5 and 15 µg/kg/day) and a weak phytoestrogen, coumestrol (CE) (10, 35, 75 and 150 mg/kg/day), as positive controls, produced increases in uterine weight in SD and F344 rats (McKim et al., 2001). However, in response to the chemical D4 (0,10,50,100, 250, 500 and 1000 mg/kg/day), the maximal uterine weight was increased 160% relative to control values in the SD rats and only 86% relative to control values in F344 rats. Thus, SD rats were more sensitive to the effects of octamethylcyclotetrasiloxane (D4) in the uterotrophic assay. McKim et

al. (2001) suggested that the metabolism of D4 may be slower in SD versus F344 rats based on pharmacokinetic data.

In a study by Christian et al. (1998), the uterotrophic assay was compared across three rat strains, Wistar-Chbb:THOM-SPF, and Wistar-CRL:(WI)BR, and SD (Charles River). When administered DES, all three strains exhibited a positive uterine response, with a similar response in both Wistar strains and a slightly lower response in SD rats. Variability of responses was associated with background spontaneous incidences of abnormally high relative uterine weights possibly due to fluctuations in estrogen occurring between pnd 21 to 25. Due to differences in control mean uterine weights between rat strains (i.e. means were lower in Wistar-Chbb:THM-SPF versus Wistar-CRL and SD), criteria for biological outliers were different in Wistar-Chbb:THOM-SPF ($\geq$ 0.15%) versus Wistar-CRL and SD rats ($\geq$ 0.20%). Christian et al. (1998) demonstrated the importance of historical control data in the determination of statistically significant effects in subsequent studies.

Sometimes genetic differences have no observable effect on an endocrine endpoint. A study by Odum et al., 1999a, investigated the effects of *p*-Nonylphenol (NP) (0 to 250 mg/kg/day orally for 3 or 11 days; and 0 to 7.12 mg/kg/day via mini-pumps implanted s.c., for 11 days) in the uterotrophic assay in Alderley Park (Wistar-derived) and SD rats. Results were similar in both strains with a positive response to both DES (0.01 mg/kg/day) and NP (250 mg/kg/day), which were of the same magnitude as in previous studies performed in Noble rats (Odum et al., 1999b). The uterotrophic effects of NP and DES were found to be independent of rat strain.

3.0 Interspecies Similarities and Differences in Endocrine Endpoints

Few studies have been conducted in a single laboratory comparing the effects of endocrine-disrupting chemicals in more than one strain within a species, and even fewer studies have been conducted in a single laboratory comparing the effects of endocrine-disrupting chemicals in more than one species. Therefore the criterion that only studies performed in the same laboratory across species would be included in this white paper could not be applied. One review paper which looked at many studies on many chemicals across many laboratories in mice versus rats was included as well as other studies comparing endocrine endpoints across species in response to endocrine-disrupting chemicals.

There are many reviews on species differences in reproductive and developmental toxicology studies. Among these is a comparison of reproductive organ weights, sperm parameters, and vaginal cytology from fifty 13-week studies involving 24 chemicals in seven different laboratories (and four routes of exposure for the National Toxicology Program in $B6C3F_1$ mice and F344 rats (Morrissey et al., 1988). Considerable interlaboratory variability was demonstrated, but overall, it was concluded that there were no differences in types of sperm head abnormalities between control and treated rats and mice, and that testis, epididymis, and cauda epididymis weights and sperm motility were the most statistically powerful endpoints evaluated. Of all the

chemicals tested, only one, methylphenidate in the rat, produced an increase in abnormal sperm without effects on any other male endpoint. The agreement in results in these endocrine endpoints in response to reproductive toxicants between rats and mice was about 58%. A combination of confounding factors and species differences may have accounted for disparity in toxicological data.

Several reports have focused on differences in endocrine endpoints between rats and mice. In the uterotrophic assay, the estrogenic activity of parabens was assessed in B6D2F1 mice and Wistar rats (Hossaini et al., 2000). The parabens tested were methyl-, ethyl-, propyl- and butyl *p*-hydroxybenzoate, and *p*-hydroxybenzoic acid, which were administered either orally or subcutaneously for three days at doses up to 1000 mg/kg/day, and E2 (0.1 mg/kg) was used as a positive control. In the mouse uterotrophic assay, there was no significant effect on uterus weight in doses up to 1000 mg/kg/day for all parabens. In the rat uterotrophic assay, 600 mg/kg/day of butyl-paraben produced a positive response. Thus the estrogenic activity of parabens was found to be weak in rats and was not observed in mice.

Two separate studies reported species differences in the ovarian toxicity of reproductive toxicants. In one study (Doerr et al., 1996), 1,3-butadiene epoxides (butadiene monoepoxide, BMO; and butadiene diepoxide, BDE) were administered at doses up to 1.43 mmol/kg/day intraperitoneally for 30 days, to young SD rats and B6C3F1 mice. BMO was ovotoxic in mice, producing decreases in follicle counts and reproductive organ weights, with no effects in rats at the doses tested. BDE was ovotoxic in both rats and mice, with a greater sensitivity to BDE in mice, resulting in reductions in uterine and ovarian weights in mice at lower concentration than in rats. In addition, follicle counts were greatly reduced in mice at lower doses of BDE than in rats. The authors speculate that metabolic differences affecting the conversion of BMO to BDE may be responsible. In another study by Takizawa et al., 1985, intraovarian injection of increasing concentrations of benzo(*a*)pyrene, a polycyclic aromatic hydrocarbon, reduction in small oocytes occurred in a dose-dependent fashion at doses ranging from 0.01 to 30 µg/ovary in C57BL/6N and DBA/2N mice and 0.8 to 240 µg/ovary in SD rats. Thus effects of benzo(a)pyrene on small oocyte number were present in both SD rats and two strains of mice.

Cadmium has also been found to induce ovarian toxicity in animals. In a study by Rehm and Waalkes (1988), the effects of cadmium were assessed in immature and mature female Syrian hamsters, four mouse strains (BALB/cAnNCr, DBA/2NCr, C57BL/6NCr, NFS/NCr) and two rat strains [F344 and Wistar-Furth (WF)] after a single injection (sc) of a dose ranging from 20 to 47.5 µmol/kg, and the reproductive tracts were examined by light microscopy. Syrian hamsters were the most sensitive to cadmium-induced ovarian hemorrhagic necrosis, in particular, shortly before ovulation. In mice, only the DBA/2NCr strain showed significant cadmium-induced ovarian hemorrhagic necrosis, and uterine lesions in any of the mouse strains were rare. Though rats showed dose and age-dependent toxicity of the ovaries, uterus, cervix, and liver, cadmium induced uterine lesions only in mature F344 rats, not WF rats. Thus species and strain differences in cadmium-induced reproductive toxicity were reported.

As female mice were more sensitive than female rats to the ovotoxic effects of 1,3-butadiene (Doerr et a., 1996), male mice were found to be more sensitive to the reproductive effects of 1,3-butadiene, while the same doses produced no effects in rats. Anderson et al. (1998) compared the effects of exposure by inhalation to 1,3-butadiene (up to 1250 ppm in rats and up to 130 ppm in mice) for 10 weeks prior to mating, in male CD-1 mice and male SD rats. Exposure in mice resulted in F_1 abnormalities and increases in early deaths. None of these effects were observed at the same exposure concentrations in SD rats.

In an earlier study by Brinkworth, Anderson, and McLean (1992), dietary restriction in CD-1 mice was found to increase abnormal sperm which may have been related to a decrease in calories, and to decrease epididymal sperm counts, which may have been due to a lack of protein. In CD rats, dietary restriction only reduced epididymal sperm count. Thus dietary changes had different impacts on spermatogenesis in mice compared to rats.

A differential sensitivity to the developmental toxicity of BPA was reported by Morrissey et al. (1987) in CD-1 mice compared to CD rats. Mice and rats were dosed daily from gd 6 to 15, with 0-640 mg/kg/day BPA (rats) and 0-1250 mg/kg/day BPA (mice). Maternal toxicity (as evidenced by significant decreases in maternal weight gain) occurred in rats at $\geq$ 160 mg/kg/day and at a much higher dose (1250 mg/kg/day) in mice. No fetal toxicity occurred at doses up to 640 mg/kg/day in rats, and in mice, at 1250 mg/kg/day, there were significant increases in the number of resorptions, and a decreases in average fetal body weight. Maternal mice were more sensitive to toxic doses of BPA than maternal rats, and fetal malformations were not observed in either rats or mice.

Species differences in endocrine receptor binding characteristics have been reported in *in vitro* experiments. Mathews et al. (2000) examined the ability of several natural and synthetic chemicals to compete with ^{3}H-E2 for binding to bacterially expressed estrogen receptor alpha D, E and F domains from five different species (human, mouse, chicken, anole, and rainbow trout) fused to glutathione S-transferase (GST). While all of these fusion proteins displayed high affinity for E2 (Kd of 0.3 to 0.9 nM), species differed in affinity of binding other estrogenic chemicals. With, for example, rainbow trout, ER fusion proteins showing much higher relative binding affinity for alpha-zearalenol, Bisphenol A, octylphenol, o,p' DDT, Methoxychlor, p,p'-DDT, o,p'-DDE, p,p'-DDE, alpha-endosulfan and dieldrin than ERs from the human, mouse, chicken and green anole (Matthews, Celius et al. 2000). Thus sequence variation in the ER ligand binding domain between strains and between species could be a major source of variation in EDSP assays, in evaluating compound which act via the ER.

4.0 Summary and Conclusions of Intraspecies and Interspecies Similarities and Differences in Endocrine Endpoints and Conclusions

Endocrine-mediated toxicity of chemicals varies among strains of animals of the same species and among different species. Endocrine endpoints vary in sensitivity to

chemicals across strains and species. The sensitivity to endocrine-active chemicals is obviously dependent on the endpoint evaluated, the chemical administered, and the genotype of the animal model. Genetic variability which exists within outbred strains, between inbred and outbred strains as well as in the animal and human populations to which the results will be applied has the potential to confound the detection and interpretation of reproductive toxicity. The following table summarizes strain-related similarities and differences in endocrine endpoints in many key research articles designed to investigate the effects of endocrine disruptors. The focus of the comparison is on rat strains, with some examples in mouse strains.

Table 2. Intraspecies Comparisons of Endocrine Endpoints

Endocrine Endpoints	Strains	Chemical	Similarities	Differences	Key References
Uterine Weight	Wistar, SD, Da/Han	Ethinyl Estradiol (EE), BPA	Wistar and SD less sensitive to BPA than Da/Han	SD less sensitive than Da/Han to EE and BPA, Wistar more sensitive than SD to EE.	[1]Diel et al., 2001
	Alderley Park, SD	NP	Results were similar in both strains with a positive response to NP (250 mg/kg/day), which were of the same magnitude as previous studies performed in Noble rats (Odum et al., 1999b)	250 mg/kg NP resulted in slightly greater uterotrophic response in Alderlery Park (1.84-fold) than in SD (1.55-fold).	[2]Odum et a., 1999a
	SD, F344	E2, BPA	Both strains showed increased uterine weight in response to E2	F344 more sensitive to BPA and E2	[3]Steinmetz et al., 1998
	SD, F344	EE, DES and octamethylcyclotetrasiloxane (D4)	EE, DES and D4 produced similar positive uterine response in SD and F344 rats.	Maximal uterine response to D4 was two-fold higher in SD than F344 rats. F344 more sensitive to EE.	[4]McKim et al., 2001
	Wistar, SD	DES	SD and Wistar(CRL) had similar control values for uterine weight; similar uterotrophic response in both Wistar strains.	Wistar(Chbb-THOM-SPF) had lower mean uterine weights in controls versus SD and Wistar(CRL), less variability in response to DES	[5]Christian et al., 1998
	SD, F344	E2,4-OH tamoxifen (4-OHT)	F344 and SD showed similar uterine weight responses to E2	F344 more sensitive than SD to E2 induced uterine epithelial cell height. SD more sensitive than F344 to induction of uterine weight and epithelial cell height by 4-OHT	[6]Bailey et al., 2002

Table 2. (continued)

Endocrine Endpoints	Strains	Chemical	Similarities	Differences	Key References
Male and female sexual development (AGD, PPS, VO)	SD, LE	p,p´-DDE; flutamide	PPS same time in LE and SD controls; p,p´-DDE had no effect on PPS or VO in either strain; flutamide decreased AGD and caused nipple retention and changes in male reproductive organ weights in both strains	p,p´-DDE produced significant decrease in AGD in male LE rats, not in SD; p,p´-DDE produced nipple retention at a lower dose in SD than LE; VO earlier in LE controls versus SD controls	[7]You et al., 1998
	SD, F344	β-E2-3-benzoate	neonatal exposure to 1.5 mg/kg/day decreased male reproductive organ weights in both F344 and SD males (pnd 90), and delayed PPS	At pnd 90, in rats exposed to 1.5 mg/kg/day, there were greater reductions in reproductive organ weights in F344 rats than in SD rats (greater responsiveness in F344, higher sensitivity in SD)	[8]Putz et al., 2001
Female reproductive tract	SD, Alderley Park	BPA	There was no effect on age of first estrus in either strain.	VO delayed in Alderley Park, but not in SD rats	[9]Tinwell et al., 2002
	F344, SD	BPA	Metabolic clearance of BPA is same	BPA increased DNA synthesis and cell proliferation in the vaginal epithelium of F344 rats but not of SD rats	[10]Long et al., 2000
	LE, Holtzmann	TCDD	1 µg/kg on gd 15 produced malformations of F1 offspring female external genitalia and increased UVD in both strains.		[11]Gray and Ostby, 1995

Table 2. (continued)

Endocrine Endpoints	Strains	Chemical	Similarities	Differences	Key References
Male reproductive tract/andrology	F344, ACI, Lewis, SD, Wistar	3,2'-dimethyl 4-amino-biphenyl (DMAB)		tumorigenic response in the prostate of F344, ACI, Lewis, CD and Wistar rats to DMAB was more sensitive in F344>ACI>Lewis>CD and Wistar rats were insensitive	[12]Shirai et al., 1990
	LE, SD	p,p´-DDE	In both SD and LE rats, there were increases in liver weight, and in E2; decreases in T4; and no change in Prl or LH	In SD rats there was a decrease in epididymis weight, no change in T, DHT, or TSH, and a decrease in FSH. In LE rats, there was an increase in epididymis weight, increases in T, DHT and TSH, and no change in FSH.	[13]O'Connor et al., 1999
	LE, Wistar	Vinclozolin	200 mg/kg/day from gd 14 to pnd 3 produced malformations of male external genitalia, nipple retention lasting into adulthood, and increased inflammation of epididymides, prostate, seminal vesicles and coagulating glands in both strains. 12 mg/kg produced transient retention of nipples/areolae in preweanling males of both strains. Similar NOAEL in both strains, 12 and 6 mg/kg bod wt. In Wistar and LE respectively.	200 mg/kg/day from gd 14 to pnd 3 reduced maternal body weights and pup weights in Wistar but not LE. Chronic inflammation of urinary bladder, and testis atrophy were noted in LE and not Wistar. At 12 mg/kg/day, nipple/areolae retention persisted into adulthood in LE, not in Wistar. Adult offspringLE also had reduced prostate, seminal vesicle, and coagulating gland weights; these effects were not seen in Wistar adult offspring..	[14]Hellwig et al., 2000

Table 2. (continued)

Endocrine Endpoints	Strains	Chemical	Similarities	Differences	Key References
	AP, SD	BPA	No difference in PPS .	50 mg/Kg BPA decreased total sperm count and daily sperm production in AP (Wistar-derived) rats but not SD.	[15]Tinwell et al., 2000
	Wistar, SD and Dark Agouti (DA)	none		DA exhibited lower absolute testis weight than the other two strains, no difference in sperm count among the three strains.	[16]Wilkinson et al., 2000
	SD versus other strains (review)	lead		SD more sensitive than other strains to testicular toxicity of lead.	[17]Apostoli et al., 1998
	F344, SD	Neonatal E2-3-Benzo-ate (EB)		F344 more sensitive and responsive than SD to reduction in TW, Epididymis, Seminal Vessicle and Coagulating gland weights.	[18]Putz et al., 2001

Table 2. (continued)

Endocrine Endpoints	Strains	Chemical	Similarities	Differences	Key References
Estrous cycle/ ovulation	F344, LE, SD	diet (feed restriction)		Prior to food restriction, Brown Norway rats had irregular estrous cycle patterns while SD, LE and F344 rats had regular estrous cycle patterns. By day 5 of food restriction, 75% of F344 rats and 100% of Brown Norway rats stopped cycling and SD and LE rats were unaffected	[19]Tropp et al., 2001
	Donryu, F344	none		In aging Donryu rats, estrous cycle abnormalities (e.g. persistent estrus) were more common than in F344 rats	[20]Ando-Lu et al., 1998
	LE, SD	atrazine		LE rats were more sensitive than SD rats to atrazine-induced disruption of the ovarian cycle.	[21]Cooper et al., 2000
	SD, F344	atrazine		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, decreased P, and increased incidence of mam-mary tumors in SD rats only, with no effect in F344 rats.	[22]Eldridge et al. (1994)

Table 2. (continued)

Endocrine Endpoints	Strains	Chemical	Similarities	Differences	Key References
Gonadal/ Pituitary Hormone levels	Han/Wistar, SD, LE	TCDD, DES	gestational exposure to DES (100 µg/kg in Han/Wistar rats; 100, 200 or 300 µg/kg in SD rats) significantly decreased prenatal T production in SD and Han/Wistar male rats	In 19.5 day old male rat fetuses, gestational exposure to TCDD (0, 0.5, 0.1, 0.5 or 1.0 µg/kg) was associated with increases in prenatal T and pituitary LH production in Han/Wistar but not LE rats.	[23]Haavisto et al., 2001
	SD, LE	atrazine		LH and Prl were suppressed in LE but not SD rats after 1 and 3 doses of atrazine.	[24]Cooper et al., 2000
	SD, F344	E2, BPA	After 21 doses of atrazine LH and Prl were suppressed in SD and LE rats. BPA had no effect on pituitary weight in either strain.	After 3 days of E2 exposure, Prl was increased 10X in F344, only 3X in SD; increased pituitary weight in F344, not in SD. BPA increased Prl 7-8X in F344, no effect on SD rats.	[25]Steinmetz et al., 1997
	SD, LE, Holtzman, F344	atrazine		Serum E2 increased by atrazine in SD rats, Only Holtzman strain with decreased P levels. Control and treated levels of E2 much lower and non-detectable in Dirunal F344 rats. Control levels of E2 higher nocturnally in LE rats. Diurnal atrazine reduced LH in Holtzman and LE but not in SD or F344. Nocturnal atrazine reduced LH in LE and F344 but not in SD or Holtzman.	[26]Cummings et al, 2000

Table 2. (continued)

Endocrine Endpoints	Strains	Chemical	Similarities	Differences	Key References
Thyroid hormone levels	LE, Han/Wistar	TCDD	T_3 levels in controls were comparable	T_4 and TSH levels were higher in Han/Wistar controls; TCDD produced a greater reduction in T_4 levels in LE than in Han/Wistar rats.	[27]Pohjanvirta et al., 1989
	SD, F344	TRH, TSH		Challenge with TSH and TRH increases levels of T_3 and T_4 in F344 rats, and increased T_4 only in SD rats.	[28]Fail et al., 1999
Fertility/ reproductive parameters	SD, LE, Holtzman, F344	atrazine	no effect of atrazine on pre- or post-implantation loss in SD and LE rats	Increase in % preimplantation loss in F344; SD and LE not affected. Increase in % postimplantation loss only in Holtzman rats only.	[29]Cummings et al., 2000
	SD, LE, F344	atrazine	full litter resorption at highest dose (200 mg/kg/day) in all three strains.	At lower doses (50 and 100 mg/kd/day) pregnancy loss in F344, not in SD or LE	[30]Narotsky et al., 2001
	F344, SD	BDCM	75 mg/kg/day significantly reduced body weights in both strains	75 mg/kg/day BDCM produced 62% litter resorption in F344, no effect on SD	[31]Bielmeier et al., 2001
	Wistar Hannover, CD	none		Control Wistar Hannover have lower pregnancy rates and litter sizes; higher % pre- and post-implantation loss and resorptions versus control CD®.	[32]Liberati et al., 2002

Table 2. (continued)

Endocrine Endpoints	Strains	Chemical	Similarities	Differences	Key References
organ weights, histopath-ology (rats)	SD, F344	E2		In ovariectomized rats exposed to E2 (silastic implants) for 10 or 20 days, pituitary weights and Prl levels were dramatically increased in F344, minimal effects in SD rats.	[33]Schechter et al., 1987
	Brown-Norway, F344, Wistar, Donryu	E2	No difference in pituitary weights of Wistar and Donryu rats, after E2 exposure.	In ovariectomized rats, control pituitary gland weights were the lowest in Brown-Norway > Wistar rats; After E2 treatment (10 mg s.c. pellet for 4 weeks), there was a significant > 3 fold increase in pituitary weights in F344 rats, a significant >0.5 fold increase in pituitary weights in Brown-Norway rats	[34]Yin et al., 2001
	F344, BN, SD	DES		F344 sensitive to DES-induced pituitary tumors; SD and BN resistant to effects of DES on pituitary gland.	[35]Wendell et al., 1996, 1997, 1998, 2000; Chun et al., 1998
	F344, WF	Cadmium		Cadmium-induced toxicity of the ovaries, uterus, cervix, and liver, cadmium induced uterine lesions in F344 not WF	[36]Rehm and Waalkes, 1988

Table 2. (continued)

Endocrine Endpoints	Strains	Chemical	Similarities	Differences	Key References
Organ weights, histopathology spermatogen-esis (mice)	Jcl:ICR and CD-1 mice	di-2-ethylhexyl-phthalate (DEHP)	Increase in liver weight, both strains	DEHP decreased testicular weight in CD-1 mice not Jcl:ICR	[37]Oishi et al., 1993
	C57BL/6N and ICR mice	BPA, E2	BPA did not affect male reproductive organ weights during any dose/exposure period	E2 (10 µg/kg from pnd 27 to 48, as a positive control) produced significant decreases in absolute and relative testes, epididymides, and seminal vesicle weights compared to controls in C57BL/6N mice, while ICR mice were unaffected.	[38]Nagao et al., 2002
	C57BL/6J (B6), CD-1, C17/Jls, and S15	E2		B6 and C17/Jls were sensitive to E2 showing a maximal suppression of testis weight and spermatogenesis even at the lowest dose of E2 (2.5 µg), with no effect on testis weight or spermatogenesis in CD-1 or S15 up to 10 µg E2.	[39]Spearow et al., 1999; 2001
	BALB/cAnNCrDBA/2NCr, C57BL/6NCr, NFS/NCr	Cadmium		cadmium-induced ovarian hemorrhagic necrosis only observed in DBA/2NCr mice	[40]Rehm and Waalkes, 1988

[1-40]Footnotes are used for the identification of references in Table 3

Thus after conducting this literature review, strain-related differences in effects on endocrine-mediated endpoints in response to a wide variety of endocrine-disrupting chemicals was obvious. The sensitivities of various strains to chemicals producing effects on various endocrine endpoints are summarized in Table 3. Since effects on many of the endpoints were also chemical specific, the chemicals are also included in Table 3.

Table 3 Summary of Agent- and Endpoint-Specific Intraspecies Differences

Endocrine Endpoint	Chemical	Sensitive* Strains	Less Sensitive/ Insensitive Strains	References (from Table 2)
Uterine Weight	EE	Wistar, Da/Han	SD	1
	BPA	Da/Han	Wistar, SD	1
	NP	AP>SD		2
	EE, DES	SD, F344		3
	D4	SD	F344	4
	E2	SD≈F344		6
	tamoxifen	SD	F344	6
AGD	p,p'-DDE	LE	SD	7
	flutamide	SD, LE		7
Nipple retention	p,p'-DDE	SD	LE	7
	flutamide	SD, LE		7
	vinclozolin	LE > Wistar		14
PPS	E2	F344, SD		8
	p,p'-DDE		SD, LE	7
VO	p,p'-DDE		SD, LE	7
	BPA	AP	SD	9
Male reproductive organ wts.	flutamide	LE, SD		7
	E2	F344, SD		8
	low dose E2	SD	F344	8
	vinclozolin	LE	Wistar	14
	BPA		C57BL/6N, ICR	38
	E2	C57BL/6N		38
	E2	B6, C17/Jls	ICR, CD-1, S15	39
	DEHP	CD-1	Jcl:ICR	37

Table 3. (continued)

Endocrine Endpoint	Chemical	Sensitive* Strains	Less Sensitive/ Insensitive Strains	References (from Table 2)
Estrous cycle/ovulat-ion	feed restriction	F344, BN	SD, LE	18
	atrazine	LE	SD	21
	atrazine	SD	F344	22
Fertility/gesta-tional effects	atrazine	Holtzman	F344, SD, LE	29
	atrazine	F344	SD, LE	30
	BDCM	F344	SD	31
Andrology	BPA	AP	SD	15
	lead	SD		17
	E2	B6, C17/Jls	CD-1, S15	39
Hormone Levels	p,p'DDE	SD (*FSH, E2, T_4*)	LE (*FSH, Prl, LH*)	13
Hormone Levels (continued)	p,p'DDE	LE (*E2, T_4, T, DHT, TSH*)	SD (*Prl, LH, T, DHT, TSH*)	13
	E2	SD (*Prl*)	F344 (*P*rl)	22
	TCDD	Han/Wistar (*T, LH*)	LE (*T, LH*)	23
	atrazine	LE (*LH, Prl*)	SD (*LH, Prl*)	24
	atrazine	Holtzman (*P*)	SD (E2, *P*)	26
	E2	F344 (*Prl*)	SD *(Prl*)	25
	BPA	F344 (*Prl*)	SD (*Prl*)	25
	TCDD	LE (T_4)		27
	TSH, TRH	SD, F344 (T_4)	SD (T_3)	28
	TSH, TRH	F344 (T_3)		28
Pituitary Weights	E2	F344	SD	33
	E2	F344>BN	Wistar, Donryu	34
	DES	F344	SD, BN	35
Histopath-ology (reproduct-ive organs)	BPA	F344 (females)	SD (females)	10
	DMAB	F344>ACI>Lewis>CD (males)	Wistar (males)	12
	vinclozolin	LE (males)	Wistar (males)	14

Table 3. (continued)

Endocrine Endpoint	Chemical	Sensitive* Strains	Less Sensitive/ Insensitive Strains	References (from Table 2)
	cadmium	F344 (females)	WF (cadmium)	40
Histopath-ology (continued)	atrazine	SD (females)	F344 (females)	22

*"Sensitive" refers to a greater response, and may not reflect dose level

Table 3 summarizes the intraspecies comparisons of the effects of endocrine-disrupting chemicals on endocrine endpoints in EDSP assays. 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. Less sensitive strains were F344 in response to D4, and Wistar in response to BPA. LE and SD rats were differentially sensitive to the effects of flutamide and p p'-DDE on AGD and nipple retention. SD and F344 rats showed sensitivity to PPS in response to E2, while SD and LE were insensitive to p,p'-DDE. AP rats were sensitive to BPA on VO, while SD and LE rats were less sensitive to BPA. Sensitivity to the effects on male reproductive organ weights was found in most rat strains and chemicals studies except for F344 (low dose E2), and Wistar (vinclozolin). Different strains of mice were differentially sensitive to the effects of E2 on male reproductive organ weights and andrology. Different strains of rats were differentially sensitive to the effects of BPA, atrazine, BDCM, p,p'-DDE, TCDD, E2, lead, cadmium, DES, vinclozolin, and DMAB, on estrous cycle, fertility, pregnancy loss, andrology, hormone levels and histopathology of the reproductive organs. Pituitary weights were affected in F344 rats after exposure to DES and E2 while in other strains pituitary weights were unaffected. Overall, there was no one strain which was sensitive to all of the endocrine-disrupting chemicals at most of the endocrine endpoints. Effects on endocrine endpoints were dependent on strain and chemical, and effects on strains were dependent on the endpoints and chemicals. There were no clear patterns indicating the optimal strain for detection of effects due to the majority of endocrine-active compounds tested. In addition, it was not clear that inbred strains or outbred strains would be the better choice in species/strain selection. In selection of the appropriate species/strain for EDSP assays, it may be important to consider the endocrine endpoints assessed and the test chemical employed. Obviously, it would be more thorough to conduct multi-strain assays to increase to chances of detecting endocrine effects, but extremely difficult to determine which strains should be chosen considering the considerable variation across strains, even under the same experimental conditions (which would minimize confounders).

The five example assays under consideration by the EDSP (Table 1) were compared against strain sensitivities (Table 3). In the one-generation, two-generation, and *in utero* lacational assays, a similar array of endpoints are assessed, from gestational indices, reproductive development, onset of puberty, estrous cyclicity, hormone levels, andrology, organ weights, and histopathology. Selecting a rat strain that is sensitive at the greatest number of endpoints is difficult, due to the different strain sensitivities observed with different chemicals. For example, if selecting one chemical, in this case, *p,p′*DDE, SD rats are sensitive at about half of the endpoints, and LE rats are sensitive at the other half of the endpoints. 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. Uterine weight, as assessed in the uterotrophic assay, which is currently in the proposed *in utero* lactational assay, is an endpoint which appears relatively sensitive across strains. In a review of uterotrophic assays performed in 19 different laboratories and two model systems (ovariectomized and immature rats), the response to EE was robust, reproducible and sensitive across laboratories, regardless of differences in strain, diet, bedding, housing, and vehicle (Kanno et al., 2001). 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, the F344 has a small litter size, thus reducing the number of animals available for multiple evaluations, and has a high incidence of spontaneous testicular tumors, which may confound potential effects on male reproductive organs. Both the adult male and pubertal male and female assays include assessments of hormone levels. Chemical effects on hormone levels were highly strain dependent, with no one strain insensitive to changes in every hormone level. With inclusion of enough different hormone level determinations, the possibility detecting a chemical-induced change in one strain would be enhanced. Inclusion of an additional rat strain (e.g. the LE rat) in the pre-validation of EDSP assays may provide more information on the strain sensitivity of the various endpoints and assays, in addition to providing more flexibility to laboratories in selecting strains for performance of the assays.

Since interspecies comparisons performed under the same experimental conditions are few, and strain is obviously also a confounder in comparing species-related differences in sensitivity to endocrine-disrupting chemicals, conclusions about comparisons are limited. There are differences in the response of the rat and mouse reproductive tracts to various reproductive toxicants. In some cases, mice appear more sensitive and in other cases rats are more sensitive. In a comparison of reproductive organ weights, sperm parameters, and vaginal cytology from fifty 13-week studies involving 24 chemicals in seven different laboratories (and four routes of exposure) for the National Toxicology Program in $B6C3F_1$ mice and F344 rats (Morrissey et al., 1988), there was considerable interlaboratory variability due to confounding factors (such as different suppliers and environmental conditions) resulting in only a 58% agreement in endocrine endpoints (i.e. either no response in either species, or at least one endpoint affected in both species) in response to reproductive toxicants. It is possible that EDSP assays involving only one species and strain, namely the SD rat, may not detect effects in endocrine endpoints that occur in mice (and vice versa). However, 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. Since we cannot identify the most relevant or sensitive animal model with the existing data, because the sensitivity depends on the endpoint(s) chosen, the chemicals evaluated, the timing, duration and route of exposure, the dose levels, and is confounded by the varying genotype by environment interactions, the main issue is whether to use inbred versus outbred strains. Inbred strains are homogeneous at all loci, and have a limited range of responses (less variability, but an effect may be missed), though using several genetically-defined inbred strains in endocrine screens may provide a wider spectrum of responsivity. If selecting a single strain for endocrine 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.

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