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TITLE: Validation of the *In Utero*/Lactational Exposure Screening Protocol With Methoxychlor

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PROPOSED STUDY IN-LIFE DATES: _____

AMENDMENTS:

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1.0 OBJECTIVE AND BACKGROUND

Concern has been expressed that the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) Tier 1 screening battery does not include an assay which exposes animals during perinatal life, during which the fetus/neonate is exquisitely sensitive to endocrine disruption. The objective of this assay is to detect reproductive and developmental effects in male and female rat offspring mediated by alterations in the estrogen, androgen, and thyroid (EAT) signaling pathways, resulting from exposure to the dam during gestation and lactation or from direct exposure to the offspring from weaning through puberty. It may be used to: (1) replace a number of protocols recommended by EDSTAC for the Tier 1 screening battery, (2) serve as a follow-up test for certain chemicals for which a full multigeneration test had been run prior to the upgrading of the protocol in 1998, and/or (3) augment the current developmental toxicity testing protocol. The endpoints were selected for their potential to respond to EAT-induced alterations of development and include those that are both sensitive to disruption and can be easily detected in the offspring.

The Food Quality Protection Act of 1996 and the Safe Drinking Water Act of 1996 required the EPA to develop and implement a screening program for determining the potential in humans for estrogenic (and anti-estrogenic) effects from pesticides. This program has been expanded on the advice of the EDSTAC to include androgenic (and anti-androgenic) effects and effects from thyroid-hormone (TH)-like (or anti-TH) substances.

The EDSTAC, assembled by the EPA in 1996, believed, to the best of its knowledge, that the recommended Tier 1 screening battery, if validated, would have the necessary breadth and depth to detect any currently known disruptors of EAT hormones. There was concern, however, that chemical substances or mixtures could produce effects from prenatal/prehatch exposure that would not be detected from pubertal or adult exposure (EDSTAC, 1998, Vol. 1, Chapter 5). Furthermore, there were differing views with the EDSTAC about whether there is scientific evidence of known endocrine disruptors or reproductive toxicants that can affect the prenatal stage of development without affecting the adult or prematuration stages, and whether effective doses and affected endpoints may differ among the three life stages.

The EDSTAC therefore recommended that EPA take affirmative steps, in collaboration with industry and other interested parties, to attempt to develop a protocol for a full life cycle (i.e., with embryonic exposure and evaluation of the adult offspring) developmental exposure screening assay that can be subjected to validation and standardization. The EDSTAC believed such an assay must involve prenatal or prehatch exposure and retention of offspring through puberty to adulthood and provide structural, functional, and reproductive assessment.

The EDSTAC recognized the difficulty in developing a developmental exposure screening assay that meets both the criteria specified above and the more general criteria for selecting T1S assays set forth in Chapter 3 of the EDSTAC report. However, the EDSTAC believed it is worth the effort. Furthermore, the EDSTAC provided brief protocols for *in utero* and *in ovo*

developmental screening assays that could be further evaluated for this purpose (an expanded discussion of an *in utero* protocol, similar to this EDSP assay, was included in EDSTAC report, 1998, Volume II, Appendix O).

Finally, the EDSTAC recommended that if such an assay were identified, validated, and standardized, the decision on whether it should be included in the T1S battery should include an evaluation of its potential to replace one or more of the recommended T1S assays and its overall impact to the cost effectiveness of the T1S battery (EDSTAC, 1998, Vol. 1, Chapter 5). 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 test, it is understood that modes of action, other than those of the estrogen, androgen, and thyroid-signalling 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. RTI, as the lead laboratory for this assay for the EDSP, is suggesting that if this protocol is implemented, it should be used in place of a number of protocols recommended by EDSTAC in the Tier 1 screening battery, such as the *in vitro* steroidogenesis and placental aromatase assays and the *in vivo* male Hershberger assay, the uterotrophic female assay, and either or both pubertal assays, as a "Tier 1.5" assay, or as a follow-up test for chemicals for which a full multigeneration test had been completed prior to the upgrading of the protocol in the OPPTS 1998 testing guidelines.

2.0 MATERIALS AND METHODS

2.1 Test Substance

Common Name:	Methoxychlor
Chemical Name:	Benzene, 1,1'-(2,2,2-trichloroethylidene)bis(4-methoxy-9C1)
Synonyms:	1,1,1-Trichloro-2,2-bis(4-methoxyphenyl)ethane; 1,1,1-Trichloro-2,2-bis(*p*-anisyl)ethane
CAS No.:	72-43-5
Molecular Formula:	C16H15Cl302
Molecular Weight:	345.65
Appearance:	Colorless crystals (technical, gray flaky powder)
Odor:	Slightly fruity

Melting Point:	87EC
Density/Specific Gravity:	1.41 @ 25EC
Solubility:	Readily soluble in aromatic, chlorinated, and ketonic solvents and vegetable oils
Vehicle:	Mazola® corn oil
Supplier:[a]	
Batch/Lot Number:[a]	
Purity:[a]	
Storage Conditions:[a]	

[a] All additional information on the test chemicals (e.g., supplier, batch/lot number, purity, storage conditions of bulk chemical and of dosing suspensions, etc.) will be added to the protocol by amendment.

2.2 Chemical Safety and Handling

See methoxychlor MSDS in Attachment.

2.3 Dose Formulation and Analysis

The dosing suspensions will be prepared at a frequency determined by stability tests performed prior to the start of the study. Suspensions will be prepared at Battelle Chemical Repository, Sequim, WA, and stored in wide-mouth, amber bottles. They will be shipped via 24-hour express delivery and logged into the RTI Materials Handling Facility prior to transfer to the Reproductive and Developmental Toxicology Laboratory for dosing. The test materials will be suspended in Mazola® corn oil (CAS No. 8001-30-7), with the concentration determined by the following formula:

$$\text{Concentration (mg/ml)} = \frac{\text{Dose (mg/kg/day)}}{\text{Dosage volume (5.0 ml/kg/day)}}$$

An aliquot of each dose level per formulation will be analyzed by Battelle. The dosing bottles will be identified at RTI by a five-digit, random number Rx code and a color code. Personnel, other than the Laboratory Supervisor, Project Toxicologist, and Study Director, will not be informed of the test chemicals or formulation concentrations until all laboratory work is completed (i.e., the study technicians will be "blind" for chemical and dose). Aliquots from the dosing bottles will be collected on the first day of dosing (gestation day [gd] 6) and on the first postnatal day [pnd] 0, and pnd 7, 14, and 21 and will be shipped to Battelle Chemical Repository, Sequim, WA, for analysis.

2.4 Animals

2.4.1 Species and Supplier

The proposed test animals will be the Sprague Dawley Derived Outbred Albino Rat Crl:CD®(SD) IGS BR supplied by Charles River Laboratories, Inc., Raleigh, NC.

2.4.2 Live Animals and Species Justification

The use of live animals has been requested by the Sponsor. Alternative test systems are not available for the assessment of effects of chemicals on reproduction and development in intact mammals for determining the potential risk for humans from endocrine-mediated effects of pesticides and other chemicals. The Charles River CD® rat has been the subject of choice on reproductive and developmental toxicology contracts at RTI since 1976, and has been used for other reproductive toxicology studies with this test material. Large historical data bases for reproductive performance and prevalence of spontaneous malformations in control rats are available from studies conducted at RTI (currently based on over 300 control litters) as well as from the supplier (Charles River, 1988). This strain of rat has been proven to have robust fertility and fecundity, and does not present any unusual endocrinological patterns. This study does not unnecessarily duplicate any previous study.

2.4.3 Total Number, Age, and Weight

Number of Females:	100 (nulliparous females procured specifically for this study)	
Number of Males:	110 (breeding stock used for multiple studies on this contract)	
Age on Receipt:	Females: ~8-10 weeks	Males: ~10-12 weeks
Female Wt. Range:	200-275 g on gd 0	

The number of animals assigned to each dose group is based on the breeding efficiency of the colony and the Sponsor's requirement for at least ten confirmed-pregnant animals per dose group. Minimum sample size requirements for statistical comparison of data among treatment groups were also considered in determining the number of animals assigned to each dose group. It is anticipated that 60 timed-mated, sperm-positive females (15 per group) will be used in this study.

2.4.4 Quality Control

The shipment of nulliparous, virgin females will be quarantined on arrival, and quality control evaluation will be initiated within one day after receipt. Within one day after receipt, five female rats will be chosen from the shipment, sacrificed, and blood collected for assessment of viral antibody status. Heat-inactivated serum will be sent to BioReliance (Rockville, MD) for their Level 1 rat antibody screen. The viral screen will consist of evaluation for the presence of antibodies against the following: Toolan H-1 virus (H-1), Sendai virus, Pneumonia virus of mice (PVM), rat coronavirus/sialodacryoadenitis (RCV/SDA), Kilham rat virus (KRV), CAR Bacillus, and Mycoplasma pulmonis (*M. Pul*.). In addition, fecal samples from representative animals will be externally examined for intestinal parasites.

2.4.5 Sentinels

After the assignment of F0 dams to treatment groups, four sperm-negative female rats will be arbitrarily selected, eartagged, and designated as sentinels (see Section 2.5.5, Breeding). They will be singly housed in the study room(s), with feed and water available *ad libitum* (as described below). They will be examined once daily by cageside observation for morbidity or mortality at the same time as the clinical observations or morbidity/mortality checks for the study animals. The clinical condition of sentinel animals will be recorded only in the event that an animal is moribund or found dead. If a sentinel animal is terminated moribund, blood will be collected at termination and serum samples frozen. During the F0 female necropsies and the last necropsy of the F1 offspring, the surviving sentinel females (two/time) will be terminated, blood samples collected, and serum samples prepared. All sentinel serum samples will be submitted to BioReliance (Rockville, MD) for serological evaluation (see above section on Quality Control).

2.4.6 Quarantine

Upon receipt, animals will be quarantined for seven days, with the prior concurrence of the RTI Animal Research Facility (ARF) veterinarian. They will be observed daily for general health status and ability to adapt to ARF husbandry conditions. They will be released from quarantine, if suitable for use (based on QC results), by the attending ARF veterinarian or his designate.

2.5 Animal Husbandry

2.5.1 Housing, Feed, and Water

During the quarantine period, animals will be randomly assigned to cages. Sperm-positive females and F1 male and female postweanlings will be singly housed in solid-bottom polycarbonate cages (8"x19"x10.5") fitted with stainless steel wire lids (Laboratory Products, Rochelle Park, NJ). Sani-Chip® cage bedding (P.J. Murphy, Forest Products, Inc., Montville, NJ) will be used in all solid-bottom cages. Breeder males will be housed in suspended cages. Pelleted feed (No. 5002 Purina Certified Rodent Chow®) will be available *ad libitum* for the male

breeder rats, the F0 females during quarantine, the F0 females during the rest of gestation and lactation, and for the retained F1 males and females. Tap water from the Durham, NC, water system will be filtered and available *ad libitum* to breeder males and females during cohabitation via an automatic water delivery system (Edstrom Industries Inc., Waterford, WI). Water will be available in plastic bottles with stainless steel sipper tubes *ad libitum* to the F0 females during quarantine, the F0 females during gestation and lactation, and for the retained F1 males and females. The analysis of the rodent chow for chemical composition and possible chemical contamination and analysis of Durham City water will be provided by the suppliers and maintained in the study records. It is anticipated that contaminant levels will be below certified levels for both feed and water and will not affect the design, conduct, or conclusions of this study. Rat chow will be stored at approximately 60-70EF, and the period of use will not exceed six months from the milling date. At all times, animals will be housed, handled, and used according to the NRC Guide (NRC, 1996).

2.5.2 Environmental Conditions

Environmental conditions in the ARF will be continuously monitored, recorded, and controlled during the course of the study by an automated system (Siebe/Barber-Colman Network 8000 System with Version 4.4.1 Signal® software (Siebe Environmental Controls (SEC)/ Barber-Colman Company, Loves Park, IL). Animal rooms used for this study will be maintained on a 12:12 hour light:dark cycle. Target conditions for temperature and relative humidity in the animal rooms will be between 64-79EF (18-26EC) and 30-70%, respectively, with 10-15 air changes per hour (NRC, 1996). Temperature and/or relative humidity excursions will be documented in the study records and the final report.

2.5.3 Animal Identification

During quarantine, male breeders and females will be individually identified by a unique eartag (National Band and Tag Co., Newport, KY). Confirmed-mated females will also be given a dam number on gd 0, which will be used as an identifier for cage cards and all study records.. All selected study weanling F1 males and females will also be uniquely identified by eartag at weaning. All data generated during the course of this study will be tracked by these numbers.

2.5.4 Limitation of Discomfort

Some adult toxicity may be caused by exposure at the high doses of each test material. Discomfort or injury to animals will be limited, in that if any animal becomes severely debilitated or moribund, it will be humanely terminated by CO_2 inhalation. All necropsies will be performed after terminal CO_2 asphyxiation. F1 pnd 4 culled pups will be euthanized by decapitation.

2.5.5 Breeding

For breeding, individual females will be placed in the home cage of singly-housed males (i.e., one male and one female). On the following morning and each morning thereafter, the females will be examined for the presence of vaginal sperm or a vaginal copulation plug (Hafez, 1970). The day on which vaginal sperm or plugs are found will be designated as gd 0. These females are presumed pregnant. The initial sperm-positive females (dams), designated the F0 generation, will be housed individually or with their litters until scheduled sacrifice. Sperm-negative females will be retained in the same male's cage and checked for sperm or vaginal plug on successive mornings until insemination occurs or the treatment groups are filled, whichever comes first. When all treatment groups are filled, four sperm-negative females will be arbitrarily designated as sentinels (see Section 2.4.5, Sentinels) and remaining sperm-negative females will be sacrificed by asphyxiation with CO_2. Selected male and female offspring, designated the F1 generation, will be housed individually as described above. The fate of all animals will be fully documented.

3.0 EXPERIMENTAL DESIGN

3.1 Study Design

The study will consist of three dose groups and one vehicle control group, each group comprised of 15 mated F0 females randomized for body weight. The F0 study females will be dosed by gavage once daily for 36 consecutive days (gd 6 through pnd 21). Table 1 presents the study design and target doses of the test chemical. A graphical representation of the study design is presented in Figure 1 below.

Tentative Study Dates[a] (to be added to the protocol by amendment)

Nulliparous females arrive at RTI:
Cohabitation of breeding pairs:
F0 gd 0:
F0 dosing begins (gd 6):
F0 dosing ends (pnd 21):
Parturition of F1 offspring (pnd 0):
Weaning of F1 offspring (pnd 21):
Sacrifice of F0 dams:
Assignment of F1 offspring to cohorts (pnd 21):
Uterotrophic cohort dosing period (pnd 21-24):
Sacrifice of uterotrophic cohort (pnd 24):
Female pubertal cohort dosing period (pnd 21-42/43):

Sacrifice of female pubertal cohort (pnd 42/43):
Male pubertal cohort dosing period (pnd 21-70):
Sacrifice of male pubertal cohort (on pnd 70):
Submission of nonaudited draft final report:
Submission of audited draft final report:

[a] The end dates are tentative and will depend on the dates of insemination and the duration of gestation and lactation of the F0 dams with F1 offspring.

Table 1. Study Design and Target Doses

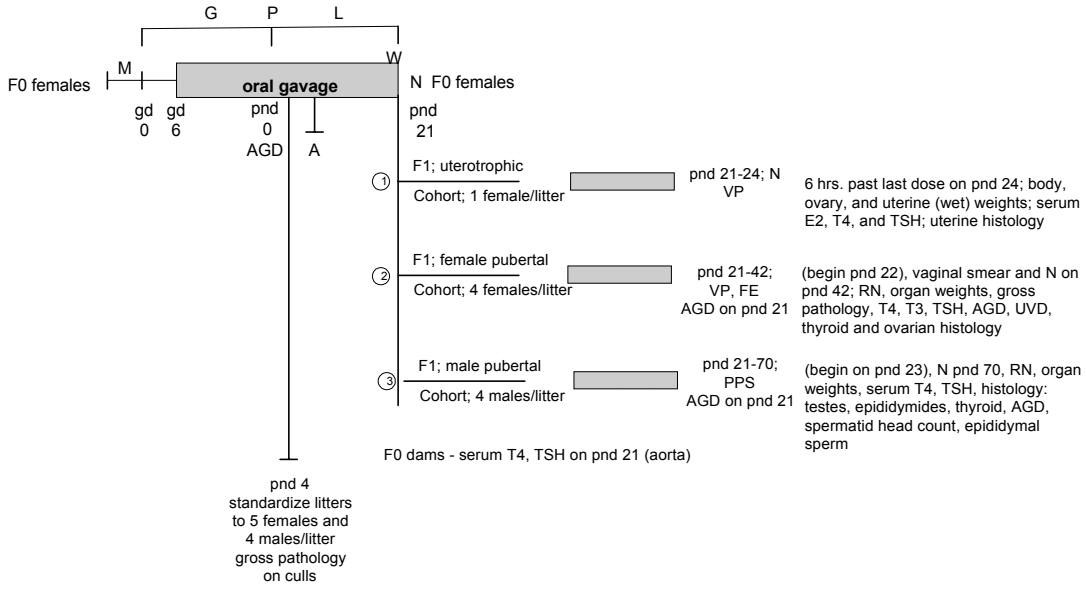
	Group Number			
	1[a]	2	3	4
F0 Females				
No.	15	15	15	15
Dose (mg/kg/day)	0	25.0	50.0	100.0
Concentration (ng/ml)	0	5.0	10.0	20.0
Dose Volume (ml/kg)	5.0	5.0	5.0	5.0
Route	gavage	gavage	gavage	gavage
F1 Pubertal Females				
No.	10	10	10	10
Dose (mg/kg/day)	0	25.0	50.0	100.0
Concentration (ng/ml)	0	5.0	10.0	20.0
Dose Volume (ml/kg)	5.0	5.0	5.0	5.0
Route	gavage	gavage	gavage	gavage
F1 Pubertal Males				
No.	10	10	10	10
Dose (mg/kg/day)	0	25.0	50.0	100.0
Concentration (ng/ml)	0	5.0	10.0	20.0
Dose Volume (ml/kg)[b]	5.0	5.0	5.0	5.0
Route	gavage	gavage	gavage	gavage
F1 Uterotrophic Females				
No.	10	10	10	10
Dose (mg/kg/day)	0	25.0	50.0	100.0
Concentration (ng/ml)	0	5.0	10.0	20.0
Dose Volume (ml/kg)	5.0	5.0	5.0	5.0
Route	sc injection	sc injection	sc injection	sc injection

[a] Corn oil, vehicle control

[b] Female rats at 21-24 days of age will weigh approximately 40-60 g. Therefore, the volume for sc

injection will be approximately 0.20-0.30 ml per female.

Figure 1. Study Design for In Utero/Lactational Exposure Assay



Key:

M = mating	RN = examination for retained nipples in F1 males and females
G = gestation	W = wean (pnd 21)
gd = gestational day	N = necropsy
P = parturition	VP = acquisition of vaginal patency (females)
pnd = postnatal day	PPS = acquisition of preputial separation (males)
AGD = anogenital distance	FE = first estrus
L = lactation	UVD = urethral vaginal distance

A = examinatioin for areolae on pnd 12-13

direct exposure to F0 dams and postweanling F1 offspring

3.2 Chemical and Dosage Selection

3.2.1 Chemical Selection

The chemical selected for this first validation study of the *in utero*/lactational exposure screening protocol is methoxychlor (MXC). MXC is an organochlorine pesticide in use as a DDT substitute to control insects. It has known endocrine effects. The *in vivo* metabolite of MXC, 2,2-bis(*p*-hydroxyphenol)-1,1,1-trichloroethane (HPTE), has selective agonist activity through the estrogen receptor (ER) alpha and antagonist activity through ER beta and the androgen receptor (AR) (Waters et al., 2000). MXC has been shown to inhibit androgen receptor-dependent transcriptional activity *in vitro* (Maness et al., 1998), so it also acts as an antiandrogen. In female mice and rats, MXC is positive in the uterotrophic assay with ovariectomized adults or weanlings, causing increased uterine weights. It stimulates ER expression in the uteri of neonatal (days 1-4) and immature (days 10-14) mice after ip injection for four days of MXC (Eroschenko et al., 1996). It also reduced the number of implants and newborns in a multigeneration study of dietary MXC (Aoyama et al., 2000). The day of vaginal opening was accelerated and body weight at acquisition was reduced by MXC administered by ip injection to female rats on pnd 10-14 (Respess et al., 1999). In male mice and rats with *in utero* exposure, MXC disrupted the morphology and growth of the developing testis (Cupp and Skinner, 2000). Perinatal and juvenile exposure to MXC reduced testicular size and Sertoli cell numbers in adult rats (Johnson et al., 2000). Perinatal exposure to dams on gd 18 to parturition and directly to pups on pnd 1-5 resulted in increased lateral prostate lobe (but not ventral lobe) weight in adult male offspring (Stoker et al., 1999). Since MXC (and its metabolite HPTE) has estrogenic, anti-estrogenic, and anti-androgenic properties, mediated through interactions with the ERα and β and the AR, because MXC and HPTE can compete for binding to the ER (Cupp and Skinner, 2000) and because it also affects circulating TSH and T4 levels (Gray et al., 1989), it was chosen as the first chemical for validation of this assay.

3.2.2 Dose Selection

MXC will be dosed by oral gavage to F0 maternal animals from gd 6 through pnd 21 and by oral gavage to the F1 male and female pubertal cohorts (pnd 21-42/43 for females, pnd 42-70 for males). It will also be administered by subcutaneous (sc) injection on pnd 21-24 to the F1 female uterotrophic cohort.

Based on a literature search and input from Dr. L. Earl Gray, Jr. (EPA NHEERL), the doses for the oral gavage dosing will be 0, 25, 50, and 100 mg/kg/day with MXC in corn oil at 5.0 ml/kg. The doses for the sc injection administration are set to the same levels (0, 25, 50, and 100 mg/kg/day, as a place holder) at the same dosing volume (5.0 ml/kg). For the uterotrophic cohort, with administration only on pnd 21-24, the F1 female pups will be approximately 40-60 g body weight. Therefore, the volume administered by sc injection will be approximately 0.2-0.3 ml/female. The justification for the doses and routes is as follows.

The doses selected represent the mid range of gavage doses identified in the literature. Doses used and effects observed are discussed by sex, males first.

Males

White et al. (2001) exposed Sprague-Dawley rats in a one-generation study design to MXC in the diet at 10, 100, and 1000 ppm (with MXC intakes calculated for this protocol of 0.7, 6.7, and 66.7 mg/kg/day), from gd 7 for F0 animals to pnd 77 for F1 offspring. The authors reported only increased splenic basal and stimulated lymphocyte proliferation response in F1 males (but not F1 females), especially following developmental exposure. Cupp and Skinner (2000, 2001) dosed pregnant rodents with MXC at 50 mg/kg/day from gd 7 through gd 15. Embryonic gonads were collected on gd 16, pnd 4, and pnd 17 during testis development. Effects on the testis were observed only on pnd 17, with gross reduction in the testicular interstitium. They confirmed the MXC effects with *in vitro* testis organ cultures, which exhibited inhibited/disrupted testicular cord formation and increased cell growth. Johnson et al. (2000) gavaged rat dams with MXC at 0, 50, 50, or 150 mg/kg/day for the last week of gestation and the first week of lactation. Male pups were dosed directly from pnd 7 to 42. The offspring males in the two highest dose groups exhibited fewer testicular spermatids and reduced numbers of Sertoli cells as adults. Stoker et al. (1999) dosed rat dams by gavage to MXC from gd 18 through pnd 5 at 50 mg/kg/day. Male offspring were examined on pnd 90. They exhibited increased prostate lateral lobe (but not ventral lobe) weight, with an increased incidence in the number and severity of inflammation in the lateral prostate. Chapin et al. (1996, 1997; Harris et al., 1996) exposed pregnant rats to MXC at 0, 5, 50, and 150 mg/kg/day for the week before and after parturition (see above), with offspring pups dosed directly from pnd 7 to pnd 21 or pnd 42. In the male offspring, anogenital distance was unaffected, but male preputial separation was delayed at 50 and 150 mg/kg/day by eight and 34 days, respectively. Epididymal sperm counts were reduced at 150 mg/kg/day, and testes and epididymal weights were reduced at 50 and 150 mg/kg/day; seminal vesicle weights were reduced at all doses. The F1 animals (15/dose group) at adulthood were mated to untreated animals twice. Males at 150 mg/kg/day impregnated 3/30 untreated females versus 21/30 in controls; litter size was unaffected.

Anderson et al. (1994, 1995) evaluated MXC in Long-Evans hooded rats under an alternative reproduction test (ART) protocol. It was administered by gavage at 0, 50, or 200 mg/kg/day to F0 males and females, starting at three weeks of age and continuing for 14 weeks in males or 18 weeks in females through gestation and lactation. For the F0 males as adults, ejaculated sperm counts, caudal epididymal sperm counts, and epididymal, ventral prostate, and seminal vesicle weights were all reduced at both doses. MXC suppressed both GnRH and hCG-stimulated testosterone levels; LH levels were significantly higher after GnRH challenge.

Gray et al. (1989) dosed rats at weaning through puberty, mating, and gestation to pnd 15 of lactation by gavage with MXC at 0, 25, 50, 100, and 200 mg/kg/day. In the males, MXC markedly reduced growth; seminal vesicle, cauda epididymis and pituitary weights; and cauda epididymal sperm content. Puberty was delayed at 100 and 200 mg/kg/day. Testicular spermatid

measures were much less affected than cauda sperm measures. Testis weight and histology were slightly affected, and testicular sperm production and sperm morphology and motility were unaffected. Endocrine function of the testes and pituitary was altered by MXC. Leydig cell testosterone production from hCG challenge was reduced, and pituitary levels of prolactin, TSH, and FSH were altered (serum levels of prolactin, FSH, and LH were unaffected). Serum TSH was reduced by 50% at 100 and 200 mg/kg/day, while pituitary levels were increased. In spite of these effects on males, the fertility of the treated males, mated with untreated females, was unaffected.

Gray et al. (1999) dosed weanling male Long-Evans hooded rats by gavage with MXC at 0, 200, 300, or 400 mg/kg/day for ten months. The treated males were then mated to untreated females. MXC delayed puberty by as much as ten weeks at the top dose, and reduced fertility and copulatory plug formation in a dose-related manner at the initial mating. During mating, treated males exhibited shorter latencies to mount and ejaculate versus control males (with the number of intromissions prior to ejaculation unaffected), indicating that MXC enhanced male arousal. Most MXC-treated males mated, but time to pregnancy was lengthened. Very low sperm counts were associated with infertility, while prolonged delays in puberty were associated with reduced fecundity. MXC at 200-400 mg/kg/day did not mimic chronic effects of exposure to 17β-estrdiol on testicular or pituitary hormone levels. MXC affected the CNS, epididymal sperm numbers, and accessory sex organs without affecting the secretion of LH, prolactin, or testosterone. Therefore, MXC did not alter pituitary endocrine function in either an estrogenic or anti-androgenic manner.

Goldman et al. (1986) investigated the effects of MXC on the pituitary and hypothalamic components of the male rat reproductive system at dose levels that did not affect the testis. Male Long-Evans rats were gavaged daily with MXC at 0, 25, or 50 mg/kg/day, starting at 21 days of age for eight weeks. There were no effects on serum LH, FSH, or prolactin levels, and no effects on pituitary concentrations of LH or FSH. Pituitary prolactin was elevated at both doses (and pituitary fragments *in vitro* released more prolactin than control fragments). The authors concluded that the reproductive effects of MXC may be mediated, at least in part, through early increased prolactin concentration and release, which in turn affects hypothalamic levels of GnRH and subsequent pituitary and gonadal adverse responses.

When MXC was administered by oral gavage to male rats at 70 days and to female rats at 14 days at 0, 100, or 200 mg/kg/day, MXC inhibited spermatogenesis, with degenerative fatty changes in the Sertoli cells. Degeneration changes in spermatogonia and spermatocytes were also observed, with some seminiferous tubules devoid of all cellular elements except spermatogonia. The epithelium of the ductus epididymis also exhibited cytoplasmic vacuolation and distention of the lumen (Bal, 1984).

Sar et al. (2001) exposed pregnant SD rats to MXC in the diet at 800 ppm (approximately 53 mg/kg/day). Inguinal mammary glands from F1 male offspring exhibited greater total

glandular area and increased numbers of branch points, lateral buds, and terminal end buds than controls (F1 female offspring mammary glands were unaffected).

Welshons et al. (1999) have reported that fetal exposure (gd 11-17) of very low doses of MXC (20 and 2000 μg/kg) result in increased prostate weight in adult male offspring.

Therefore, the doses selected should result in effects on the male testis and accessory sex organs, in delay in preputial separation, effects on testicular spermatid and epididymal sperm counts, and serum hormone levels, including effects on TSH from the pituitary and T4 from the thyroid.

Females

The effects of MXC on female reproduction have been more extensively researched and reported. Exposure of MXC at 0, 10, 100, or 1000 ppm in the diet (0, 0.7, 6.7, or 66.7 mg/kg/day, respectively) to F0 SD rats did not produce increased splenic lymphocyte proliferation under either basal or stimulated conditions in F1 females (F1 males did respond with increased proliferation) (White et al., 2001). When Sar et al. (2001) exposed pregnant SD rats to 800 ppm MXC in the diet (approximately 53 mg/kg/day), there was no effect on F1 female offspring inguinal mammary glands when evaluated for total glandular area and number of branch points, lateral buds, and terminal end buds (inguinal mammary blands for F1 male offspring were affected).

Both rats and mice (either ovariectomized adult or intact immature females) respond to short-term daily dosing of MXC by increased uterine weights (Aoyama et al., 2000 and Respess et al., 1999 in rats; Eroschenko, 1997 and Eroschenko et al., 2000 in mice). Female SD pups were administered MXC on pnd 10-14 by ip injection of 0, 0.3, 3, or 300 mg/kg/day. Pups were sacrificed on pnd 15, 23, 31, and 70. Day of vaginal opening was accelerated by four days, and body weight at acquisition was reduced (by 25 g) at 300 mg/kg/day. Ovarian and uterine weights were increased at 300 mg/kg/day on pnd 15 (Respess et al., 1999).

One-day-old female mice (five to eight/group) were administered MXC by ip injection for 14 days at 0.1, 0.5, or 1.0 mg MXC (corresponding to 14-71, 68-357, or 135-714 mg/kg/day, respectively). Three months later, the females were paired with proven breeder, untreated males and checked daily for vaginal copulation plugs. Maternal females were necropsied 18 days after insemination. All mice from the three MXC groups mated, with dose-related, decreased numbers of pregnant animals on gd 18. The mean number of live fetuses/litter was reduced at 0.5 and 1.0 mg MXC. Ovarian corpora lutea were reduced only at 1.0 mg MXC. No effects were observed at 0.1 mg MXC. The authors concluded that MXC did not affect mating but did affect initiation and/or maintenance of pregnancy. Therefore, the neonatal exposure to MXC may affect the hypothalamic-pituitary-ovarian axis as well as the uterine environment (Swartz and Eroschenko, 1998).

MXC was administered to pregnant mice on day 1, 2, 3, or 4 of pregnancy at 400 or 800 µg/g body weight (400 or 800 mg/kg). At 400 mg/kg on day 1 or 2, MXC induced delays in implantation. At 800 mg/kg/day on day 1 or 2, only 50% of the females exhibited implanted conceptuses, and the number of embryos/female was significantly reduced. Administration of lower doses of MXC or at later times did not affect implantation. However, embryonic development and transport were delayed at 400 and 800 mg/kg/day, administered on days 3 or 4. Reciprocal embryo transfers with embryos from MXC-treated dams (800 mg/kg on day 1), transferred into untreated females, resulted in no implantations (control donor embryos exhibited a 79% implantation rate). The authors concluded that MXC acts as an estrogen agonist in the uterus and oviduct but acts as an antiestrogen in the ovary. MXC also affects normal preimplantation embryonic development (Hall et al., 1997). Swartz and Vial (1996) have also reported that exposure to MXC early in pregnancy disrupts implantation.

Cummings and Perreault (1990) also reported that MXC administered by gavage to rats on days 1-3 of pregnancy (sperm positive = day 0) at 0, 100, 200, or 500 mg/kg/day resulted in accelerated embryo transport from the oviducts into the uterus on days 2 and 3 at 200 and 500 mg/kg/day. The top dose also reduced the total number of embryos recovered on the third day, 100 mg/kg/day also accelerated embryo transport, and 200 mg/kg/day reduced the number of total embryos recovered. This acceleration of embryonic transport appears to be the primary cause of MXC-induced preimplantation embryonic loss when exposure to MXC occurs after fertilization.

Gavage dosing of dams from gd 14 to pnd 7 (Chapin et al., 1996, 1997; Harris et al., 1996) to MXC at 0, 5, 50, and 150 mg/kg/day resulted in dose-dependent amounts of MXC and metabolites in milk and plasma of both dams and pups. Lactating mice were administered MXC by ip injection for 14 days (pnd 1-14) at 0, 1.0, 2.0, or 5.0 mg of technical grade MXC. At pnd 15, suckling female pups were necropsied. Stimulatory changes in the vagina and uterine horns indicated that MXC was excreted in milk and remained biologically active in the suckling mice. Higher MXC doses also caused “cellular atypia” in the uterine horns (Appel and Eroschenko, 1990).

Sexually mature CD-1 virgin female mice were administered technical grade MXC by oral gavage at 0, 1.25, 2.5, or 5.0 mg for five days/week for two or four weeks (Martinez and Swartz, 1991) or to just 50 mg for five days/week for four weeks (Martinez and Swartz, 1992). Twenty-four hours after the last dose, the females were necropsied. MXC caused dose-dependent, persistent vaginal estrus and reduced ovarian weights. Ovaries from females at 2.5 and 5.0 mg exhibited an inreased number of atretic large follicles (Martinez and Swartz, 1991) and increased lipid accumulation in interstitial and thecal cells at 5.0 mg MXC (Martinez and Swartz, 1992). The authors concluded that MXC appeared to mimic estrogen-induced effects on the female reproductive system, and that the exposed ovarian cells appeared to be unable to synthesize and secrete steroids.

Mouse neonates were administered 14 daily ip injections of 0, 0.05, 0.1, 0.5, or 1.0 mg MXC. Exposure to 0.5 or 1.0 mg MXC increased reproductive tract weights three-fold due to

excessive fluid accumulation, induced vaginal cornification, and accelerated vaginal opening by ten days (similar to 10 μg 17β-estradiol). The surface alterations in the vagina and uterus induced by MXC (cornified cells without complex surface microridges, uterine cells with dense microvilli growth, atypical morphology and separation) were different than those induced by estradiol (Eroschenko, 1991).

Cummings and Laskey (1992, 1993) administered MXC to female rats at a range of doses during days 1-8 of pregnancy; the females were terminated on day 9. Ovaries were removed and incubated. Incubation medium and serum from the rats were analyzed for progesterone, estradiol, and testosterone *ex vivo*. *In vivo* MXC treatment reduced serum progesterone but had no effect on ovarian secretion of progesterone *in vitro*. Conversely, MXC had no effect on serum estradiol levels (testosterone was undetectable in serum) but induced a reduction in the rates of ovarian estradiol and testosterone secretion. Cummings and Gray (1989) have also shown that MXC blocks pregnancy in female rats in a dose- and time-dependent pattern. Exposure on gd 1-3 (preimplantation) resulted in decreased implantations and uterine weight, while exposures on gd 4-8 (peri-implantation) increased resorptions to 100%, decreased uterine weight, and reduced serum progesterone without altering the number of implantations, ovarian weight, or corpora lutea (effect levels for both dosing regimens were >200-500 mg/kg/day). However, Cummings and Gray (1987) reported that MXC affects the decidual cell response of the uterus but not other progestational parameters in the female rat.

Immature female rats were administered MXC (or other compounds) by oral gavage at 250 mg/kg 24 hours prior to evaluation of uterine peroxidase activity. MXC alone increased uterine peroxide activity by increasing RNA and protein synthesis, as did estradiol alone. Co-administration of progesterone or tamoxifen blocked this stimulation induced by both MXC and estradiol (Cummings and Metcalf, 1995a). The same authors exposed immature female rats to MXC (500 mg/kg) or estradiol (E2; 10 μg/rat), and uteri were evaluated for the presence of estrogen-induced protein (IP), also known as creatine kinase. Both MXC and E2 stimulated IP. The induction of IP by MXC was time- and dose-dependent. This induction by MXC or E2 was blocked by actinomycin D (which blocks DNA-dependent RNA synthesis) or cycloheximide (which inhibits pattern synthesis), indicating the induction requires RNA and protein synthesis. Progesterone did not block the induction of IP by either E2 or MXC. In fact, Cummings (1997) has proposed MXC as a model for environmental estrogens. Interestingly, MXC and E2 do not exhibit additivity or synergism in the reproductive tract of ovariectomized mice (Eroschenko et al., 2000).

Neonatal female mice received 14 days ip injections of 0, 0.05, 0.1, 0.5, or 1.0 mg (approximately 0, 7-35, 14-71, 68-357, or 135-714 mg/kg/day) of technical MXC. At three, six, and 12 months, vaginal smears were collected and ovaries were examined (E2 at 10.0 μg, ip, was used as the positive control). All MXC doses (and E2) increased the duration of vaginal cornification. MXC at 0.5 and 1.0 mg and E2 induced ovarian atrophy, relative ovarian weight depression, and depletion of corpora lutea. However, MXC doses of 0.05 or 0.1 mg produced opposite effects: ovaries remained heavy, large, and filled with corpora lutea. At all MXC doses,

except 1.0 mg, follicular cysts were present. The authors concluded that the stimulatory effects of MXC at low doses and the inhibiting effects of MXC at high doses mimicked the effects of E2 at low and high doses and were probably due to alterations of the hypothalamic-hypophyseal (anterior pituitary) function (Eroschenko et al., 1995). Prenatal exposure (gd 11-17) to low doses (0.01 or 10 mg/kg) of MXC in mice alters the uterine response to estrogen as adults (Howdeshell et al., 1999).

MXC also affects endometriosis in rats. Endometriosis was surgically induced in sixty female rats on pnd 0. On pnd 21, all rats were ovariectomized and the size of fully-developed endometriotic implants measured. Also starting on pnd 21, these rats were treated daily for three weeks with MXC, 250 mg/kg ± 2 mg/rat of progesterone (E2 at 1.0 µg/rat was also used). On day 42, the rats were terminated and the size of the endometriotic implants remeasured. Ovariectomy plus treatment altered the growth of endometrosis. Progesterone or vehicle produced full regression. Both E2 and MXC increased the size of the endometriotic implants; exposure to MXC or E2 + progresterone did not alter the growth (Cummings and Metcalf, 1995b).

Chapin et al. (1996, 1997; Harris et al., 1996; Johnson et al., 2000) dosed F0 maternal rats with MXC by gavage from gd 14 to pnd 7 (starting one week before and ending one week after parturition) at 0, 5, 50, or 150 mg/kg/day. F1 offspring were directly dosed at the same dose levels as their dams from pnd 7 to pnd 21 (weaning) or to pnd 42. In the female offspring, anogenital distance was unaffected, but vaginal opening was accelerated in all groups. Adult F1 female estrous cyclicity was disrupted at 50 and 150 mg/kg/day. Females in these groups also exhibited reduced rates of pregnancy and delivery. Uterine weights, corrected for pregnancy, were reduced in all treated pregnant females. All groups of treated females exhibited uterine dysplasia and less mammary gland alveolar development. Estrous levels of FSH were lower in all groups, and estrous progesterone levels were lower at 50 and 150 mg/kg/day, attributed to fewer corpora lutea secondary to ovulation defects. The author concluded that 5 mg/kg/day is not a NOEL or a NOAEL, and that effects on female puberty, ovarian weights, uterine weights, and female hormone data imply that the sites of MXC action are both central and peripheral.

Shimizu et al. (2000) evaluated MXC in teratogenicity studies in rats (Jcl:SD) and rabbits (Kbl:JW). Rats were dosed by gavage on gd 6 through 19 at 0, 1, 50, or 150 mg/kg/day, and rabbits were dosed by gavage on gd 6 through 27 at 0, 1, 15, or 45 mg/kg/day. At the two highest dose groups in both species, there was decreased maternal body weight gains and feed consumption during the dosing period. At 150 mg/kg/day in rats, gravid uterine weight was reduced, and resorptions and fetal deaths were increased, resulting in decreased number of live fetuses. Fetal body weights were reduced, but anogenital distance was unaffected at 150 mg/kg/day; there were no treatment-related fetal rat abnormalities at any dose. In rabbit fetuses, fetal body weights were reduced at 45 mg/kg/day. Rabbit fetuses in the mid and high dose groups exhibited increased incidences of 13th rib pairs and of 27 presacral vertebrae (both designated as fetal skeletal variations in the presence of maternal toxicity). Therefore, MXC was not teratogenic in either species but did result in *in utero* deaths at 150 mg/kg/day in rats.

It is clear that 150 mg/kg/day is too high, resulting in *in utero* deaths when administered to the dams starting on gd 6 (Shimizu et al., 2000) and as planned in this protocol, although it is well tolerated in dams and perinatal offspring when administered starting on pnd 14 (Chapin et al., 1996, 1997; Harris et al., 1996; Johnson et al., 2000). Therefore, the top dose for this study will be 100 mg/kg/day, an effective dose (Gray et al., 1989). The low dose chosen for this study will be 25 mg/kg/day, which resulted in demonstrable effects by Gray et al. (1989). The mid dose chosen, 50 mg/kg/day, has also been shown to produce adverse effects in the offspring (Gray et al., 1989; Goldman et al., 1986; Anderson et al., 1994, 1995; Johnson et al., 2000).

3.3 Allocation and Treatment of F0 Maternal Animals

All sperm-positive F0 female rats (dams) will be assigned to treatment groups by a stratified randomization method designed to provide uniform mean body weights across dosage groups at the initiation of gestation (gd 0). Methoxychlor in vehicle (three dose levels) or the vehicle alone will be administered by gavage daily, once in the morning, from gd 6 through pnd 21 (day of birth designated pnd 0), at a 5.0 ml/kg dosing volume per time. The volume of dosing formulation given to presumed pregnant females will be adjusted, based on each animal's most recent body weight or the current weight on a scheduled weighing day. The dosing formulations will be administered using a 16-gauge, two-inch curved dosing needle (Perfektum®, Popper and Sons, New Hyde Park, NY), fitted to a syringe of appropriate volume. The route of administration (gavage) was specified by the Sponsor.

3.4 F0 Dams and F1 Litters Prior to Weaning

3.4.1 F0 Maternal Gestation, Parturition, and Lactation

Clinical observations of F0 maternal animals will be documented at least once daily on gd 0-5 (prior to dosing period), at least twice daily, at dosing, one to two hours postdosing throughout the dosing period (gd 6 through pnd 21), and once daily during lactation. The examining technicians will be unaware of the test materials or of dosage levels. Observations will be made for (but not limited to):

- Any response with respect to body position, activity, coordination, or gait
- Any unusual behavior such as head flicking, compulsive biting or licking, circling, etc.
- The presence of:
    1. Convulsions, tremors, or fasciculations
    2. Increased salivation
    3. Increased lacrimation or red-colored tears (chromodacryorrhea)
    4. Increased or decreased urination or defecation (including diarrhea)
    5. Piloerection
    6. Mydriasis or miosis (enlarged or constricted pupils)

7. Unusual respirations (fast, slow, labored, audible, gasping, or retching)
8. Vocalization

All F0 dams will be weighed in the morning on gd 0 and daily in the morning during the dosing period, gd 6 through pnd 21, for calculation of dosing volume. Maternal body weights will be reported for gd 0, 6, 9, 12, 15, 18, and 20 and on pnd 0, 4, 7, 14, and 21. F0 maternal weight gains will be calculated for gd 0-6 (pretreatment), 6-9, 9-12, 12-15, 15-18, 18-20, pnd 0-4, 4-7, 7-14, 14-21, gd 0-20 (gestation period), gd 6-20, and pnd 0-21 (treatment period).

F0 maternal feed consumption will be evaluated in the morning for gd 0-6 (pretreatment), gd 6-9, 9-12, 12-15, 15-18, 18-20, pnd 0-4, 4-7, 7-14, 14-21, gd 0-20 (gestation period), gd 6-20, and pnd 0-21 (treatment period).

Beginning on gd 20, each female will be examined twice daily (a.m. and p.m.) for evidence of littering. Dosing will continue through parturition through pnd 21. If the dam is in the process of littering at the usual time of dosing, she will not be dosed at that time but will be dosed at the next scheduled morning dosing time. Signs of dystocia or other signs of difficulty at parturition will be recorded. Dams that have not produced a litter by calculated gd 26 will be necropsied. Nonpregnant uteri will be stained in 10% ammonium sulfide (Salewski, 1964) to determine pregnancy status. Any dams whose whole litters are born dead or die prior to pnd 21 will be sacrificed, the number of uterine implantation scars will be recorded, and a sample of mammary tissue (one abdominal mammary gland with nipple, surrounding skin, and underlying mammary tissue) and both ovaries and pituitary will be retained in buffered neutral 10% formalin for possible future examination.

3.4.2 Necropsy of F0 Females

On pnd 21 of each F1 litter, each F0 dam will be euthanized by CO_2 asphyxiation. Blood will be collected from the abdominal aorta for T4 and TSH determinations. The thoracic and abdominal organs will be examined for grossly evident morphological changes, and uterine implantation scars will be counted and recorded (Salewski, 1964). F0 maternal carcasses and nonretained tissues will be discarded.

3.4.3 F1 Progeny

3.4.3.1 Birth and Perinatal Observations

All pups will be counted, sexed, and examined externally as soon as possible on the day of birth (pnd 0) to determine the number of total, viable, and stillborn members of each litter. Each live pup will be weighed individually and anogenital distance measured (by vernier calipers or microscopically with an eyepiece grid and platform micrometer). Grossly malformed pups will be sacrificed and examined externally and viscerally. Pups that are stillborn or die before pnd 4 will be examined externally and viscerally by Staples' technique (Staples, 1974), and any

abnormal tissues or specimens will be retained in buffered neutral 10% formalin until the study report is finalized.

Pups will be examined daily and will be counted, weighed, sexed, and examined externally on pnd 0, 2, 4, 7, 10, 14, 17, and 21. Pups which die or are sacrificed moribund on pnd 5-21 will be necropsied; any abnormal tissues or specimens will be retained in buffered neutral 10% formalin until the study report is finalized. Survival indices will be calculated on pnd 0, 4, 7, and 14 and at weaning on pnd 21. The body weights and sexes of the F1 pups will be recorded on an individual basis, but the pups will not be uniquely identified at this stage. All pups will be examined for physical abnormalities at birth and throughout the lactation and postwean period.

The presence or absence of retained nipples and areolae on the ventrum will be recorded for all F1 male and female offspring on pnd 11-13. Any males with one or more nipples or areolae will be uniquely marked within the litter (dye on tail) until weaning.

3.4.3.2 Standardization of Litter Sizes

On pnd 4, the size of each litter will be adjusted to nine pups (five females and four males), if possible. There will be a minimum of ten litters per dose group. Natural litters with ten or fewer pups will not be standardized. Natural litters which cannot be standardized to five females and four males will be discarded, if possible. Pups from larger litters will not be fostered to smaller litters, since the unit for statistical comparison is the litter; fostering will prevent litter-based analyses. A gross necropsy will be performed on all culled pups. The F0 dams will be allowed to rear their remaining F1 young to pnd 21.

3.5 Treatment and Evaluation of F1 Weanlings

All litters will be weaned on pnd 21.

3.5.1 Uterotrophic Cohort

On pnd 21, one female from each litter will be chosen for the uterotrophic cohort. Females will be dosed sc from pnd 22-24 and evaluated on pnd 24. Clinical observations of F1 females assigned to the uterotrophic cohort will be documented at least twice daily (at dosing and one to two hours postdosing) throughout the dosing period (pnd 22 through pnd 24). The examining technicians will be unaware of the test materials or dosage levels. Observations will be made for (but not limited to):

- Any response with respect to body position, activity, coordination, or gait
- Any unusual behavior such as head flicking, compulsive biting or licking, circling, etc.
- The presence of:
  1. Convulsions, tremors, or fasciculations

2. Increased salivation
3. Increased lacrimation or red-colored tears (chromodacryorrhea)
4. Increased or decreased urination or defecation (including diarrhea)
5. Piloerection
6. Mydriasis or miosis (enlarged or constricted pupils)
7. Unusual respirations (fast, slow, labored, audible, gasping, or retching)
8. Vocalization

Beginning on pnd 22, each F1 female will be dosed with the same dose level as her mother but via subcutaneous injection on pnd 22 through 24. Each animal will be weighed every other day prior to treatment and the body weight recorded. Each female will be examined daily for vaginal patency on pnd 21 through 24 (see Section 3.5.2). Treatments are administered sc daily using an 22-gauge needle and a 1 cc glass (disposable) tuberculin syringe for each treatment, from pnd 22 and continuing through pnd 24. The treatments will be administered on a mg/kg body weight basis, adjusted based on the most recent body weight, and the volume of the dose administered will be recorded each day. Dosing solutions/suspensions will be well mixed to keep the chemical in suspension prior to and throughout dosing.

Females will be necropsied six hours after the last dose on pnd 24. Body weight and ovary and uterus weight (wet) will be determined. Blood will collected from the abdominal aorta for determination of serum estradiol, T4, and TSH. The uterus will be evaluated histopathologically.

3.5.2 F1 Weanling Female Pubertal Cohort

The four remaining females from each litter will be divided into dosed (n=2) and nondosed (n=2) groups on pnd 21. At this time, anogenital distance (AGD) will be measured and recorded with the individual pup weight using a Vernier calipers (precision to 0.1 mm). The test compound or vehicle will be administered via gavage from pnd 21-42, based on daily body weights. Clinical observations (see Section 3.5.6.1 above) of F1 females assigned to the pubertal cohort will be documented at least twice daily (at dosing and one to two hours postdosing) throughout the dosing period (pnd 21 through pnd 42). All F1 females will be weighed in the morning on pnd 21 and every other day in the morning during the dosing period on pnd 22 through pnd 42 for adjustment of dosing volume, based on the most recent body weight. F1 female weight gains will be calculated for pnd 21-22, 22-24, 24-26, 26-28, 28-30, 30-32, 32-34, 34-36, 36-38, 38-40, 40-42, and 21-42 (treatment period). F1 female body weights will also be recorded on the day of acquisition of vaginal patency.

Beginning on pnd 22, each F1 study female will be examined daily for vaginal patency. The appearance of a small “pin hole,” a vaginal thread, as well as complete vaginal opening should all be recorded on the days they are observed. The day of complete vaginal patency is the endpoint used in the analysis for the age of vaginal opening. However, if a sufficient number of animals within any treatment group show persistent threads for greater than three days, a separate

analysis should be conducted using the age at which the thread was first observed. Body weight at acquisition of complete vaginal patency will be recorded.

Beginning on the day of vaginal opening and continuing until pnd 42/43, daily vaginal smears will be obtained from each F1 female, stained with Toluidine Blue, and evaluated under low- and high-power light microscopy for the presence of leukocytes, nucleated epithelial cells, or cornified epithelial cells to determine the age at the first complete vaginal cycle and/or any effects on estrous cyclicity. This provides a means to determine the age of first estrus and/or the first vaginal cycle, and distinguishes pseudoprecocious puberty from true precocious puberty. The vaginal smears will be classified as diestrus (presence of leukocytes), proestrus (presence of nucleated epithelial cells), estrus (presence of cornified epithelial cells), or metestrus (presence of approximately equal numbers of leukocytes and large folded epithelial cells with translucent nuclei). Prolonged estrus shall be defined as exhibiting cornified cells with no leukocytes for three or more days; and prolonged diestrus as the presence of leukocytes for four or more days. On pnd 42/43, all F1 females will be examined for nipples/areolae at necropsy.

3.5.3 F1 Weanling Male Pubertal Cohort

The four males from each litter will be divided into dosed (n=2) and nondosed (n=2) groups on pnd 21. At this time, AGD will be measured. AGD will be recorded with the individual pup weight using a Vernier calipers (precision to 0.1 mm). The test compound or vehicle will be administered via gavage from pnd 21 to pnd 70, based on daily body weights. Clinical observations (see Section 3.5.6.1 above) of F1 males assigned to the pubertal cohort will be documented at least twice daily (at dosing and one to two hours postdosing) throughout the dosing period (pnd 21 through pnd 70). All F1 males will be weighed in the morning on pnd 21 and every other day in the morning during the dosing period on pnd 22 through pnd 70 for adjustment of dosing volume, based on the most recent body weight. F1 male weight gains will be calculated for pnd 21-22, 22-24, 24-26, 26-28, 28-30, 30-32, 32-34, 34-36, 36-38, 38-40, 40-42, 42-44, 44-46, 46-48, 48-50, 50-52, 52-54, 54-56, 56-58, 58-60, 60-62, 62-64, 64-66, 66-68, 68-70, and 21-70 (treatment period). F1 male body weights will also be recorded on the day of acquisition of preputial separation (see below).

Beginning on pnd 23, each F1 study male will be examined daily for preputial separation. The appearance of partial and complete preputial separation or a persistent thread of tissue between the glans and prepuce should all be recorded if and when they occur. In addition, the body weight at complete preputial separation should be recorded. However, if a sufficient number of animals within any treatment group show persistent threads for greater than three days, a separate analysis should be conducted using the age at which the thread was first observed.

3.6 Necropsy of F1 Offspring

3.6.1 Terminal Blood Collection

At scheduled necropsy of the F1 females (pnd 24 or 42/43) and males (pnd 70), after terminal anesthesia (CO_2 asphyxiation), the animals will be weighed and the maximum amount of blood will be taken by external cardiac puncture and placed in a labeled tube. The blood will be allowed to clot and centrifuged under refrigeration at approximately 1400 x g for approximately ten minutes. The resulting serum will be frozen at approximately -20○C for subsequent analysis of E2 (uterotrophic cohort), thyroxine (T4), triiodothyronine (T3), and thyroid stimulating hormone (TSH) at RTI.

3.6.2 Gross Necropsy and Organ Weights

Once each F1 animal is bled (see Section 3.7.1), it will be necropsied and internal thoracic and abdominal organs and cavities examined. Any abnormalities will be documented.

At necropsy, F1 females assigned to the female pubertal cohort will be examined externally for the number of nipples, AGD, and urethral-vaginal distance (UVD). The following organs will be dissected out and weighed: liver, kidneys (paired), adrenal glands (paired), ovaries (paired), uterus (see below), and thyroid (with attached portion of trachea). For the uterine dissection, care must be taken to remove mesenteric fat from the uterine horns and not damage the uterus so that the uterine fluid is retained. The uterus and cervix will be separated from the vagina and the weight of the uterus with fluid recorded. The uterus will then be placed on a paper towel, slit to allow the fluid contents to leak out, gently blotted dry, and reweighed. All organs will be weighed to the nearest 0.1 mg. Adrenal glands will be weighed immediately (to minimize drying out of tissues). The thyroid and ovaries will be processed for microscopic evaluation. The adrenals will be weighed immediately to prevent drying out prior to weighing.

At scheduled necropsy, F1 males will be shaved on the ventrum and examined externally for retained nipples. The thoracic and abdominal organs and cavities will be examined and any abnormalities documented. The following organs will be dissected out and weighed: paired testes, epididymides (whole left and right separately), prostate (intact and separated into ventral and dorsolateral lobes), seminal vesicles with coagulating glands (and fluid), Cowper's glands, glans penis, levator ani plus bulbcavernosus muscle complex, liver, adrenal glands (paired), kidneys (paired), and thyroid (taken with attached portion of trachea). All organs will be weighed to the nearest 0.1 mg. Adrenal glands will be weighed immediately (to minimize drying out of tissues). During necropsy, care must be taken to remove mesenteric fat with small surgical iris scissors from these tissues such that the fluid in the sex accessory glands is retained. Small tissues such as the adrenals, as well as tissues that contain fluid, will be weighed immediately to prevent partial drying prior to weighing.

At the time of sacrifice, one testis from each F1 male will be frozen at approximately -20°C for subsequent enumeration of testicular homogenization-resistant spermatid heads from high dose and control males. In addition, one cauda epididymis from each F1 male will be immediately removed, weighed, and seminal fluid from the cauda will be assessed for sperm

number. Sperm number will be assessed using an HTM-IVOS (Version 10.8 S) automated sperm analysis system (Hamilton-Thorne Research, Beverly, MA).

3.6.3 Histology and Pathology

For F1 females, the ovaries, uterus, and thyroid with attached portion of trachea will be placed in Bouin's fixative for 24 hours, after which they will be rinsed and stored in 70% alcohol. Once the tissues are fixed, the trachea will be carefully removed fro the fixed thyroid (to retain glandular integrity). The tissues will then be embedded in paraffin. They will then be sectioned at 3-5 microns and stained with hematoxylin and eosin (H and E) for subsequent histological evaluations. Optional tissues for histopathology include the vagina (if warranted), which will be processed as above. Stained sections will be evaluated by a Board Certified veterinary pathologist for pathologic abnormalities and potential treatment-related effects. Thyroids should be evaluated for morphologic changes, such as altered follicular epithelial height, the relative number and staining characteristics of colloid, the extent of thyroid vascular supply, and the density, size, and shape of the thyroid follicles. Ovarian histology should include an evaluation of corpus luteum development and the presence of atretic follicles.

For F1 males, one testis, one epididymis, and the thyroid with attached portion of trachea will be dissected out and placed in Bouin's fixative for 24 hours, after which they will be rinsed and stored in 70% alcohol (with the trachea removed from the thyroid; see above) until embedded in paraffin. They will then be sectioned at 3-5 microns and stained with hematoxylin and eosin (H and E) for subsequent histological evaluations. Optional tissues for histopathology include the liver, paired kidneys, adrenal glands (paired), and pituitary, as indicated by altered organ weight (change of "significant magnitude"), which will be processed as above. Stained sections will be evaluated by a Board Certified veterinary pathologist for pathologic abnormalities and potential treatment-related effects. Thyroids should be evaluated for morphologic changes, such as altered follicular epithelial height, the relative number and staining characteristics of colloid, the extent of thyroid vascular supply, and the density, size, and shape of the thyroid follicles. The other testis per male will be frozen for subsequent evaluation. The one testis and epididymis per male (fixed) will be evaluated for spermatogenesis, spermiogenesis, status of seminiferous tubules in the testis, and sperm in the epididymis, as well as the structural integrity of these organs. The whole, unfixed epididymis per male will be used for evaluation of sperm count. The one frozen testis per male will be used to enumerate homogenization-resistent spermatid head for calculation of daily sperm production (DSP) and efficiency of DSP (Robb et al., 1978; Sharpe et al., 1995).

4.0 STATISTICAL ANALYSES

The unit of comparison will be the pregnant female, the litter, or the retained F1 offspring, as appropriate. Treatment groups will be compared to the concurrent control group using either parametric ANOVA under the standard assumptions or robust regression method (Zeger and Liang, 1986; Royall, 1986; Huber, 1967), which does not assume homogeneity of variance or normality. The homogeneity of variance assumption will be examined via Levene's test (Levene, 1960), which is more robust to the underlying distribution of the data than the traditional Bartlett's test. If Levene's test indicates lack of homogeneity of variance ($p<0.05$), robust regression

methods will be used to test all treatment effects. The robust regression methods use variance estimators that make no assumptions regarding homogeneity of variance or normality of the data. They will be used to test for overall treatment group differences, followed by individual tests for exposed vs. control group comparisons (via Wald Chi-square tests), if the overall treatment effect is significant. The presence of linear trends will be analyzed by GLM procedures for homogenous data or by robust regression methods for nonhomogenous data (SAS Institute Inc., 1989a,b, 1990a,b,c, 1996a,b, 1997). Standard ANOVA methods, as well as Levene's test, are available in the GLM procedure of SAS® Release 6.12 (SAS Institute Inc., 1989, 1996a,b, 1997), and the robust regression methods are available in the REGRESS procedure of SUDAAN® Release 7.5.3 (Shah et al., 1997).

If Levene's test does not reject the hypothesis of homogeneous variances, standard ANOVA techniques will be applied for comparing the treatment groups. The GLM procedure in SAS® 6.12 will be used to evaluate the overall effect of treatment and, when a significant treatment effect is present, to compare each exposed group to control via Dunnett's Test (Dunnett, 1955, 1964). For the litter-derived percentage data (e.g., periodic pup survival indices), the ANOVA will be weighted according to litter size. A one-tailed test (i.e., Dunnett's test) will be used for all pairwise comparisons to the vehicle control group, except that a two-tailed test will be used for parental and pup body weight and organ weight parameters, feed consumption, percent males per litter, and anogenital distance.

Frequency data such as offspring survival indices will not be transformed. All indices will be analyzed by the chi-square test for independence for differences among treatment groups (Snedecor and Cochran, 1967) and by the Cochran-Armitage test for linear trend on proportions (Cochran, 1954; Armitage, 1955; Agresti, 1990). When chi-square reveals significant ($p<0.05$) differences among groups, then a Fisher's exact probability test, with appropriate adjustments for multiple comparisons, will be used for pairwise comparisons between each treatment group and the control group. Acquisition of developmental landmarks (e.g., vaginal patency and preputial separation), as well as anogenital distance, will also be analyzed by analysis of covariance (ANCOVA; in addition to ANOVA analysis) using body weight at acquisition or measurement as the covariate. For correlated data (e.g., body and organ weights at necropsy, with more than one pup/sex/litter), SUDAAN® software (Shah et al., 1997) will be used for analysis of overall significance, presence of trend, and pairwise comparisons to the control group values.

A test for statistical outliers (SAS Institute, Inc., 1990b) will be performed on F0 maternal body weights, feed consumption (in g/day), and retained F1 male or female body and organ weights. If examination of pertinent study data do not provide a plausible biologically sound reason for inclusion of the data flagged as "outlier," the data will be excluded from summarization and analysis and will be designated as outliers. If feed consumption data for a given animal for a given observational interval (e.g., pnd 0-7 or 7-14 during the lactational exposure period) are designated outliers or unrealistic, then summarized data for this animal encompassing this period (e.g., pnd 0-21 for the lactational exposure period) also will not include this value. For all statistical tests, p # 0.05 (one- or two-tailed) will be used as the criterion for significance.

5.0 RETENTION OF SPECIMENS AND RECORDS

All specimens and records which remain the responsibility of RTI will be retained in the RTI archives for two years at the performing laboratory's expense. Beyond two years, continued retention will be at additional cost to the Sponsor.

6.0 QUALITY CONTROL/QUALITY ASSURANCE PROCEDURES

Quality control (QC) and quality assurance (QA) procedures will follow those outlined in the Quality Assurance Project Plan (QAPP) prepared for this study.

7.0 REPORTING

An executive summary will be prepared describing the number and strain of rats used in the study, the doses and chemicals tested, and the effects with levels of statistical significance for all endpoints. Electronic and hard copies of spreadsheets containing the raw data from all animals will be provided for each endpoint. In addition, the spreadsheet should include treatment means, standard deviation, standard error, coefficient of variation, and sample number below each endpoint. Data presented should include animal number and treatment, block and day of necropsy (if study conducted in blocks or animals killed on pnd 42 and 43), age and weight at vaginal opening, ovarian, uterine (with and without fluid), adrenal, liver, and body weights at weaning and necropsy, body weight change from pnd 22 to necropsy, and serum T4 and TSH. A data summary table containing the mean, standard deviation, standard error, coefficient of variation, and sample size for each treatment group should be provided for all endpoints. Organ weights may be presented after covariance adjustment for necropsy body weight, but this should not replace presentation of the unadjusted data. Summaries of any histopathologic findings with photomicrographs of significant observations will also be provided.

8.0 PERSONNEL

Study Director:	Julia D. George, Ph.D.
Project Toxicologist:	Rochelle W. Tyl, Ph.D., DABT
ARF Veterinarian:	Donald B. Feldman, D.V.M., ACLAM
ARF Manager:	Frank N. Ali, M.B.A., RLATG, ILAM
Laboratory Supervisor:	Melissa C. Marr, B.A., RLATG

Data Analyst and Reproductive Toxicity Supervisor:	Christina B. Myers, M.S.
Statistical Advisor:	Gayle. S. Bieler, M.S.
Research Data Entry Assistant:	Timothy W. Wiley, B.S.
Research Biologist:	William R. Ross, B.A.
Biologists:	Vickie I. Wilson Lawson B. Pelletier, RVMT, LAT
Biological Laboratory Assistants:	Charlene N. Beauman, B.S. Marian C. Rieth, RVMT Robin T. Krebs, LAT Melody P. Gower
Endocrinology:	Patricia A. Fail, Ph.D. Carol S. Sloan, M.S. Kristi D. Vick, B.S.
Andrology:	Patricia A. Fail, Ph.D. Carol S. Sloan, M.S. Kristi D. Vick, B.S. Timothy W. Wiley, B.S.
Histology:	Tsai-Ying Chang, B.S. HT-ASCP
Pathology:	John C. Seely, D.V.M., ACVP (EPL, Inc.)

Additional study team members to be determined.

9.0 STUDY RECORDS TO BE MAINTAINED

Protocol and any Amendments

List of any Protocol Deviations

List of Standard Operating Procedures

Animal Requisition and Receipt Records

Quarantine Records

Temperature and Humidity Records for the Animal Room(s)

Animal Research Facility Room Log(s)

Durham City Water Analysis (analyzed monthly, reported annually)

Feed Type, Source, Lot Number, Dates Used, Certification, Analytical Results

Dosage Code Records Containing Five-Digit Rx Code, Color Code, and Concentration

F0 Mating Records

F0 Maternal Gestational and Lactational Records

Dose Formulation Receipt and Use Records

F1 Distribution into Groups

F1 Dosing Forms

F1 Postwean Dosing Period:	Body Weights
	Clinical Signs
	Acquisition of Vaginal Patency
	Acquisition Preputial Separation
	Estrous Cyclicity
F1 Necropsy Records:	Body weight, organ weights, gross observations, required (and optional, if done) organ histopathology

Statistical Analysis Records

Histopathology Report

Serum Estradiol Analysis (E2)

Serum Thyroid Hormone Analyses (T4, TSH)

Correspondence

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ATTACHMENT

Material Safety Data Sheet (MSDS): Methoxychlor