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TITLE: Assessment of Pubertal Development and Thyroid Function in Juvenile Female CD® (Sprague-Dawley) Rats After Exposure to Selected Chemicals Administered by Gavage on Postnatal Days 22 Through 42/43

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AMENDMENTS:

Number	Date	Section(s)	Page(s)
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Attachment: Material Safety Data Sheets (MSDSs):

1.0 OBJECTIVE AND BACKGROUND

The objective of this study is to examine the sensitivity of pubertal assays to the effects of a wide variety of chemicals that are known to affect the endocrine system through different pathways and/or mechanisms of action.

The Food Quality Protection 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 Endocrine Disruptor Screening and Testing Advisory Committee (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, recommended the use of a female 20-day pubertal assay with thyroid to evaluate test materials for effects on the thyroid, hypothalamic-pituitary-gonadal (HPG) axis, aromatase, 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 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 estrus 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). 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 endocrine disruptor screening program is a female pubertal assay (see FR Vol. 63, No. 248, pp. 71541-71568, December 28, 1998). This assay is the most comprehensive assay in the proposed Tier 1 battery of assays, as it is capable of detecting substances that alter thyroid function, that are aromatase inhibitors, estrogens, antiestrogens, and that are agents which interfere with the hypothalamus-pituitary-gonadal axis. 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.

EPA has already tested the response of the pubertal assays to a variety of chemicals, but at only one dose per chemical (Rocca and Borst, 2000; Rocca and Pepperl, 2000a,b,c). EPA is in the process of testing the response of two chemicals (vinclozolin and methoxychlor) at multiple doses to determine the sensitivity of the assays to subtle effects of estrogens and antiandrogens.

Although the experiments that have been completed or are in progress are believed to be sufficient to demonstrate the usefulness of these pubertal assays for a wide variety of chemicals, EPA feels that additional multiple-dose studies across an array of chemicals will provide greater confidence in the reliability and relevance of the assays. Therefore, EPA has decided to test ten additional chemicals that have various modes of action. Some chemicals will be tested in both sexes, some chemicals will be tested in only males, while others will be tested only in females.

2.0 MATERIALS AND METHODS

2.1 Test Substances

2.1.1 Atrazine

CAS Number: 1912-24-9

2.1.2 Propylthiouracil

CAS Number: 51-52-5

2.1.3 Ketoconazole

CAS Number: 65277-42-1

2.1.4 Fenarimol

CAS Number: 60168-88-9

2.1.5 Octylphenol

CAS Number: 140-66-9

2.1.6 Bisphenol A

CAS Number: 80-05-7

2.1.7 Letrozole

CAS Number: 112809-51-5

NOTE: All additional information on the test chemicals (e.g., supplier, batch/lot number, purity, appearance, molecular formula, molecular weight, 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 MSDSs of all chemicals 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:

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

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 (postnatal day [pnd] 22) and on the first pnd 28, 35, and 42 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 study does not unnecessarily duplicate any previous study.

2.4.3 Total Number, Age, and Weight

For the seven-group component of this study (see Table 1), 20 timed-pregnant female rats (designated the F0 generation) will be purchased for this study, at ten to 12 weeks of age upon arrival. They will arrive at RTI on gestational day (gd) 14 (based on the vendor's designation of the day of insemination as gd 1), which is gd 13 (based on the performing laboratory's designation of the day of insemination as gd 0). One hundred five (105) female rat offspring, designated the F1 generation, will be placed on study at weaning (pnd 21), weighing approximately 40-50 grams (i.e., seven groups of 15 F1 weanling females each). For the nine-group component of this study (see Table 1), 25 timed-pregnant female rats will be purchased as described above. One hundred thirty-five (135) female rat offspring, as described above, will be placed on study at weaning (i.e., nine groups, 15 F1 weanling females each).

2.4.4 Quality Control

The shipment of pregnant females will be quarantined on arrival, and quality control evaluation will be initiated within one day after receipt. Within one day after receipt, two 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 selection of F1 weanling study females, four unselected female rats (or, if necessary, one to four remaining males) will be randomly selected, eartagged, and designated as sentinels. 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 F1 female necropsies, the surviving sentinel females (and/or males) 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

The initial F0 timed-pregnant females will be quarantined for approximately one week (gd 13-20), 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 the 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. Pregnant and lactating females and F1 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 cages. Pelleted feed (No. 5002 Purina Certified Rodent Chow®) and tap water from the Durham, NC water system, in plastic bottles with stainless steel sipper tubes, will be available *ad libitum* for the pregnant F0 females during quarantine, for the F0 females during the rest of gestation and lactation, and for the retained F1 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 60-70°F, 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 64-79°F (18-26°C) 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

All F0 maternal rats will be individually identified by ear tag after arrival at RTI. All selected study weanling F1 females will also be uniquely identified by eartag at weaning, as well

as receiving a female study number. 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 and discarded.

3.0 EXPERIMENTAL DESIGN

3.1 Study Design

The study will be conducted in two components. Each component will consist of two dose groups per test material (and three test materials) and one vehicle control group, each group comprised of 15 weight-matched F1 female weanlings, for each of the two components of this study. The F1 study females will be dosed by gavage once daily for 21-22 consecutive days (pnd 22 to pnd 42 or 43). Table 1 presents the study design and target doses of the test chemicals. 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)

F0 timed-pregnant females arrive at RTI:
Parturition of F1 offspring (pnd 0):
Weaning of F1 offspring (pnd 21):
Sacrifice of F0 dams:
Dosing (pnd 22 - pnd 42/43):
Sacrifice of F1 females (on pnd 42 or 43)::
Submission of nonaudited draft final report:
Submission of audited draft final report:

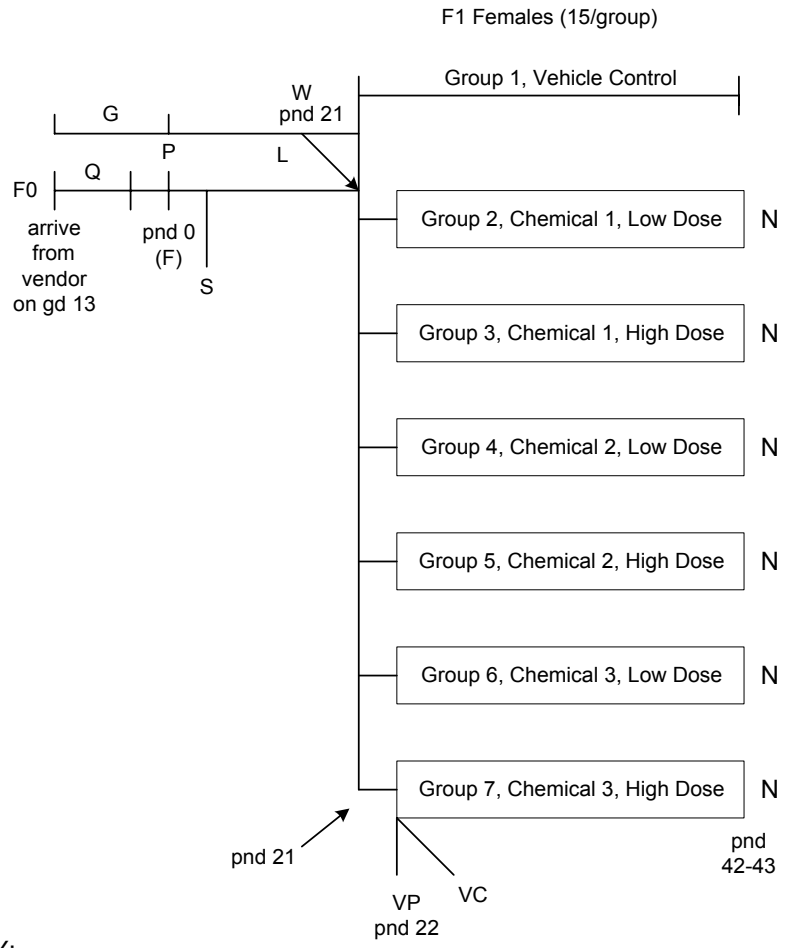
[a] The end dates are tentative and will depend on the duration of gestation and lactation of the F0 dams with F1 offspring.

Table 1. Study Design and Target Doses

Group No.	No. F1 Females	Chemical	Dose (mg/kg/day)	Concentration (mg/ml)	Dose Volume (ml/kg)
COMPONENT 1					
1	15	–[a]	0	0.0	5.0
2	15	Atrazine	75	15.0	5.0
3	15		150	30.0	5.0
4	15	Propylthiouracil	2	0.4	5.0
5	15		25	5.0	5.0
6	15	Letrozole	0.3	0.06	5.0
7	15		3.0	0.6	5.0
COMPONENT 2					
8	15	Fenarimol	50	10.0	5.0
9	15		250	50.0	5.0
10	15	Octylphenol	200	40.0	5.0
11	15		400	80.0	5.0
12	15	Bisphenol A	100	20.0	5.0
13	15		400	80.0	5.0
14	15	Ketoconazole	50	10.0	5.0
15	15		100	20.0	5.0
16	15	–[a]	0	0.0	5.0

[a] Corn oil, vehicle control

Figure 1. Study Design for Female Pubertal Assay



KEY:

——— No exposures to F0 dams or F1 offspring during gestation or lactation

▭ Direct once daily gavage dosing of F1 females starting on pnd 22 (see text)

Q = Quarantine (seven days, gd 13-20)

G = Gestation

P = Parturition (pnd 0)

L = Lactation

W = Wean (pnd 21) F1 pups; euthanize and discard F0 dams

F = Foster pups, if necessary, to maximize retention of F1 female pups

S = Standardize litters to ten with maximum number of F1 female pups (discard culled pups)

VP = Acquisition of vaginal patency (evaluation will begin on pnd 22)

VC = Vaginal cytology (evaluation will begin on the day of VP)

N = Necropsy (see text)

3.2 Dosage Selection

The U.S. EPA selected all seven chemicals for evaluation and selected the high and low target doses for three of them (atrazine, which affects the hypothalamus; ketoconazole, which inhibits steroidogenesis; and octylphenol, a weak "environmental estrogen" which binds to the estrogen receptor). The RTI Project Toxicologist selected the high and low target doses for propylthiouracil (which affects the thyroid, causing hypothyroidism), fenarimol (a weak aromatase inhibitor), bisphenol A (a weak "environmental" estrogen which binds to the estrogen receptor) and Letrozole, an aromatase inhibitor. The rationale for the selection of doses by the RTI Project Toxicologist is as follows.

3.2.1 Propylthiouracil (PTU)

The target doses selected were 2 and 25 mg/kg/day. Duarte et al. (2000) gavaged rats (180±10 g) daily with PTU doses of 1-50 mg/200 g body weight (equivalent to 5-250 mg/kg) for one to 30 days. The high dose, 250 mg/kg, after 30 days resulted in profound hypothyroidism (based on T3 and T4 levels). O'Connor et al. (1999) employed SD rats and ip injection (which, at least partially, circumvents first-pass metabolism in the liver) of 0, 0.025, 0.25, 1.0, or 10.0 mg/kg/day (in 0.25% methyl cellulose) and reported effects on thyroid gland weight and histology and serum thyroid hormone analyses. The authors considered these parameters as "the most reliable endpoints for identifying thyroid gland toxicants in a short-duration screening battery." Radovsky et al. (2000) reported morphometric effects of PTU on regions of the developing brain of rat offspring on pnd 11 from gavage administration to CD (SD) rat dams on gd 6 through pnd 10 at 38 mg/kg/day (in a developmental neurotoxicity study). Wilen et al. (1981) exposed female SD rats to PTU from weaning to acquisition of vaginal opening, at 0.001, 0.01, or 0.1% in the diet, equivalent to 10, 100, and 1000 ppm (and 18, 18, and 180 mg/kg/day, respectively). The growth rate was significantly reduced at all doses. Age at vaginal opening was delayed, body weight and vaginal opening was reduced, and pubertal body weights were reduced at 0.1%. Serum T4 and T3 were greatly reduced at all PTU doses. In previous pubertal assays in female rats (Rocca and Pepperl, 2000c; Rocca and Borst, 2000), the single oral PTU dose in both studies was 240 mg/kg/day, with profound systemic and thyroid-mediated toxicity. All remaining studies identified in the open literature used dosed drinking water as the route of administration of PTU.

3.2.2 Fenarimol

The target doses selected were 50 and 250 mg/kg/day. A single (or three or five) oral dose(s) to rats of 62.5 or 125 mg/kg/day caused dose-dependent liver growth (Palut et al., 1992). Intraperitoneal injection of 150 mg/kg/day in mice resulted in liver enlargement and induction of P450 CYP2B1 isoforms (Hrelia et al., 1994). Fenarimol, administered by gavage at 250 mg/kg/day (in olive oil) in rats for 26 weeks induced liver enzymes in partially hepatectomized animals (Palut et al., 1997). Fenarimol has also been shown to induce infertility in offspring male rats when administered to the Wistar dams during gestation and lactation. The authors suggested that fenarimol acted centrally (i.e., via the hypothalamus) to decrease male sexual behavior,

thereby decreasing male fertility (Hirsch et al., 1986, 1987). Paolini et al. (1996) administered fenarimol ip at 200 or 400 mg/kg to male and female SD rats. A complex pattern of P450 CYP induction, with sex- and organ-related differences, was reported in the kidney and liver. The authors considered fenarimol as "a possible cotoxic, comutagenic, carcinogenic, and promoting" fungicide. Fenarimol was also administered by oral gavage at 35 mg/kg/day to Sprague Dawley and Wistar male and female rats in a reproductive toxicity assessment. Dosing to one or the other sex was for 28 days prior to mating and seven days during mating, with females dosed through gestation and lactation. In both rat strains, fertility was reduced when only males were treated, and treatment of only females resulted in dystocia and impairment of parturition, resulting in reduction in live litter size at birth and in early postpartum survival of the offspring (Eli Lilly and Co., 1992).

3.2.3 Bisphenol A (BPA)

The target doses selected were 100 and 400 mg/kg/day. Kwon et al. (1999) dosed maternal SD rats with BPA from gd 11 through pnd 21 at 0, 3.2, 32, or 320 mg/kg/day (DES at 15 mg/kg/day was the positive control). F1 female offspring were evaluated at four months of age. BPA did not affect estrous cyclicity or ovarian morphology at any dose evaluated. There were also no apparent adverse effects from BPA on female rat pubertal development or reproductive function (Kwon et al., 2000).

Markey et al. (2001) administered BPA to immature female CD-1 mice at 0, 0.1, 5, 75, and 100 mg/kg/day for three days. They reported that BPA caused a uterotrophic response (increased uterine wet weight) at 100 mg/kg/day; it induced lactoferrin (as estrogen-dependent uterine protein) at 75 and 100 mg/kg/day and accelerated vaginal opening at 0.1 and 100 mg/kg/day, but not at the intermediate doses.

Yamasaki et al. (2000) administered BPA sc (subcutaneously, which circumvents first pass hepatic metabolism) at 0, 8, 40, and 160 mg/kg/day or orally at 0, 40, 160, and 800 mg/kg/day for three days, beginning on pnd 18. Rats were sacrificed 24 hours after the last dose. BPA by sc injection produced a uterotrophic effect at all doses. BPA by oral administration increased uterine relative weight at 160 and 800 mg/kg/day, and wet and blotted weights only at 800 mg/kg/day. The study was repeated with essentially the same results (Yamasaki et al., 2000).

Nagao et al. (1999) treated male and female rat pups with BPA sc at 300 μg/g (equivalent to 300 mg/kg) on pnd 1-5 and reported no alterations in function or histopathology of reproductive organs, and no changes in the volume of the SDN-POA (sexually dimorphic nucleus of the preoptic area of the brain; usually four-fold larger in males). The positive control (estradiol benzoate) at 2 μg/g (2 mg/kg) affected all male and female reproductive endpoints evaluated.

Ashby and Tinwell (1998) administered BPA by gavage (or by sc injection) to immature female rats for three days at 400, 600, or 800 mg/kg/day. BPA by sc injection accelerated vaginal patency at 600 and 800 mg/kg/day; BPA administered orally had no effect on vaginal patency.

Liaw et al. (1997) dosed pregnant SD rats by gavage on gd 2 through pnd 10 at 0, 160, or 320 mg/kg/day. The size of the male offspring SDN-POA on pnd 10 was unaffected by BPA. In offspring females on pnd 10, the size of the SDN-POA was 85% larger than in control females.

Laws et al. (2000) reported that BPA (200 mg/kg/day, po) induced a significant uterotrophic response within three days in prepubertal rats; doses up to 400 mg/kg/day from pnd 21-35 failed to accelerate puberty. Female body weights at necropsy at ≤400 mg/kg/day were unaffected.

Tyl et al. (2001) exposed CD (SD) rats to BPA continuously in the feed through three offspring generations at concentrations from 15 ppb to 7500 ppm. Systemic toxicity was obvious at 7500 and 750 ppm (resulting in approximate BPA intakes of 500 and 50 mg/kg/day, respectively); reproductive effects (reduced absolute and relative ovarian weights and reduced litter sizes at birth) occurred only at 7500 ppm. The NOAEL (no observable adverse effect level) for systemic toxicity was therefore 5 mg/kg/day (75 ppm), and the reproductive effects NOAEL was 50 mg/kg/day (750 ppm). Animals exposed by dietary (or drinking water) exposure can tolerate higher intake levels (since they "self-dose" by eating or drinking intermittently throughout the exposure period) than they can from gavage exposure (one bolus dose once per day), most likely due to the capacity to metabolize compounds (primarily in the liver) over time from intermittent exposures versus a large bolus dose. Therefore, the Project Toxicologist selected BPA target doses of 100 and 400 mg/kg/day administered by gavage, which should be tolerated by the rats and provide some "estrogenic" effects, certainly at 400 mg/kg/day and likely at 100 mg/kg/day.

3.2.4 Letrozole

There are two classes of P450 aromatase inhibitors: (1) Type I agents are steroids and irreversible inhibitors, and (2) Type II agents are nonsteroidal and reversible inhibitors. Letrozole (CGS 20267) is a Type II aromatase inhibitor which can be administered orally and interferes with the aromatase heme (Murphy, 1998). It has a high affinity for its target and has no effects on adrenal steroidogenesis (Reddy, 1998; Bhatnagar et al., 1990; Brueggemeier, 2001). The usual human dose is 2.5 mg (oral tablet or i.v. injection), equivalent to approximately 0.05 mg/kg in men (Bhatnagar et al., 1992; Soufi et al., 1997a) and women (Soufi et al., 1997b; Elisafet et al., 2001). Most of the papers involving animal models in the published literature employed dosed drinking water as the route of administration of letrozole. However, a few studies were identified which employed gavage administration and rats as the subject species. Schieweck et al. (1993) dosed adult female rats with 3 to 3000 µg/kg (the last dose is 3 mg/kg/day) by gavage once daily for six weeks. After six weeks of treatment, the ED50 for suppression of tumor volume was 300 µg/kg/day (0.3 mg/kg/day), accompanied by disruption of ovarian cyclicity (at the top dose, rats were in constant diestrus), reduced uterine weight, and suppressed serum estradiol at ≥ 300 µg/kg/day. Serum LH was elevated three- to four-fold at ≥ 300 µg/kg/day. Intact male rats were orally dosed with letrozole at 1 mg/kg/day for seven days. There were no effects on GnRH-induced calcium signals, serum LH concentration, or the weight of the ventral prostate or testes

(along with other data, this indicates that these parameters are not under estradiol control but are under DHT control) (Tobin and Canny, 1998). Sintra et al. (1998) administered letrozole to cycling female rats by gavage at doses up to 5.0 mg/kg/day for four weeks. The maximum effects were observed at 5.0 mg/kg/day and included uterine weight reduced to that after ovariectomy, and ovarian tissue estradiol levels inhibited by approximately 80%. As anticipated, the levels of ovarian androstenedione (the substrate for aromatase) were increased by an order of magnitude, and plasma LH and FSH were also increased (associated with an increase in ovarian weight). Bhatnagar et al. (1993) administered letrozole by gavage at 1.0 mg/kg/day once daily, for 14 days, to adult female cycling rats. In the treated rats, serum estradiol was reduced by 76%, and serum LH was elevated to 378% of controls. FSH was unchanged (125% of control value). Uterine weight was reduced by 60%. Bhatnagar et al. (1990) administered letrozole orally to ACTH-treated rats at 4 mg/kg (1000 times higher than the ED50 for aromatase inhibition), with no effects on plasma levels of adrenal corticosterone or aldosterone. Oral administration of letrozole to adult female rats at 1.0 mg/kg for 14 days completely interrupted ovarian cyclicity and suppressed uterine weight to that observed 14 days after ovariectomy. In adult female rats bearing DMBA-induced mammary tumors, 0.1 mg/kg letrozole, administered orally once daily for 42 days, caused almost complete regression of the tumors (Bhatnagar et al., 1990).

Therefore, the Project Toxicologist recommends the target oral doses of letrozole as 0.3 mg/kg/day (low) and 3.0 mg/kg/day (high), which should result in loss of estrous cyclicity, reduced uterine weight, possibly increased ovarian weight, reduced circulating estradiol, and possibly increased LH at the high dose with lesser effects at the low dose.

3.3 F0 Dams and F1 Litters Prior to Weaning

3.3.1 F0 Maternal Parturition and Lactation

Beginning on gd 20, each female will be examined twice daily (a.m. and p.m.) for evidence of littering. Females who are littering at morning and afternoon checks will have this information recorded on the gestational sheet. 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 euthanized by CO_2 and discarded. Any dams whose whole litters are born dead or die prior to pnd 21 will be sacrificed, and the number of uterine implantation scars will be recorded.

3.3.2 Necropsy of F0 Females

On pnd 21 of each F1 litter, each F0 dam will be euthanized by CO_2 asphyxiation. 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.

3.3.3 F1 Progeny

3.3.3.1 Mortality, Body Weights, and Clinical Observations. All pups will be counted, sexed, weighed, and examined as soon as possible on the day of birth (designated as pnd 0) to determine the number of viable and stillborn members of each litter. Thereafter, litters will be evaluated for survival, sex, gross observations, and body weights on pnd 4, 7, 14, and 21. Any pup which appears moribund or dies during lactation will be necropsied, when possible, to investigate the cause of death and discarded. No organs will be weighed or saved.

3.3.3.2 Standardization of Litter Sizes. On pnd 4, the size of each litter will be adjusted to ten pups, maximizing the number of female pups retained. Natural litters with ten or fewer pups will not be culled. If necessary, F1 female pups from litters with more than six females will be fostered to litters containing fewer than six females on pnd 4. All culled pups will be sacrificed by decapitation. The F0 dams will be allowed to rear their remaining F1 young to pnd 21. On pnd 21, each litter will be weaned.

3.4 Selection of F1 Weanling Females

When each F1 litter has reached pnd 21, the F1 females from each pnd 21 (wean) date will be weight ranked across litters (outliers, i.e., heaviest and lightest pups, will be eliminated from selection). The selected females will then be eartagged and distributed across the seven groups by stratified randomization (e.g., one of the seven heaviest selected females will go into each of the seven treatment groups, etc.). Of the remaining F1 females, four will be eartagged and selected as sentinels. If not enough unselected females are available, then the remaining F1 males will be used as sentinels (to obtain a total of four); see Section 2.4.5.

3.5 Treatment of F1 Weanling Females

Beginning on pnd 22, each F1 female will be dosed with one of the test materials at one of the dose levels or the vehicle control (corn oil for all chemicals). Each animal will be weighed every day prior to treatment and the body weight recorded. Treatments are administered daily by oral gavage using an 18-gauge gavage needle (1 inch length with 2.25 mm ball) and a 1 cc glass (disposable) tuberculin syringe for each treatment, from pnd 22 and continuing through pnd 42/43. This duration of treatment is unnecessary to detect estrogenic chemicals but is required for the detection of pubertal delay and antithyroid effects. Xenobiotics are administered in corn oil vehicle at a dosing volume of 5.0 ml/kg body weight at 0700-1000 daily. 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. It is important that any dosing solutions/suspensions be well mixed to keep the chemical in suspension prior to and throughout dosing.

3.6 Observation of F1 Weanling Females

3.6.1 Clinical Observations

Clinical observations of F1 female study animals will be documented at least once daily on pnd 21 (prior to dosing period) and at least twice daily, at dosing and one to two hours postdosing, throughout the dosing period (pnd 22 through pnd 42 or 43). The examining technicians will be unaware of the test materials or of dosage levels. Observations will be made for (but not limited to):

a. Any response with respect to body position, activity, coordination, or gait
b. Any unusual behavior such as head flicking, compulsive biting or licking, circling, etc.
c. 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

3.6.2 F1 Weanling Female Body Weights

All F1 females will be weighed in the morning on pnd 21 and every day in the morning during the dosing period on pnd 22 through pnd 42/43, for adjustment of dosing volume based on the most recent body weight. Body weights will be reported and statistically analyzed for pnd 21, 22, 28, 34, 40, 42, and 43. F1 female weight gains will be calculated and analyzed for pnd 21-22, 22-28, 28-34, 34-40, 40-42, 42-43, and 22-42/43 (treatment period). F1 female body weights will also be recorded on the day of acquisition of vaginal patency (see Section 3.6.3).

3.6.3 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.

3.6.4 Estrous Cyclicity

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 first estrus, 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; prolonged diestrus as the presence of leukocytes for four or more days.

3.7 Necropsy of F1 Offspring Females

3.7.1 Terminal Blood Collection

At scheduled necropsy of the F1 females, after terminal anesthesia (CO_2 asphyxiation), the females 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 subdivided into two aliquots and frozen at approximately -20°C:

a. One milliliter from each animal for analysis of thyroxine (T4) and thyroid stimulating hormone (TSH) at RTI.

b. Remaining serum from each animal shipped frozen, for possible subsequent analyses, to:

Ralph L. Cooper, Ph.D.
Chief, Endocrinology Branch, MD-72
RTD, NHEERL, US EPA
Research Triangle Park, NC 27711
Cooper.ralph@epa.gov
Phone: 919-541-4084 Fax: 919-541-5138

3.7.2 Gross Necropsy and Organ Weights

Once each F1 female is bled (see Section 3.7.1), she will be necropsied and internal thoracic and abdominal organs and cavities examined. Any abnormalities will be documented. The following organs will be dissected out: ovaries (paired), uterus (see below), thyroid (with attached portion of trachea), and pituitary. 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, and gently blotted dry and reweighed.

Optional weights of the following organs will be taken if warranted: liver, kidneys (paired), pituitary (fixed), adrenal glands (paired). All organs will be weighed to the nearest 0.1 mg. Adrenal glands, if taken, will be weighed immediately (to minimize drying out of tissues). The pituitaries will be weighed after fixation of the head minus the lower jaw, and the thyroid will be weighed after fixation and removal of the attached trachea.

3.7.3 Histology and Pathology

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 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 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.

4.0 STATISTICAL ANALYSES

All data for a single chemical (two doses) and concurrent vehicle control group (weaning body weight, body weights and weight gains, age and weight at vaginal opening, body and organ weights at necropsy, and serum hormones) will be analyzed using either parametric ANOVA under the standard assumptions or robust regression methods (Zeger and Liang, 1986; Royall, 1986; Huber, 1967) which do not assume homogeneity of variance or normality. The homogeneity of variance assumption will be examined via Levene's test (Levene, 1960). 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 linear trends across dose as well as overall treatment group differences (via Wald chi-square tests). Significant overall treatment effects will be followed by single degree-of-freedom *t*-tests for exposed vs. control group comparisons, if the overall treatment effect is significant. 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® Version 6 (SAS Institute, Inc., 1989a,b; 1990a,b,c; 1996; 1997) or 8 (SAS Institute, Inc., 1999a,b,c,d,e; 2000) will be used to test for linear trend, 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). Standard ANOVA methods, as well as Levene's Test, are available in the GLM procedure of SAS®, and the robust regression methods are available in the REGRESS procedure of SUDAAN® Release 7.5.4 (Shah et al., 1997) or Release 8.0 (RTI, 2001). Organ weights will also be analyzed by Analysis of Covariance (ANCOVA) using the body weight at necropsy as the covariate. When statistically significant effects are observed, treatment means will be examined further using LSMeans.

The unit of comparison will be the weanling F1 female offspring on study.

A test for statistical outliers will be performed in the UNIVARIATE procedure of SAS® Version 6 (SAS Institute, Inc., 1989a,b; 1990a,b,c; 1996; 1997) or 8 (SAS Institute, Inc., 1999a,b,c,d,e; 2000) on F1 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. For all statistical tests, $p \leq 0.05$ (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 V. Rieth, RVMT Dee A. Wenzel, RVMT, LATG Robin T. Krebs, ALAS
Endocrinology:	Patricia A. Fail, Ph.D. Carol S. Sloan, M.S. Kristi D. Vick, 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 from Vendor
F0 Maternal Gestational and Lactational Records
Dose Formulation Receipt and Use Records
F1 Female Distribution into Groups
F1 Dosing Forms
F1 Female Postwean Dosing Period: Body Weights
Clinical Signs
Acquisition of Vaginal Patency
Estrous Cyclicity
F1 Female Necropsy Records: Body weight, organ weights, gross observations, required (and optional, if done) organ histopathology, TSH and T4 serum levels
Statistical Analysis Records
Histopathology Report
Serum Thyroid Hormone Analyses (T4, TSH)
Correspondence

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ATTACHMENT

Material Safety Data Sheets (MSDSs)