FINAL REPORT

Volume 1 of 2
(Text, Figures 1-3, Tables 1A-9 and Appendices A-H)

STUDY TITLE

VALIDATION OF THE PUBERTAL FEMALE ASSAY IN RATS (WA 4-14)

EPA CONTRACT NUMBER

68-W-01-023

STUDY NUMBER

WIL-431004

STUDY DIRECTOR

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STUDY INITIATION DATE

5 January 2005

STUDY COMPLETION DATE

13 December 2005

PERFORMING LABORATORY

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COMPLIANCE STATEMENT

This study, designated WIL-431004, was conducted in compliance with the United States Environmental Protection Agency (EPA) Good Laboratory Practice Standards (40 CFR Part 792, 18 September 1989 and 40 CFR Part 160, 16 October 1989); the standard operating procedures of WIL Research Laboratories, LLC, and the protocol as approved by the Sponsor.

13 DEC 2005

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Date

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1. EXECUTIVE SUMMARY

1.1. PURPOSE AND OBJECTIVE

The purpose of the study was to participate in an interlaboratory validation of the female pubertal assay and present the results from WIL Research Laboratories, LLC to be used for the subsequent determination of whether independent laboratories arrive at the same conclusion about the ability of substances to interact with the endocrine system using this study design.

The objective of this study was to quantify the effects of test substances on pubertal development and thyroid function in the juvenile/peripubertal female rat.

1.2. STUDY DESIGN

Breeder females (experimentally naive time-mated female Crl:CD®(SD)[1] rats) that were between gestation days 7 and 10 were obtained for this study from Charles River Laboratories, Inc., Portage, Michigan. The breeder females were housed in plastic maternity cages containing nesting material (heat-treated laboratory-grade pine shavings) in a controlled environment. Breeder females were observed twice daily for changes in general appearance and behavior and were allowed to deliver naturally. Litters were standardized to 8 to 10 pups per litter on postnatal day (PND) 4. Offspring clinical observations, body weights, viability and sex were recorded at appropriate intervals. Any unthrifty litters or runted pups were excluded from the study on PND 4 (prior to culling) or on PND 21 (prior to randomization). Breeder females were euthanized and discarded following weaning of the offspring.

On PND 21, all surviving female pups (male pups were used for a pubertal male assay; Bowman, 2005) were weaned and randomly assigned to 1 of 7 groups, with subsequent selection of 15 rats/group with body weights in the middle of the distribution per group and across groups. Selected juvenile rats were housed 2 to 3 animals per cage in plastic

[1] Prior to 1 January 2005, this strain of rat was designated the Crl:CD®(SD)IGS BR rat.

maternity cages in an environmentally controlled room. Beginning on PND 22, the juvenile rats were administered the vehicle (corn oil) or 1 of 3 test substances (DE-71 [30 or 60 mg/kg], 2-chloronitrobenzene [25 or 100 mg/kg] or methoxychlor [12.5 or 50 mg/kg]), once daily by oral gavage until PND 42. The dosage volume for all groups was 2.5 mL/kg, and dosing occurred between 7:00 a.m. and 8:57 a.m. All animals were observed twice daily for appearance, behavior, mortality and moribundity. Detailed physical examinations, post-dose observations and body weights were recorded daily from PND 21 to PND 42. Each female pup was observed daily for vaginal perforation beginning on PND 22 and continuing until vaginal perforation was complete. For estrous cycle evaluations, vaginal lavages were performed daily, beginning on the day that vaginal perforation was complete and continuing until the day of necropsy. Approximately 2 hours following dosing on PND 42, all females were euthanized by exposure to carbon dioxide for up to 60 seconds followed by decapitation. Trunk blood was collected from each female and centrifuged; serum levels of thyroxine (T_4) and thyroid stimulating hormone (TSH) were determined. A complete necropsy was conducted on all females; selected tissues were weighed and examined microscopically. All in-life and post mortem activities were conducted blind to treatment group.

1.3. RESULTS

1.3.1. DE-71

There was a slight delay (1.6 days) in the mean age of complete vaginal opening at 60 mg/kg/day DE-71 compared to the concurrent controls, which is consistent with a mean delay of 1.8 days in pubertal Wistar rats at this dose of DE-71 (Stoker, 2004). The increased follicular epithelial height and decreased colloid area of the thyroid in both DE-71 dose groups observed in the current study is also consistent with this previous report in pubertal Wistar rats dosed with 60 mg/kg/day DE-71 (Stoker, 2004). Although the mean thyroid weights in the current study were not elevated in the DE-71 groups compared to the control group, the mean serum T_4 was statistically significantly decreased and the mean serum TSH was statistically significantly increased at 30 and

60 mg/kg/day DE-71. Decreased serum T_4 in rats treated with similar doses of DE-71 is consistent with previous reports (Stoker, 2004; Zhou, 2001). The increase in mean serum TSH was also observed previously in the 20-day female pubertal assay, although the increase was not statistically significant in that assay. The mechanism of serum T_4 reduction is not completely known, although DE-71 is an inducer of hepatic biotransformation enzymes, suggesting that T_4 glucuronidation and elimination was one factor contributing to the reduction (Zhou, 2001). In this study, mean absolute and PND 21 body weight-adjusted liver weights were statistically significantly increased in a dose-related manner in the DE-71 treated groups, supporting a possible effect on enzyme induction.

1.3.2. 2-CHLORONITROBENZENE

There was a transient decrease in mean body weight gain during the first week of dose administration, but in the following 2 weeks the 100 mg/kg/day 2-chloronitrobenzene group of animals recovered and had final body weights similar to controls. There was a statistically significant delay in the mean age of vaginal opening (2.6 days later than the control group) and a corresponding increase in mean body weight at that age in the 100 mg/kg/day group. There is no indication that 2-chloronitrobenzene impacts the number of estrous cycles or cycle length at the dosage levels tested; rather, the differences in the estrous measurements calculated from this bioassay are likely secondary to the delay in vaginal opening. Serum TSH was increased 14% and 29% in the 25 and 100 mg/kg/day 2-chloronitrobenzene groups compared to the control group. The relevance of this apparent increase in serum TSH is unclear in the absence of any effects on T_4, thyroid weights or thyroid histopathology. There was a statistically significant increase in absolute and adjusted mean liver weights in both 2-chloronitrobenzene dose groups, and a statistically significant decrease in mean absolute and PND 21 body weight-adjusted weight of the adrenal glands in the high dose group compared to the control group.

1.3.3. METHOXYCHLOR

Treatment with 50 mg/kg/day methoxychlor decreased mean body weight gains slightly, resulting in a 5.8% decrease in mean final body weight compared to the control group (not statistically significant). Complete vaginal opening was observed an average of 7 days earlier in the 50 mg/kg/day methoxychlor group (mean age of PND 27.1) compared to the control group. Secondary to the younger age, the mean body weight at that age in these high dose females was also significantly decreased (36%) compared to the control group. There is no indication that methoxychlor impacts the number of cycles or cycle length at the dosage levels tested, rather differences in the estrous measurements calculated from this bioassay are likely secondary to the acceleration in vaginal opening exclusively. There were no effects on the serum T_4, serum TSH or thyroid histopathology that were related to methoxychlor treatment. Slight hypertrophy of the uterus was observed in the methoxychlor groups. However, due to lack of differences in histologic appearance of the ovary, and the lack of statistically significant organ weight changes, the slight differences described in the uterus of the methoxychlor animals were likely a result of biologic variation.

1.4. CONCLUSIONS

The most sensitive endpoints in this study at the doses tested were serum T_4, serum TSH, thyroid histopathology and absolute and adjusted mean liver weights for DE-71 (30 mg/kg/day), absolute and adjusted mean liver weights for 2-chloronitrobenzene (25 mg/kg/day), and vaginal opening for methoxychlor (50 mg/kg/day). The results of the current study are consistent with available data from previous studies and are considered acceptable for interlaboratory comparisons for the purposes of validating this female pubertal bioassay across laboratories.

2. INTRODUCTION

2.1. GENERAL STUDY INFORMATION

This report presents the data from "Validation of the Pubertal Female Assay in Rats (WA 4-14)".

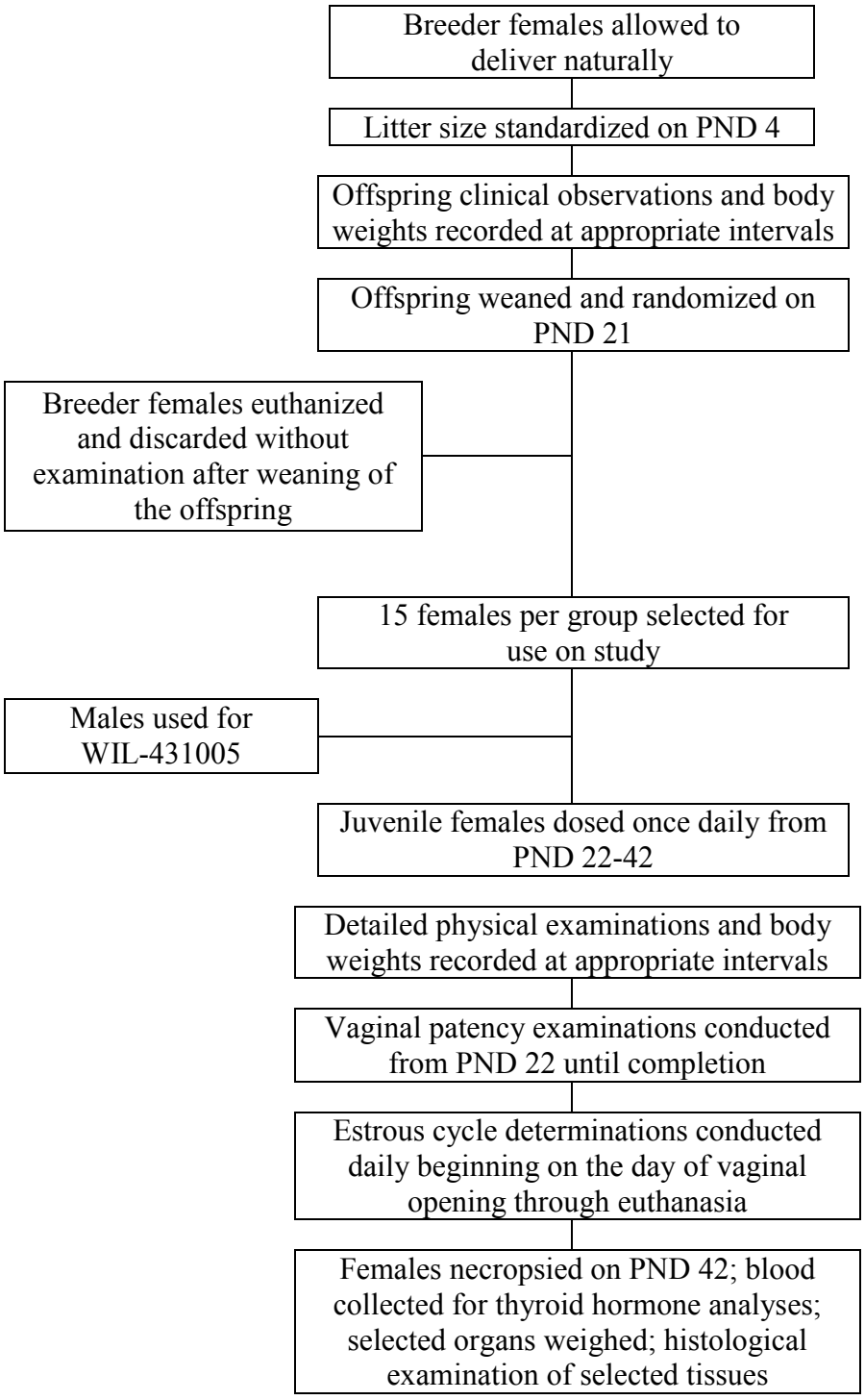
The following computer protocols were used for data collection during the study:

Computer Protocol	Type of Data Collected
WIL-431004P	Breeder female and pretest offspring data
WIL-431004	Main study data

2.2. KEY STUDY DATES

Date(s)	Event(s)
6 January 2005	Experimental starting date (breeder female receipt)
17-21 January 2005	Breeder female parturition
9 February - 3 March 2005	Dosing period (PND 22-42)
9 February - 3 March 2005	Developmental indices (vaginal patency)
13 February - 3 March 2005	Estrous cycle determinations
1-3 March 2005	Necropsies (PND 42)
19 July 2005	Experimental termination date (last TSH analysis)

3. STUDY DESIGN



4. EXPERIMENTAL PROCEDURES - MATERIALS AND METHODS

4.1. TEST AND VEHICLE CONTROL SUBSTANCES

4.1.1. TEST SUBSTANCES IDENTIFICATION

The test substances, DE-71, 2-chloronitrobenzene and methoxychlor, were received from Marine Sciences Laboratory of Battelle Northwest, Sequim, Washington, as follows:

Identification	Quantity Received	Physical Description	Date of Receipt
DE-71[1] Lot no. 4550OD23D Exp. date: 3 Nov 10 CAS no. Mixed [WIL log no. 6385A]	1 bottle Total gross weight: 572.4 g	Clear, amber, viscous liquid	15 Dec 04
2-Chloronitrobenzene[2] Lot no. 09019MC Exp. date: 1 Nov 10 CAS no. 88-73-3 [WIL log no. 6390A]	1 bottle Total gross weight: 1023.3 g	Clear, yellow liquid	17 Dec 04
Methoxychlor[3] Lot no. 102K1373 Exp. date: 13 Oct 09 CAS no. 72-43-5 [WIL log no. 6387A]	1 bottle Total gross weight: 350.0 g	Light orange powder with lumps	16 Dec 04

[1] = Sponsor-determined purity: 100.0% bromodiphenylether (BDE; 48.7% tetraBDE, 49.5% pentaBDE, 1.8% hexaBDE)
[2] = Sponsor-determined (manufacturer) purity: 100.34% (99.80%)
[3] = Sponsor-determined (manufacturer) purity: 95.49% (95.30%)

Certificates of Analysis for the test substances were provided by the Sponsor and are presented in Appendix A. A correction factor of 1.02 was used for the methoxychlor dose formulations; the DE-71 and 2-chloronitrobenzene dosing formulations were not adjusted for purity. The test substances were stored at room temperature, and were considered stable under this condition. A reserve sample of the methoxychlor (approximately 1 g) was collected on 11 January 2005 and reserve samples of the DE-71

and 2-chloronitrobenzene (approximately 2.5 g and 1.6 g, respectively) were collected on 14 January 2005 and stored in the Archives of WIL Research Laboratories, LLC.

4.1.2. VEHICLE CONTROL SUBSTANCE IDENTIFICATION

The vehicle used in preparation of the test substance formulations and for administration to the control group was corn oil, received from Marine Sciences Laboratory of Battelle Northwest, Sequim, Washington, as follows:

Identification	Quantity Received	Physical Description	Date of Receipt
Corn oil[1] Lot no. AO-001 Exp. date: 28 Aug 05 CAS no. 8001-30-7 WIL Log no. 6389A	14 bottles[2]	Yellow liquid	16 Dec 04
Corn oil Lot no. AO-002 Exp. date: 28 Aug 05 CAS no. 8001-30-7 WIL Log no. 6389B	8 bottles	Clear yellow liquid	26 Jan 05

[1] = Peroxide content less than 3 mEq/mL as determined by WIL Research Laboratories, LLC (WIL-431005, Appendix C).

[2] = Eight bottles used for pre-initiation method development. The remaining six bottles were mixed with lot no. AO-002. This new batch was designated 6389A/B-S1 and was used in formulations for dose administration. Peroxide content of this combined batch was less than 3 mEq/mL as determined by WIL Research Laboratories, LLC (WIL-431005, Appendix C).

Reserve samples of the vehicle were collected on 17 January 2005 (log no. 6389A, approximately 1 g) and 2 February 2005 (log nos. 6389B and 6389A/B-S1, approximately 1 mL each) and stored in the Archives of WIL Research Laboratories, LLC.

4.1.3. PREPARATION

The bottles of corn oil from the 2 lots remaining after pre-initiation method development (lot nos. AO-001 and AO-002) were combined in a 20-L carboy that had previously been washed, rinsed with acetone and dried. The combined batch (designated 6389A/B-S1) was then stirred for at least 15 minutes. For the control group (Group 1), a sufficient amount of this corn oil was dispensed into four 250-mL amber glass bottles. Each bottle was used for approximately 1 week and stirred continuously throughout preparation, sampling, dispensation and dose administration. The vehicle was stored refrigerated.

All test substance formulations were weight/volume (test substance/vehicle) mixtures and were prepared once for pre-initiation method development and once for use on study. The formulations prepared for use on-study as described below were poured into four 250-mL amber glass bottles per group, which were capped tightly with screw-cap lids and refrigerated. Each 250-mL bottle of formulation prepared for use on study was used for dose administration for approximately 1 week. The formulations were mixed using a magnetic stirrer throughout the sampling and dose administration procedures. The formulations were stored refrigerated.

For the DE-71 groups (Groups 2 and 3), the test substance was warmed to approximately 40°C until it was liquefied and then agitated for approximately 2 minutes by inverting. The test substance was then heated to approximately 50°C and an appropriate amount of DE-71 for each group was weighed into a calibrated glass jar. Vehicle was added to each container to bring the formulations nearly to the calibration mark. The formulations were mixed using a magnetic stirrer until a solution was formed. Vehicle was then added to each container to bring the formulations to the calibration mark. The formulations were then stirred vigorously with an overhead stirrer for approximately 30 minutes.

For the 2-chloronitrobenzene groups (Groups 4 and 5), the test substance was warmed to approximately 40°C until it was liquefied. An appropriate amount of 2-chloronitrobenzene for each group was weighed into a tared, 1-L volumetric flask.

Approximately half of the final volume of the vehicle was added and the contents were immediately agitated in order to dissolve the test substance. Vehicle was then added to each container to bring the formulations to the calibration mark.

For the methoxychlor groups (Groups 6 and 7), an appropriate amount of test substance was ground to a fine powder with a mortar and pestle. An appropriate amount of the ground test substance for each group was weighed and quantitatively transferred to a 1-L volumetric flask. Vehicle was added to each flask to bring the formulations to the calibration mark. A stir bar was added and the formulation was mixed until uniform. If necessary, the formulations were sonicated for up to 90 minutes, without exceeding 40°C, in order to dissolve the test substance. After sonicating, the solutions were manually agitated to ensure that the contents were uniform.

The vehicle was visually inspected by the study director on 3 February 2005 and found to be visibly homogeneous and acceptable for administration. The pre-initiation and dosing formulations were visually inspected by the study director on 28 January and 3 February 2005 (DE-71), 21 January and 3 February 2005 (2-chloronitrobenzene) and 23 January, 1 February and 4 February 2005 (methoxychlor) and were found to be visibly homogeneous. The test substance formulations were analytically confirmed to contain the amounts of test substance specified in the protocol (see Section 4.1.5.).

4.1.4. ADMINISTRATION

Dose administration was performed blind to treatment group by assigning each group a letter designation. The test and vehicle control substance formulations were administered orally by gavage, via an appropriately-sized stainless steel ball-tipped dosing cannula (Popper and Sons, Inc., New Hyde Park, New York), once daily from PND 22-42. A dosage volume of 2.5 mL/kg was used. Individual dosages were based on the body weights recorded before dosing on each day to provide the correct mg/kg/day dose. All animals were dosed between 7:00 am and 8:57 am each day; the time of dose

administration was recorded for each animal. The juvenile females were assigned to study groups as follows:

Group Number	Letter Code	Test Substance	Dosage Level (mg/kg/day)	Dosage Concentration (mg/mL)	Dosage Volume (mL/kg)	Number of Females
1	A	Corn Oil	0	0	2.5	15
2	B	DE-71	30	12	2.5	15
3	C	DE-71	60	24	2.5	15
4	D	2-Chloronitrobenzene	25	10	2.5	15
5	E	2-Chloronitrobenzene	100	40	2.5	15
6	F	Methoxychlor	12.5	5	2.5	15
7	G	Methoxychlor	50	20	2.5	15

Dosage levels were selected based on the results of previous studies and were provided by the Sponsor representative.

The animal model selected, the Crl:CD®(SD) rat, is recognized as appropriate for prepubertal studies and has been proven to be susceptible to the effects of reproductive toxicants. WIL Research Laboratories, LLC has historical control data for the Crl:CD®(SD) rat.

4.1.5. SAMPLING AND ANALYSIS

Stability of the test substance solutions bracketing the dosage concentrations used on study were provided by the Sponsor and are presented in Appendix B; the test substance formulations were considered to be stable for 42 days under refrigerated conditions. All test substance formulations were considered solutions; therefore, homogeneity was not assessed. Samples (1 mL each) for concentration analysis were collected approximately 1 inch below the surface of the pre-initiation formulations and the formulations for dose administration. In addition, 5 gram samples of the vehicle (corn oil) were analyzed in triplicate for peroxide content from the first lot (pre-initiation formulations) and from the combination of lots (dose administration formulations).

Concentration and peroxide content analyses were conducted by the Analytical Chemistry Department, WIL Research Laboratories, LLC. The methodology and results of these analyses are presented in Appendix C. The test substance formulations contained the amounts of test substance specified in the protocol, and the vehicle control contained less than 3 mEq/mL peroxide.

4.2. BREEDER FEMALE RECEIPT AND QUARANTINE

Forty-three experimentally naive, time-mated Crl:CD®(SD) rats were received in good health from Charles River Laboratories, Inc., Portage, Michigan, on 6 January 2005 (gestation days 7-10). Each female was examined by a qualified technician on the day of receipt and weighed the day after receipt. Each rat was uniquely identified by a Monel® metal eartag displaying the animal number and placed in quarantine for the duration of the gestation period. During the quarantine period, the rats were observed twice daily for changes in general appearance and behavior.

4.3. ANIMAL HOUSING

Upon arrival all breeder dams were housed individually in plastic maternity cages (16" x 7 3/8" x 8 1/8") with nesting material (heat-treated laboratory-grade pine shavings, Northeastern Products Corp., Warrensburg, New York). The females were housed in these cages until lactation day 21, the scheduled day of euthanasia. Following weaning on PND 21, pups selected for use on study were housed together (2-3 pups) by litter in plastic maternity cages with bedding material (heat-treated, laboratory-grade pine shavings, as described above), which was changed at least 3 times each week. Animals were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996). The animal facilities at WIL Research Laboratories, LLC are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

4.4. DIET, DRINKING WATER AND MAINTENANCE

The basal diet used in this study, PMI Nutrition International, LLC, Certified Rodent LabDiet® 5002, is a certified feed with appropriate analyses performed by the manufacturer (under cGMPs) and provided to WIL Research Laboratories, LLC. The batch of feed used on this study was analyzed for isoflavone content and had a total genistein-equivalent content (aglycone) of approximately 319 ppm (Owens et al., 2003). The batch of feed was approved for use on study by the Sponsor, as the genestein-equivalent content (aglycone) was higher than the protocol-specified 300 ppm. The analytical method summary, results and calculation of the total genistein-equivalent content are presented in Appendix D. Feeders were changed and sanitized once per week. Feed samples were retained at ≤10°C for possible future analysis. These samples will be discarded after issuance of the final report. Municipal water supplying the facility is sampled for contaminants according to the standard operating procedures. The results of the feed lot analysis were placed in the study records and the results of the water analyses are maintained at WIL Research Laboratories, LLC. No contaminants were thought to be present in animal feed or water at concentrations sufficient to interfere with the objectives of this study. Reverse osmosis-purified (on-site) drinking water, delivered by an automatic watering system, and the basal diet were provided ad libitum during the study.

4.5. ENVIRONMENTAL CONDITIONS

Animals were housed throughout the quarantine period, pre-test procedures and during the study in an environmentally controlled room. The room temperature and humidity controls were set to maintain daily averages of 71 ± 3°F (22 ± 2°C) and 45 ± 5% relative humidity. Room temperature and relative humidity were monitored using the Metasys DDC Electronic Environmental control system and were recorded approximately hourly. These data are summarized in Appendix E. Actual mean daily temperature ranged from 70.4°F to 70.7°F (21.3°C to 21.5°C) and mean daily relative humidity ranged from 30.8% to 48.8% during the study. Light timers were calibrated to provide a 14-hour light (5 a.m.

to 7 p.m.)/10-hour dark photoperiod. Air handling units were set to provide approximately 10 fresh air changes per hour.

4.6. PRETEST PROCEDURES

The breeder dams were allowed to deliver naturally. Cages were checked daily in the morning for new births until parturition was complete. The day parturition was initiated was designated PND 0. All pups were individually identified by application of tattoo markings on the tail following completion of parturition. To maximize uniformity in growth rates, the litters were standardized to 8-10 pups per litter on PND 4. Offspring clinical observations and body weights were recorded weekly. Offspring viability was recorded daily and offspring sex was recorded at birth and on PND 1, 4, 7, 14 and 21. Any unthrifty litters or runted pups were excluded from the study on either PND 4 (prior to culling) or on PND 21 (prior to randomization).

4.7. ASSIGNMENT OF ANIMALS TO TREATMENT GROUPS

Once pretest deliveries were complete, the number of projected PND 21 calendar days was reduced to 3 days, while still allowing for adequate numbers of animals available for randomization onto study. The litters and corresponding maternal females not selected for the projected PND 21 calendar day randomization procedures were transferred to the stock colony or euthanized by carbon dioxide inhalation after PND 10. Female offspring were weaned on PND 21; male rats were also weaned on PND 21 and used for a male pubertal assay (Bowman, 2005). On PND 21, all surviving eligible female pups were individually weighed to the nearest 0.1 g. At the conclusion of the pretest period, all female pups judged to be suitable test subjects were assigned to one of seven groups at random using a WIL Toxicology Data Management System (WTDMS^{TM}) computer program, which randomized the animals based on stratification of the PND 21 body weights in a block design with the condition that litter mates were not placed in the same group. All eligible animals were assigned to groups for each day of randomization and then, on the last PND 21 randomization calendar day, the overall PND 21 mean body

weight for each group was determined. In order to keep the mean PND 21 body weight and variances across groups as similar as possible, the animals with PND 21 body weights at the heavy and light end of the distribution were removed from the study on the last PND 21 randomization calendar day (euthanized by carbon dioxide inhalation and discarded without examination). Therefore, the experimental design for WIL-431004 consisted of 7 test substance-treated groups (3 test substances at 2 dosage levels each) and 1 vehicle control group composed of 15 rats each. Body weight values for the selected study females ranged from 41.0 g to 51.6 g on PND 21.

5. PARAMETERS EVALUATED

5.1. CLINICAL OBSERVATIONS AND SURVIVAL

All juvenile rats selected for use on study were observed twice daily, once in the morning and once in the afternoon, for appearance, behavior, moribundity and mortality. A detailed physical examination was conducted on the day of randomization and daily prior to dose administration. All animals were also observed for signs of toxicity approximately 1 hour following dose administration each day. All significant clinical findings were recorded at these observation periods.

5.2. BODY WEIGHTS

Individual body weights (to the nearest 0.1 g) were recorded daily from PND 21-42, inclusively. Group mean body weights were calculated for each of these days. Group mean body weight changes were calculated for each daily interval, and also for PND 22-42.

5.3. VAGINAL PATENCY

Each female pup was observed for vaginal perforation beginning on PND 22 (Adams et al., 1985). The day on which the vaginal lumen was first observed to open was recorded for each pup. Examination of the females was continued and the appearance of a small "pin hole", a vaginal thread and complete vaginal opening were recorded on the days they were observed. Individual body weights were recorded on the day of complete vaginal opening.

5.4. ESTROUS CYCLES

Starting on the day of complete vaginal opening and continuing daily until necropsy, vaginal lavages were performed daily and the slides were evaluated to assess the regularity and duration of the estrous cycle of each female. Vaginal smears were classified as diestrus (presence of leukocytes), proestrus (presence of nucleated epithelial cells) or estrus (presence of cornified epithelial cells). Age at first estrus and animals that stopped cycling were also noted.

5.5. SCHEDULED NECROPSY

On PND 42, all females were euthanized by exposure to carbon dioxide (up to 60 seconds) followed by decapitation; all females were euthanized by 1:00 p.m. to minimize variability in thyroid hormone levels due to normal diurnal fluctuation. A complete necropsy was conducted on all animals starting approximately 2 hours following dose administration. The necropsy included examination of the external surface, all orifices, the cranial cavity, the external surfaces of the brain and spinal cord, and the thoracic, abdominal and pelvic cavities, including viscera. Selected tissues were retained for all animals as described in Section 5.2.3.

5.5.1. THYROID/PITUITARY HORMONE ANALYSIS (T_4 AND TSH)

Immediately following euthanasia, trunk blood (minimum of 2 mL) was collected for thyroid hormone analysis. Serum was isolated by centrifugation (4°C for approximately 10 minutes) and each serum sample volume was divided approximately equally into two siliconized microcentrifuge tubes and stored frozen at ≤-20°C until analysis of thyroid-stimulating hormone (TSH) and thyroxine (T_4). The Clinical Pathology Department, WIL Research Laboratories, LLC conducted T_4 hormone analyses by a solid-phase chemiluminescent enzyme immunoassay (Immulite®, Diagnostic Products Corporation, Los Angeles, California) and the Metabolism Department, WIL Research Laboratories, LLC conducted the TSH analysis using a radioimmunoassay (RIA) procedure (Amersham BioSciences, Piscataway, New Jersey). Multiple quality control samples were run dispersed within each assay. For the TSH RIA assay, rat TSH standard (RP3) from the National Institute of Diabetes and Digestive and Kidney Diseases was diluted in assay buffer to 3, 6 and 9 ng/mL as quality control samples. For all hormone analyses, all serum samples analyzed were within the range of the respective standard curve. In general, all samples were analyzed in a single assay per hormone and quality control samples were in the expected range based on manufacturer specifications and previous experience. The intra-assay variability (based on coefficient of variation of each of the QC samples) was less than 15% for each assay.

5.5.2. TISSUE COLLECTION AND ORGAN WEIGHTS

The following organs from all females euthanized at scheduled termination were weighed (to the nearest 0.1 mg):

Adrenal glands[a]	Pituitary gland
Kidneys[a]	Thyroid [b,c]
Liver	Uterus[d]
Ovaries[a]	

[a] = Paired organs were weighed together.
[b] = Includes parathyroid glands; designated as "Thyroid Glands" on all tables.
[c] = Weighed after fixation in 10% neutral-buffered formalin.
[d] = Wet and blotted weights were recorded as described below.

Wet and blotted uterine weights were measured for all animals at the scheduled necropsy. Each uterus was harvested in the same sequence in which dosing occurred. The pubic symphysis was opened and each ovary and uterine horn was detached from the dorsal abdominal wall. The ovaries were separated from the uterine horns at the oviduct/uterus junction. The urinary bladder and ureters were removed from the ventral and lateral side of the uterus and vagina. The fibrous adhesion between the rectum and vagina was then detached until the junction of the vaginal orifice and perineal skin was identified. The uterus and vagina were detached from the body by incising the vaginal wall just above the junction with the perineal skin. The excess fat and adnexa were trimmed away. The vagina was then removed from the uterus, leaving the cervix intact and attached to the uterus for uterus weight measurement. Care was taken during uterus harvesting such that the luminal contents were retained. A record was made if any luminal contents were lost. The uterus was transferred to a uniquely marked and tared plastic Petri dish with care to avoid desiccation before weighing. The Petri dish was lined with saline-moistened filter paper (or equivalent) and covered to minimize desiccation.

Immediately following collection of the wet weight, each uterus was individually processed by opening the uterine wall and carefully blotting the excess fluid. Both uterine horns were pierced and cut longitudinally with small surgical scissors, placed on

filter paper and gently pressed with another piece of dry filter paper to absorb the luminal fluid. The procedure was not so severe as to render the tissue unacceptable for histopathologic analysis.

Small tissues such as the adrenals and pituitary, as well as tissues that contain fluid were weighed immediately to prevent tissues from drying out prior to weighing.

To minimize systematic bias in the weighing procedures, organ harvesting and weighing procedures were divided as equally as possible among the prosecting and weighing technicians, such that all animals from a group were not processed by a single individual.

The following GLP deviation occurred. On 7 March 2005, correction fluid was used to correct animal numbers incorrectly recorded on form T3-044 (Individual Organ Weights) for female no. 73415-15 in the control group and female no. 73403-04 in the 50 mg/kg methoxychlor group.

5.5.3. TISSUE FIXATION AND PROCESSING

The ovaries and uterus were placed in Bouin's fixative for approximately 24 hours, after which they were rinsed and stored in 70% alcohol until histological processing. The thyroid, with attached trachea, was fixed in 10% neutral-buffered formalin for at least 24 hours. Then the thyroid was dissected from the trachea, blotted and weighed to the nearest 0.1 mg and placed in 70% ethanol until histological processing. The ovaries, uterus and thyroid were then embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for subsequent histological evaluations.

5.5.4. MICROSCOPIC EVALUATION

The thyroid, ovary and uterus were evaluated microscopically for pathologic abnormalities and potential treatment-related effects.

Three sections of the paired thyroid lobes from each animal were qualitatively evaluated for follicular epithelial height and colloid area as described by Capen and Martin (1989), using a 5 point grading scale (1=shortest/smallest; 5=tallest/largest) and any

abnormalities/lesions noted. There was great variability in the microscopic appearance of the thyroid follicles in some animals, both within an individual section of thyroid and between sections of the same lobe. In general, when variability was noted, follicles toward the center of the thyroid lobes were given greater emphasis than those follicles at the periphery of the lobe, and sections of thyroid that were closer to the center of the lobe were given greater emphasis than those sections that were obtained closer to the periphery. Other microscopic changes were also recorded when present.

In the ovaries, presence or absence of corpora lutea and primordial follicles, numbers of viable primary and tertiary/antral follicles, numbers of atretic (any size) follicles, changes in type or morphologic appearance of corpora lutea, and other changes in morphology were recorded. For endpoints requiring counting, such as numbers of primary, tertiary/antral and atretic follicles, the total number of each type of structure in both ovaries was determined, and the result recorded as follows: grade 1, 1-5 structures; grade 2, 6-10 structures; grade 3, 11-15 structures; grade 4, 16-20 structures; grade 5, >20 structures. If none of the structure of interest was present, the finding was recorded as 'absent'.

Uteri were examined for hypoplasia/hypertrophy/hyperplasia of the stroma, epithelium and/or myometrium and uterine glandular development, in addition to other changes unrelated to the estrous cycle. Although uteri were opened longitudinally at necropsy, an attempt was made to compare the relative thickness of the uterine wall.

Because the control group was not identified during the microscopic examination, and because the animals appeared microscopically to be in different stages of the estrous cycle at the time of necropsy, it was difficult to determine whether the hypertrophic changes observed were normal for the apparent stage of estrus. As such, an attempt was made to 'stage' the cycle, based only on the uterine morphology, taking into consideration the ovarian morphology and, if present, vaginal epithelial changes. If the

evidence for the cycle stage bordered between two stages, the later stage was assigned. The following criteria were used to stage the estrous cycle in these animals.

-*Estrus*: presence of apoptotic epithelium in the uterus. May be accompanied by sloughed cornified vaginal epithelium and/or evidence of recent ovulation (complete or incomplete basophilic corpora lutea composed of very small luteal cells, ova or other debris in oviduct lumen).

-*Diestrus* (includes both diestrus 1 and diestrus 2): presence of small numbers of leukocytes in the uterine endometrium, low columnar-cuboidal epithelium, short, non-tortuous glands. May be accompanied by thin vaginal epithelium, leukocytes in vaginal wall, and/or presence of new basophilic corpora lutea in the ovary.

-*Proestrus*: presence of tall columnar epithelium lining the uterus, multiple leukocytes in the myometrium/stroma, tortuous endometrial glands, stromal edema, increased vascularity/thickness of myometrium. May be accompanied by other changes, including thick and/or mucified vaginal epithelium, and presence of viable antral follicles in the ovary.

The stage of the cycle determined from the microscopic exam was then compared to the estrous cycle data obtained from vaginal smears on the day of necropsy. Differences in the stage of the cycle determined from the vaginal smear vs. the microscopic examination of the uterus were apparent, but fell into 3 categories. The most common difference was diestrus diagnosed from the smear, vs. proestrus diagnosed from the microscopic exam. The changes used to diagnose proestrus microscopically in the uterus included stromal edema, increased epithelial height, increased vascularity, and increased depth/tortuosity of glands; if any of these changes was present, then, by convention, the later stage was assigned. It is likely that many of these animals were in (late) vaginal diestrus, diagnosed as (early) proestrus microscopically. The other differences noted in stage determinations were 1) diestrus diagnosed one day following vaginal estrus from the smear, versus estrus diagnosed microscopically and 2) estrus diagnosed from the vaginal smear, versus

proestrus diagnosed microscopically. The only criterion used to diagnose estrus microscopically in the uterus was the presence of apoptotic epithelial cells. Animals in late proestrus/early estrus will not show apoptosis; thus, these animals microscopically were determined to be in proestrus. Because apoptosis can still be observed microscopically as the animal enters early diestrus, it is likely that the animals determined to be in vaginal diestrus from the smears were in early diestrus but still had remaining apoptotic cells in the uterus. Thus, the apparent differences in estrous cycle staging were likely the result of differences in tissues used to stage the cycle, and conventions used in determining the stage.

The pathologist reading the slides was presented only with the coded identity of the group to which a sample belonged. After the raw data were collected, the codes were translated to test substances and dose groups for the purposes of the pathologist's interpretation and report. Microscopic examinations were conducted by Karen S. Regan, DVM, DACVP, DABT, Consulting Pathologist.

5.6. STATISTICAL METHODS

All statistical tests were performed using appropriate computing devices or programs. Analyses were conducted using two-tailed tests (except as noted otherwise) for minimum significance levels of 5% and 1%, comparing each test substance-treated group to the control group. Each mean was presented with the standard deviation (S.D., Appendices G, I and L only), standard error of the mean (S.E.) and the number of animals (N) used to calculate the mean. The coefficients of variation were calculated by the sponsor, and are presented with S.D. in Appendix H. The tables in Appendix H are intended to be an extension of the regular report tables, as such, the footnotes used in Appendix H correspond to those footnotes in the regular report tables. Due to the different rounding conventions inherent in the types of software used, the means, standard deviations, coefficients of variation and standard errors of the mean on the summary and individual tables may differ by ±1 in the last significant figure. Upon review of the individual data

for biological plausibility, no data points were identified as potential outliers; therefore, no statistical outlier tests were conducted.

All endpoint measures (PND 21 and 42 body weight, body weight gain from PND 22 to 42, age and body weight at vaginal opening, body and organ weights at necropsy and serum hormones) were analyzed per test substance (control and 2 dosage levels). All endpoints were analyzed for heterogeneity of variance using Levene's test (Levene, 1960). For the PND 21 and 42 body weights, body weight gains from PND 22-42 and serum hormone values, if the data were homogeneous, a parametric 1-way analysis of variance (ANOVA) was used to determine intergroup differences (Snedecor and Cochran, 1980). If the ANOVA revealed statistically significant ($p<0.05$) intergroup variance, Dunnett's test (Dunnett, 1964) was used to compare the test substance-treated groups to the control group. If the data were not homogeneous and normal, the data were analyzed by the Kruskal-Wallis nonparametric ANOVA test (Kruskal, 1952). If the results of this ANOVA were statistically significant, Dunn's test (Dunn, 1952) was applied to the data to compare all test substance-treated groups to the control group.

For organ weights and age and body weight at vaginal opening, if the data were homogenous using Levene's test, the data were analyzed using a parametric 1-way ANOVA followed by Dunnett's test, as described above. In accordance with the statistical tree presented in Appendix F, if the data were not homogeneous using Levene's test, 1 of 5 methods of transformation were applied to the data in order to achieve homogeneity. The methods of transformation were applied to heterogeneous data in the following order: Log_{10} (x+1), x^2, $\sqrt{x}$, 1/x and rank sum. In addition, organ weights and age and body weight at vaginal opening (using transformed data when necessary) were also analyzed by Analysis of Covariance (ANCOVA) using the body weight on PND 21 as the covariant. If the ANCOVA revealed statistically significant ($p<0.05$) intergroup variance, Dunnett's test (Dunnett, 1964) was used to compare the LSmean of each test substance-treated group to the control group (SAS Institute, 1999-2001). If data could not be transformed to homogeneity using any of the 5 methods listed above, only the

arithmetic mean was presented; that is, no statistics or adjusted data (LSmeans) were presented.

5.7. DATA RETENTION

The Sponsor has title to all documentation records, raw data, specimens or other work product generated during the performance of the study. All work product generated by WIL Research Laboratories, LLC, including raw paper data and specimens, are retained in the Archives at WIL Research Laboratories, LLC, as specified in the study protocol.

Reserve samples of the test and vehicle control substances, pertinent electronic storage media and the original final report are retained in the Archives at WIL Research Laboratories, LLC in compliance with regulatory requirements.

6. RESULTS

6.1. CONTROL FEMALES

Fifteen females were assigned to the control group. This control group was used as a comparison to evaluate the effects of each test substance on pubertal development and thyroid function.

6.2. DE-71 TREATED-FEMALES

6.2.1. IN-LIFE DATA

Tables 1A, 1B, 2A, Figure 1 and Appendices G, H and I (overall summary tables, coefficients of variation and individual tables, respectively)

There were no clinical observations in animals treated with 30 or 60 mg/kg/day DE-71 at the daily examinations or approximately 1 hour following dose administration. Mean daily body weight and body weight gain (PND 22-42) in both DE-71 dose groups were similar to the control group for the duration of dose administration. The mean age (PND) of complete vaginal opening was 34.9 ± 0.56 and 35.7 ± 0.61 in the 30 and 60 mg/kg/day DE-71 groups, respectively, compared to 34.1 ±0.59 in the control group (no statistical significance). Eight of 15 females in the 60 mg/kg/day DE-71 group were ≥PND 36 at complete vaginal opening, compared to 4 of 15 vehicle control group females. Corresponding to the slight mean delay of 1.6 days in the 60 mg/kg/day group, there was a statistically significant ($p<0.05$) increase in the mean body weight compared to controls on day of complete vaginal opening. The PND 21-adjusted mean age and body weight at vaginal opening for each group was consistent with the unadjusted means. The slight delay in vaginal opening at 60 mg/kg/day in this study is consistent with a mean delay of 1.8 days (statistically significant) demonstrated previously in Wistar rats at the same dose of DE-71 using a similar study design (Stoker, 2004).

The later age of vaginal opening in the 60 mg/kg/day dose group likely resulted in the slight delay in the mean age of first estrus in that group compared to the control group (PND 37.1 ± 0.82 compared to 35.7 ± 0.88, respectively) and lower mean number of days

from vaginal opening to PND 42 in this group compared to the control group (6.3 ± 0.61 days compared to 7.9 ± 0.59 days, respectively). The mean age at first estrus and mean number of days between vaginal opening and PND 42 were similar between the 30 mg/kg/day DE-71 group and the control group. The total number of days in proestrus for each animal and the number of females in continuous diestrus or continuous estrus on PND 42 were similar between the DE-71 treated animals and control animals, therefore DE-71 did not appear to affect the number of estrous cycles prior to PND 42.

6.2.2. NECROPSY, HISTOPATHOLOGICAL AND HORMONE DATA

Tables 2A, 2B, 3 and Appendices G, H, I and J (overall summary tables, coefficients of variation, individual tables and photographs of microscopic lesions, respectively)

The final mean body weight on the day of necropsy was similar between vehicle control and DE-71 treated females. In the 30 and 60 mg/kg/day dose groups, mean absolute and PND 21 body weight-adjusted liver weights were significantly ($p<0.01$) increased in a dose-related manner compared to the control group. This weight change is consistent with the induction of hepatic enzymes by DE-71, as demonstrated previously (Zhou, 2001). There were no significant differences in mean absolute or PND 21 body weight-adjusted organ weights for the uterus (wet and blotted), ovaries, pituitary, adrenal glands, kidneys, or thyroid glands weights with DE-71 treatment compared to controls. A slightly lower uterus weight was noted in the 60 mg/kg/day DE-71 group compared to the control group. This slight change was likely due to no females in that group that were in estrus on PND 42 compared to the control group, which had 4 females in estrus on PND 42.

There was an increased mean follicular epithelial cell height (1 = shortest; 5 = tallest) and decreased mean colloid area (1 = least; 5 = most) in DE-71-treated animals compared to control animals (see following text table and Appendix J). The changes in colloid area were dose-related. Similar changes in these 2 parameters have been previously reported in pubertal rats dosed with 60 mg/kg/day DE-71 (Stoker, 2004). There were no other

effects considered DE-71-related in the thyroid and there were no treatment-related macroscopic or microscopic findings in the ovaries. Follicular degeneration in the thyroid, previously reported in pubertal animals treated with similar doses of DE-71 (Stoker, 2004) was not observed in the current study. Microscopic findings similar to those reported as degeneration in that study were observed in this study in animals in all groups, including the control group, and were interpreted as artifactual.

Diagnosis	**0**	**30 mg/kg/day**	**60 mg/kg/day**
Follicular Epithelial Height (total)	15	15	14
Grade 1	3	0	1
Grade 2	12	6	3
Grade 3	0	8	9
Grade 4	0	1	1
Grade 5	0	0	0
Mean	**1.8**	**2.7**	**2.7**
Colloid Area (total)	15	15	14
Grade 1	0	0	0
Grade 2	0	3	5
Grade 3	6	9	8
Grade 4	7	3	0
Grade 5	2	0	1
Mean	**3.7**	**3.0**	**2.8**

Mean serum T_4 (µg/dL) levels in the 30 and 60 mg/kg/day DE-71 dose groups were statistically significantly ($p<0.01$) lower (1.40 ± 0.118 and 0.96 ± 0.049, respectively) compared to the control group (5.23 ± 0.361). Conversely, the mean serum TSH (ng/mL) levels were increased 40% and 72%, respectively, compared to the control group; the differences were statistically significant ($p<0.05$ or $p<0.01$). A DE-71 induced decrease in T_4 and increase in TSH in the female pubertal assay was demonstrated previously; however, the TSH data were not statistically significant in that study (Stoker, 2004).

6.3. 2-CHLORONITROBENZENE-TREATED FEMALES

6.3.1. IN-LIFE DATA

Tables 4A, 4B, 5A, Figure 2 and Appendices G, H and I (overall summary tables, coefficients of variation and individual tables, respectively)

In the 100 mg/kg/day 2-chloronitrobenzene group, 4 of 15 animals were observed near the end of the dose administration period (PND 36 through 42) with wet clear material around the mouth approximately 1 hour following dosing. The mean body weight gain was decreased in animals dosed with 100 mg/kg/day for the first 3 days of dose administration (PND 22-23 through 24-25) compared to controls. This decreased body weight gain resulted in decreased mean daily body weights (5% to 10.5%) from PND 23 through 29. For the rest of the dose administration period in the 100 mg/kg/day dosage group and for the entire dose administration period in the 25 mg/kg/day dosage group, mean daily body weights and body weight gains were generally similar or slightly increased compared to the control group. The mean final body weight and body weight gain from PND 22 to 42 were slightly increased in the treated groups compared to the control group, but the difference was not statistically significant.

There was a 2.6 day delay in the mean age that vaginal opening was complete in the 100 mg/kg/day 2-chloronitrobenzene group compared to controls (PND 36.7 ± 0.64 and 34.1 ± 0.59, respectively); this difference was statistically significant ($p<0.01$). Secondary to this delay was an increased mean body weight on the day of attaining this developmental landmark (statistically significant, $p<0.01$). The PND 21 body weight-adjusted mean age and body weight upon vaginal opening in the 100 mg/kg/day dose group were consistent with the absolute data (also statistically significant).

The mean age (PND) of first estrus in the 100 mg/kg/day dosage group (37.3 ± 0.69) was also delayed compared to controls (35.7 ± 0.88), with a corresponding decrease in mean number of days between vaginal opening and PND 42 (5.3 ± 0.64 compared to 7.9 ± 0.59 days, respectively). Animal no. 73415-17 (100 mg/kg/day group) completed vaginal

opening on PND 42 and the vaginal cytology indicated diestrus stage of the cycle, so no first day of estrus was obtained for this animal. There was also one animal in the 25 mg/kg/day dose group that did not obtain first estrus by euthanasia even though that animal had completed vaginal opening by PND 33. The mean postnatal day of complete vaginal opening, mean age at first estrus and mean number of days between vaginal opening and PND 42 were similar between the 25 mg/kg/day 2-chloronitrobenzene group and the control group. Although the incidence of animals in the 100 mg/kg/day dose group with more days in proestrus (5/15) compared to the control and 25 mg/kg/day dose groups (0/15) listed in Table 4B is suggestive of more cycles in this group, closer examination of the individual cycles of each animal in Appendix I demonstrates that there is no difference in the average number of cycles across groups. In addition, the number of females in continuous diestrus or continuous estrus on PND 42 was similar between the treated and control animals; therefore, 2-chloronitrobenzene did not appear to affect the estrous cycle prior to PND 42.

6.3.2. NECROPSY, HISTOPATHOLOGICAL AND HORMONE DATA

Tables 5A, 5B, 6 and Appendices G, H and I (overall summary tables, coefficients of variation and individual tables, respectively)

The mean final body weight on PND 42 was not significantly different between control and 2-chloronitrobenzene treated groups. Mean absolute and PND 21 body weight-adjusted liver weights were significantly increased ($p<0.01$) in a dose-related manner in the 25 and 100 mg/kg/day dose groups compared to control. This increased liver weight is consistent with previous data demonstrating that 2-chloronitrobenzene induces liver necrosis, inflammation and hepatocytomegaly (IUCLID, 2003). There was also a statistically significant ($p<0.05$) decrease in mean absolute and PND 21 body weight-adjusted weight of the adrenal glands in the high dose group compared to the control group. There were no statistically significant differences in the mean absolute or adjusted organ weights for uterus (wet and blotted), ovaries, pituitary, kidney, or thyroid gland weights with 2-chloronitrobenzene treatment compared to control, as such any

differences observed were considered incidental and not related to treatment. The wet/blotted uterine weight in the 100 mg/kg/day group was slightly decreased compared to the control group. This slight decrease was attributed to slightly fewer animals (2 of 15) in that group in estrus on PND 42 compared to the control group (4 of 15).

There were no macroscopic or microscopic effects on the thyroid, uterus or ovaries that were attributed to 2-chloronitrobenzene treatment. In the ovaries of two 25 mg/kg/day group females luteinized follicles were observed, characterized as large follicles containing a zona pellucidum and completely filled with luteinized and non-luteinized cells. Normal follicles and corpora lutea were present in the ovary with the luteinized follicle. The relationship of this finding to the test article is unclear. The study pathologist has observed luteinized follicles in control animals in other studies. Additionally, luteinized follicles did not occur in the highest dose group. For these reasons, the luteinized follicles were not considered test article-related. The lack of definitive microscopic changes in the ovary is consistent with the lack of statistically significant changes in the mean absolute ovary weights in any treated group when compared to controls. The mean follicular epithelial cell height and colloid areas of the thyroid were graded similarly between the treated and control groups. All observations were considered incidental and not related to treatment.

Mean T_4 levels (μg/dL) were not significantly different with 25 and 100 mg/kg/day treatment compared to the control group (5.13 ± 0.213 and 4.87 ± 0.216 compared to 5.23 ± 0.361, respectively). Mean serum TSH (ng/mL) was increased in a dose dependent manner with 25 and 100 mg/kg/day 2-chloronitrobenzene (14% and 29%, respectively) compared to controls; the difference between the high dose and control was statistically significant ($p<0.05$). The relevance of this increase in serum TSH is unclear in the absence of any effects on T_4, thyroid weights or thyroid histopathology.

6.4. METHOXYCHLOR-TREATED FEMALES

6.4.1. IN-LIFE DATA

Tables 7A, 7B, 8A, Figure 3 and Appendices G, H and I (overall summary tables, coefficients of variation and individual tables, respectively)

There were no clinical observations in animals treated with 12.5 or 50 mg/kg/day methoxychlor at the daily examinations or approximately 1 hour following dose administration. The overall mean body weight change from PND 22-42 was slightly decreased with 50 mg/kg/day methoxychlor compared to vehicle control (101.6 ± 2.14 g compared to 110.6 ± 3.35 g, not statistically significant). This slight decrease in mean body weight gain in the high-dose group (primarily beginning on PND 35) resulted in a 5.8% decrease in mean final body weight on PND 42 compared to the control group (not statistically significant). Mean daily body weight and body weight gain (PND 22-42) in the 12.5 mg/kg/day dose group was similar to the control group for the duration of dose administration.

Complete vaginal opening was observed an average of 7 days earlier in the 50 mg/kg/day methoxychlor group compared to the control group (PND 27.1 ± 0.15 and 34.1 ± 0.59, respectively). Secondary to the younger age, the mean body weight in these high dose females measured on the day of complete vaginal opening was also decreased (36%) compared to the control group (statistically significant, $p<0.01$). No statistics could be performed on the absolute or adjusted age of vaginal opening since the data could not be transformed to homogeneity per protocol. However, the lack of homogeneity in this endpoint is entirely attributed to the treatment of 50 mg/kg/day methoxychlor. The accelerated vaginal opening in the 50 mg/kg/day group in the current study is consistent with the 5.5 day acceleration to a mean age of 27.4 observed previously (George, 2003). The mean age of complete vaginal opening and associated mean body weight were similar between the 12.5 mg/kg/day methoxychlor group and the control group.

Related to the acceleration in complete vaginal opening in the 50 mg/kg/day dose group was the earlier mean age of first estrus in this dose group compared to the control group (PND 32.5 ± 0.79 compared to 35.7 ± 0.88, respectively), the higher number of days between vaginal opening and PND 42 (14.9 ± 0.15 compared to 7.9 ± 0.59, respectively), and the apparent increase in number of estrous cycles prior to PND 42 as indicated by Table 7B. In addition, there were no animals in continuous diestrus on PND 42 in the 50 mg/kg/day methoxychlor group compared to 4/15 and 3/15 in the control and low dose groups, respectively (likely attributable to the accelerated age of vaginal opening).

6.4.2. NECROPSY, HISTOPATHOLOGICAL AND HORMONE DATA

Tables 8A, 8B, 9 and Appendices G, H and I (overall summary tables, coefficients of variation and individual tables, respectively)

The final mean body weight was decreased by 5.8% in the 50 mg/kg/day methoxychlor group compared to the control group. There were no significant differences in absolute or PND 21 body weight-adjusted organ weight data between the treatment groups and the control group. Mean serum T_4 and TSH levels in the treated groups were similar to the control group. Treatment with 12.5 or 50 mg/kg/day methoxychlor did not cause any macroscopic or microscopic evidence of treatment-related effects in the thyroids; mean follicular epithelial cell height and colloid areas of the thyroid were similar between the treated and control groups.

In the ovaries of two 12.5 mg/kg/day group females luteinized follicles were observed, characterized as large follicles containing a zona pellucidum and completely filled with luteinized and non-luteinized cells. Normal follicles and corpora lutea were present in the ovary with the luteinized follicle. The relationship of this finding to the test article is unclear. The study pathologist has observed luteinized follicles in control animals in other studies. Additionally, luteinized follicles did not occur in the highest dose group. For these reasons, the luteinized follicles were not considered test article-related. The lack of definitive microscopic changes in the ovary is consistent with the lack of

statistically significant changes in the mean absolute ovary weights in any treated group when compared to controls.

Based on the microscopic determination of the stage of the estrus cycle (criteria listed in the methods), not the vaginal smear, there was a slight increase in the number of animals in the estrogenic phase (proestrus/estrus) of the cycle in the 12.5 and 50 mg/kg/day dose groups (11, 15 and 15 animals in the control, 12.5 and 50 mg/kg/day dose groups, respectively). A slight increase in the degree of uterine hypertrophy was also observed in the methoxychlor groups. Nine, 7 and 5 females in the control, 12.5 and 50 mg/kg/day groups, respectively, had uterus hypertrophy of grade 2 or less, while 6, 8 and 10 females in the same respective groups had uterine hypertrophy of grade 3 or greater. The slight increase in the degree of uterine hypertrophy in the methoxychlor groups correlates with the increased number of animals in the estrogenic phase of the cycle. Consistent with the increase in uterine hypertrophy, the absolute and adjusted mean uterus weights, with and without fluid, were also slightly higher in the methoxychlor groups (range of 11% and 23%) but the differences compared to controls were not statistically significant.

7. DISCUSSION

7.1. DE-71

There was a slight delay (1.6 days) in the mean age of complete vaginal opening at 60 mg/kg/day DE-71 compared to the concurrent controls, which is consistent with a mean delay of 1.8 days in pubertal Wistar rats at this dose of DE-71 (Stoker, 2004). The increased follicular epithelial height and decreased colloid area of the thyroid in both DE-71 dose groups observed in the current study are also consistent with this previous report in pubertal Wistar rats dosed with 60 mg/kg/day DE-71 (Stoker, 2004). Although the mean thyroid weights in the current study were not elevated in the DE-71 groups compared to the control group, the mean serum T_4 was statistically significantly decreased and the mean serum TSH was statistically significantly increased at 30 and 60 mg/kg/day DE-71. Decreased serum T_4 in rats treated with similar doses of DE-71 is consistent with previous reports (Stoker, 2004; Zhou, 2001). The increase in mean serum TSH was also observed previously in the 20-day female pubertal assay, although the increase was not statistically significant in that assay. The mechanism of serum T_4 reduction is not completely known, although DE-71 is an inducer of hepatic biotransformation enzymes, suggesting that T_4 glucuronidation and elimination was one factor contributing to the reduction (Zhou, 2001). In this study, mean absolute and PND 21 body weight-adjusted liver weights were statistically significantly increased in a dose-related manner in the DE-71 treated groups, supporting a possible effect on enzyme induction.

7.2. 2-CHLORONITROBENZENE

There was a transient decrease in mean body weight gain during the first week of dose administration, but in the following 2 weeks the 100 mg/kg/day 2-chloronitrobenzene group of animals recovered and had final body weights similar to controls. There was a statistically significant delay in the mean age of vaginal opening (2.6 days compared to control) and a corresponding increase in mean body weight at that age in the 100 mg/kg/day dose group. There is no indication that 2-chloronitrobenzene impacts the

number of estrous cycles or cycle length at the doses tested; rather, these differences in the estrous measurements calculated from this bioassay are likely secondary to the delay in vaginal opening.

Serum TSH was increased 14% and 29% in the 25 and 100 mg/kg/day 2-chloronitrobenzene groups compared to control. The relevance of this increase in serum TSH is unclear in the absence of any effects on T_4, thyroid weights or thyroid histopathology. However, slightly increased TSH levels (6% and 21%) compared to controls were observed in the concurrent male pubertal rat study (not statistically significant) at these same dose levels (Bowman, 2005).

There was a statistically significant and dose-responsive increase in absolute and adjusted mean liver weights in both 2-chloronitrobenzene dose groups, consistent with previous data demonstrating that 2-chloronitrobenzene induces liver necrosis, inflammation and hepatocytomegaly (IUCLID, 2003). There was also a statistically significant decrease in mean absolute and PND 21 body weight-adjusted weight of the adrenal glands in the high dose group compared to control. Although this decrease in adrenal weights may be induced by 2-chloronitrobenzene, this has not been reported in previous repeat-dose toxicity studies (IUCLID, 2003).

7.3. METHOXYCHLOR

Treatment with 50 mg/kg/day methoxychlor decreased mean body weight gains slightly resulting in a 5.8% decrease in mean final body weight compared to the control group (not statistically significant). In general, this is consistent with the statistically significant effect on mean body weight and body weight gain observed previously (George, 2003). Complete vaginal opening was observed an average of 7 days earlier in the 50 mg/kg/day methoxychlor group compared to the control group. Secondary to the younger age, the mean body weight at that age in these high dose females was also significantly decreased (36%) compared to the control group. The accelerated vaginal opening to a mean age of

PND 27.1 in the 50 mg/kg/day group in the current study is consistent with the 5.5 day acceleration to a mean age of 27.4 observed previously (George, 2003).

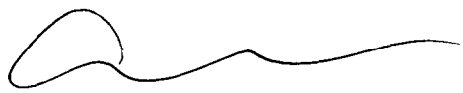
Methoxychlor has been shown to alter fertility in rats when dosed from weaning through puberty and gestation (Gray, 1989), and to produce ovulation defects accompanied by microscopic and organ weight changes in the ovary when administered to perinatal/juvenile animals (Chapin, 1997) or adult mice (Eroschenko, 1995). In this study, all animals appeared to be cycling normally. The differences in the estrous measurements calculated from this bioassay are likely secondary to the acceleration in vaginal opening exclusively. There were no effects on the serum T_4, serum TSH or thyroid histopathology that were related to methoxychlor treatment. The differences described in the uterus were only slight. A lack of significant histopathologic changes in the reproductive tract of rats treated in the peripubertal period with doses of methoxychlor similar to those used in this study was reported previously (George, 2003). Because of the lack of differences in histologic appearance of the ovary, and the lack of statistically significant organ weight changes, the slight differences described in the uterus of the methoxychlor animals were likely a result of biologic variation.

8. CONCLUSIONS

The most sensitive endpoints in this study at the doses tested were serum T_4, serum TSH, thyroid histopathology and absolute and adjusted mean liver weights for DE-71 (30 mg/kg/day), absolute and adjusted mean liver weights for 2-chloronitrobenzene (25 mg/kg/day), and vaginal opening for methoxychlor (50 mg/kg/day). The results of the current study are consistent with available data from previous studies and are considered acceptable for interlaboratory comparisons for the purposes of validating this female pubertal bioassay across laboratories.

9. KEY STUDY PERSONNEL AND REPORT SUBMISSION

Report Submitted By:

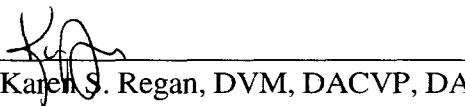


13 DEC 2005

Christopher J. Bowman, PhD, DABT
Staff Toxicologist, Developmental and
Reproductive Toxicology
Study Director

Date

Pathologist of Record:

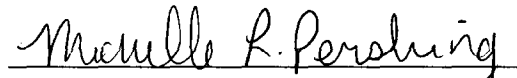


12 Dec 2005

Karen S. Regan, DVM, DACVP, DABT
Consulting Pathologist

Date

Report Prepared By:

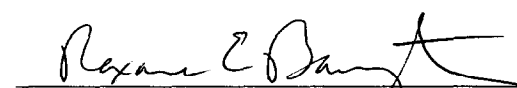


13Dec05

Michelle L. Pershing, MS
Senior Study Analyst

Date

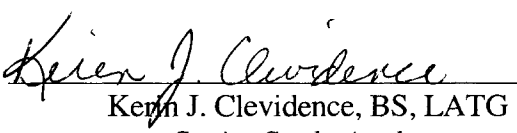
Report Reviewed By:



13 Dec 05

Roxanne E. Baumgartner, DVM, DACVP, DABT
Senior Pathologist

Date



13 Dec 2005

Kevin J. Clevidence, BS, LATG
Senior Study Analyst

Date

KEY STUDY PERSONNEL AND REPORT SUBMISSION (CONTINUED)

13Dec.2005

Mark D. Nemec, BS, DABT
Director, Developmental and
Reproductive Toxicology

Date

13Dec.2005

for: Donald G. Stump, PhD, DABT
Associate Director, Developmental and
Reproductive Toxicology

Date

13 Dec 2005

Evelyn Tanchevski, BS
Group Supervisor, Study Analysis and Reports

Date

Study Personnel:

Susan C. Haley, BS	Manager, Clinical Pathology
Sally A. Keets, AS	Senior Operations Manager, Vivarium
Carol A. Kopp, BS, LAT	Manager, Gross Pathology and Developmental Toxicology Laboratory
Michael A. Safron, AS, HT (ASCP)	Manager, Histology
Theresa M. Rafeld	Group Supervisor, Formulations Laboratory
Daniel W. Sved, PhD	Director, Metabolism and Analytical Chemistry
Bennett J. Varsho, BS, DABT	Operations Manager, Developmental, Reproductive and Neurotoxicology
Robert A. Wally, BS, RAC	Acting Manager, Reporting and Regulatory Technical Services

10. QUALITY ASSURANCE UNIT STATEMENT

10.1. PHASES INSPECTED

Date(s) of Inspection(s)	Phase Inspected	Date(s) Findings Reported to Study Director	Date(s) Findings Reported to Management	Auditor(s)
05-Jan-2005	Protocol Review	05-Jan-2005	16-Feb-2005	J.Tooman
03-Feb-2005	Test Article Preparation	04-Feb-2005	25-Mar-2005	C.O'Neill K.Dobbs
04-Feb-2005	Protocol Amendment I Review	04-Feb-2005	25-Mar-2005	L.Goodrich
08-Feb-2005	Body Weights, Clinical Observations	08-Feb-2005	25-Mar-2005	K.Dobbs
08-Feb-2005	PND 21 Randomization	08-Feb-2005	25-Mar-2005	K.Dobbs
09-Feb-2005	Test Article Administration	09-Feb-2005	25-Mar-2005	K.Dobbs
09-Feb-2005	Computerized Randomization Using MPUPJT -PND 21	09-Feb-2005	25-Mar-2005	E.Crawford
10-Feb-2005	Test Article Administration (unscheduled audit)	10-Feb-2005	25-Mar-2005	K.Dobbs
23-Feb-2005	Animal Care/Equipment	23-Feb-2005	25-Mar-2005	S.Solomon
01-Mar-2005	Blood Collection	01-Mar-2005	23-Apr-2005	K.Dobbs
01-Mar-2005	Necropsy	01-Mar-2005	23-Apr-2005	K.Dobbs
05-Apr-2005	Trimming of Tissues	06-Apr-2005	28-May-2005	K.Dobbs
07-May-2005, 12-May-2005, 13-May-2005	Study Records (I-1)	13-May-2005	29-Jun-2005	L.Rush
11-May-2005, 13-May-2005	Study Records (I-2)	13-May-2005	29-Jun-2005	L.Rush
12-May-2005, 13-May-2005	Study Records (I-3)	13-May-2005	29-Jun-2005	L.Rush
13-May-2005, 14-May-2005	Study Records (I-4)	14-May-2005	29-Jun-2005	L.Rush
14-May-2005, 24-May-2005, 26-May-2005	Study Records (N-1)	26-May-2005	29-Jun-2005	L.Rush
27-Jun-2005	Study Records (Rx-1)	27-Jun-2005	25-Jul-2005	L.Rush

Date(s) of Inspection(s)	Phase Inspected	Date(s) Findings Reported to Study Director	Date(s) Findings Reported to Management	Auditor(s)
27-Jun-2005	Study Records (H-1)	27-Jun-2005	25-Jul-2005	L.Rush
29-Jun-2005	Study Records (P-1)	29-Jun-2005	25-Jul-2005	L.Rush
11-Jul-2005, 12-Jul-2005	Study Records (A-1 to A-4)	12-Jul-2005	25-Aug-2005	E.Crawford
14-Jul-2005	Study Records (C-2)	22-Jul-2005	25-Aug-2005	L.Rush
20-Jul-2005	Study Records (C-1)	20-Jul-2005	25-Aug-2005	E.Crawford
20-Aug-2005	Draft AC Appendix - DE-71	21-Aug-2005	22-Sep-2005	E.Crawford
20-Aug-2005	Draft AC Appendix - Methoxychlor and 1-Chloro-2-nitrobenzene	21-Aug-2005	22-Sep-2005	E.Crawford
29-Aug-2005, 01-Sep-2005, 02-Sep-2005	Draft Final Report	02-Sep-2005	17-Oct-2005	L.Rush

This study was inspected in accordance with the U.S. EPA Good Laboratory Practice Standards (40 CFR Parts 160 and 792), the standard operating procedures of WIL Research Laboratories, LLC and the Sponsor's protocol and protocol amendments with the following exceptions. The data located in Appendices A (Certificates of Analysis), B (Analyses of Test Substances) and H (Coefficients of Variation) were the responsibility of the Sponsor. The data located in Appendix D (Feed Lot Analyses) were the responsibility of the manufacturer and were collected according to cGMP standards. Quality Assurance findings, derived from the inspections during the conduct of the study and from the inspections of the raw data and draft report, are documented and have been reported to the study director. A status report is submitted to management monthly.

The raw data and draft report were audited by the WIL Quality Assurance Unit prior to submission to the Sponsor to assure that the Final Report accurately describes the conduct and the findings of the study. Quality control (QC) and quality assurance (QA)

procedures followed those outlined in the Quality Assurance Project Plan (QAPP) that was prepared for this study (Appendix K).

The raw data, the retention sample(s), if applicable, and the final report will be stored in the Archives at WIL Research Laboratories, LLC or another location specified by the Sponsor.

10.2. APPROVAL

This study was inspected according to the criteria discussed in Section 10.1.

Report Audited By:

Elizabeth S. Crawford, BS
Compliance Specialist

Lori A. Rush, BS, LAT, RQAP-GLP
Sponsor Specialist, Quality Assurance

13 Dec 2005
Date

Report Released By:

Heather L. Osborn, BS, RQAP-GLP
Manager, Quality Assurance

13 Dec 2005
Date

11. REFERENCES

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12. DEVIATIONS FROM THE PROTOCOL AND QAPP

This study was conducted in accordance with the protocol and protocol amendments, except for the following.

- **Protocol Section 6.2** states that controls will be set to maintain an average daily relative humidity of 45 ± 5%. On 16 January 2005, the average relative humidity was 37.4%, on 17 January 2005 the average relative humidity was 39.9%, on 20 January 2005 the average relative humidity was 30.8%, on 21 January 2005 the average relative humidity was 37.6%, on 27 January 2005 the average relative humidity was 38.6% and on 3 February 2005 the daily relative humidity was 37.7%.
- **Protocol Section 7.7.1** states that clinical observations will be recorded approximately 1 hour following dosing. However, observations were recorded 44 minutes following dosing on 19 February 2005 (60 mg/kg DE-71 group) and on 22 February 2005 (60 mg/kg DE-71 and 50 mg/kg methoxychlor groups).
- According to the QAPP (Appendix K), balances are required to be checked using standard check weights encompassing the weight range to be employed. This was not performed at the time of fixed thyroid weight collection on 2, 3 and 7 March 2005.

These deviations did not negatively impact the quality or integrity of the data nor the outcome of the study.