Huntingdon
Life Sciences

STUDY NO. 04-4275
SPONSOR STUDY NO. WA 4-14, Task 4

ASSESSMENT OF PUBERTAL DEVELOPMENT AND THYROID FUNCTION IN JUVENILE FEMALE RATS

Final Report

Submitted to: Batelle
505 King Avenue
Columbus, Ohio 43201

Attn: Jerry D. Johnson, Ph.D., DABT

Date: 30 December 2005

STATEMENT OF COMPLIANCE

This study was conducted in accordance with the most recent versions of the US Environmental Protection Agency Good Laboratory Practice Standards (40 CFR Part 160) and the Organization for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice ENV/MC/CHEM(98)17 with the following exceptions:

1. A signed and dated formulation report was not provided by the Sponsor for stability and homogeneity analyses of the dosing formulations. Stability results were provided by the Sponsor for 1-Chloro-2-nitrobenzene and Methoxychlor at a dose volume that covered the lowest dosing formulation concentration.

2. The protocol did not identify the name and contact information of the Principal Investigator for the Statistical Analysis report.

GLP compliance for dose formulation stability and homogeneity analyses was the responsibility of the Sponsor.

This study was also performed according to protocol, at the Test Facility according to Huntingdon Life Sciences' Standard Operating Procedures (SOPs).

Edward R Frizell MD — 30 Dec 05

Edward R. Frizell, M.D., Ph.D. — Date
Study Director

SIGNATURE PAGE

SCIENTIST

The following Scientist was responsible for the overall conduct of this study. Departmental supervisory personnel are listed on the personnel page of this report (Appendix Q).

Edward R. Frizell, M.D., Ph.D.
Study Director

30 Dec 05
Date

SCIENTIFIC REVIEW

The following Scientist has reviewed and approved this report.

Keith P. Hazelden, B.Sc., CBiol., MIBiol.
Director, Reproductive and Developmental Toxicology

30 Dec 05
Date

QUALITY ASSURANCE STATEMENT

Listed below are the dates that this study was inspected by the Quality Assurance Unit of Huntingdon Life Sciences, East Millstone, New Jersey, and the dates that findings were reported to the Study Director and Management. This report reflects the raw data as far as can be reasonably established.

Type of Inspection	Date(s) of Inspection	Reported to Study Director and Management
GLP Protocol Review-Draft Number 2	12-14 Jan 05	14 Jan 05
GLP Protocol Review- Draft Number 4	20 Jan 05	20 Jan 05
Dose Preparation	22 Mar 05	11 Apr 05
Dose Administration and Training Records	31 Mar 05	1 Apr 05
Vaginal Opening Evaluations and Protocol Amendment Number 1	7 Apr 05	11 Apr 05
Terminal Blood Collection and Necropsy	20 Apr 05	22 Apr 05
Draft Report Tables and In-Life Study Data	24 May-9 Jun 05	9 Jun 05
Hormone Analysis Procedure, Report Appendix and Data	13, 19-21 Jul 05	21 Jul 05
Validation and Dose Confirmation Data and Reports	4 & 5, 8-11 Aug 05	12 Aug 05
Thyroxine (T4) Repeat Analysis Data	15 Aug 05	15 Aug 05
Draft Final Report and Study Data	20 & 21, 23-29 Sep 05	29 Sep 05
Statistical Report	30 Sep 05	30 Sep 05
Dose Confirmation Data and Report – Peroxide Number Evaluation	22 Nov 05	22 Nov 05
Hormone Analysis Sub-Report	19, 21 Dec 05	21 Dec 05

Melissa Elliott, B.S., RQAP-GLP
Quality Assurance Auditor

30 Dec 05
Date

SUMMARY

This study was intended to detect the endocrine disruptive potential of xenobiotics in pubertal female rats, as part of an inter-laboratory validation project designed to demonstrate the inter-laboratory reproducibility and reliability of the assay. Secondary objectives were to identify any areas of ambiguity in the design description, and to make a qualitative estimate of inter-laboratory variability for the various endpoints.

The litters from 44 time-mated Sprague-Dawley females were culled on Postnatal Day (PND) 4. For each litter all the female pups were kept, and enough males were retained as to allow for 10 pups/litter. Each group was derived from nominally 15 litters. F_1 females were utilized for the assessment of pubertal development and thyroid function. F_1 males were not utilized.

Fifteen F_1 females per dose group (Set A) were weaned at PND 21 and dosed orally once daily, from PND 22 to 42/43 (inclusive) with 25 or 100 mg/kg/day of 1-Chloro-2-nitrobenzene and 30 or 60 mg/kg/day of DE-71. Fifteen F_1 females per dose group (Set B) were also weaned at PND 21 and dosed orally once daily, from PND 22 to 42/43 (inclusive) with 12.5 or 50 mg/kg/day of Methoxychlor. Each Set (A and B) had an additional Vehicle control group of 15 F_1 females that received Corn oil from PND 22 to 42/43 (inclusive). Designation of experimental groups and dosing formulations with a letter code was implemented in order to ensure that the study was carried out in a 'blind' fashion.

The following parameters were evaluated: clinical signs of toxicity, body weights, food consumption, age and body weight at vaginal opening, age at first estrous and vaginal cytology. Necropsies were performed 2 hours post dose on PND 42/43. Blood was collected for hormone analyses (Tetraiodothyronine (T_4) and Thyroid Stimulating Hormone (TSH)) by decapitation, and selected tissues were collected, weighed and processed for macroscopic and microscopic pathology.

The administration of the test substances at any of the dose levels applied was well tolerated by the F1 females.

The administration of 1 Chloro-2-nitrobenzene at 25 or 100 mg/kg/day was associated with statistically significant increases in absolute liver weight, decreased weights of the ovaries and pituitary gland, and decreased T4 serum levels. Treatment with 1 Chloro-2-nitrobenzene at 100 mg/kg/day was also associated with increased TSH levels, increased body weight at the onset of vaginal opening (VO), a 5.8-days delay in VO and a 6.4 days delay in the age at first estrus.

Treatment with DE-71 at 30 or 60 mg/kg/day was associated with statistically significant increases in absolute liver weight, decreased T4 and increased TSH serum levels. A

statistically significant decrease in the weight of the ovaries was observed at 60 mg/kg/day DE-71. There were no DE-71-related effects on the onset of vaginal opening, or on estrous cycles evaluations.

The administration of Methoxychlor at 12.5 or 50 mg/kg/day was associated with statistically significant decreases in absolute kidney weights, as well as with advanced age at the onset of vaginal opening (1.2-days and 6.2-days at 12.5 and 50 mg/kg/day, respectively). Methoxychlor at 50 mg/kg/day was associated with decreased ovarian weight and irregular estrus cycles characterized by extended estrus.

Executive Summary- **Report Title: Assessment of Pubertal Development and Thyroid Function in Juvenile Female Rats.** **Test Substances: 1-Chloro-2-Nitrobenzene, DE-71, or Methoxychlor.**

Duration of Dosing: 21-22 days (Postnatal days 22 to 42/43)
Species/Strain: Rat, Sprague-Dawley
Initial Age: Postnatal day (PND) 22 (1st dose)
Date of First Dose (PND 22): 31 March 2005
Day of Necropsy: PND 42/43

Method of Administration: Gavage, once daily
Vehicle/Formulation: Corn oil/ Solution

Study No. 04-4275

GLP Compliance: Yes

		Set A [a]			
Daily Dose	**Corn oil (Set A) 0 mg/kg/day**	**1-Chloro-2-Nitrobenzene 25 mg/kg/day**	**1-Chloro-2-Nitrobenzene 100 mg/kg/day**	**DE-71 30 mg/kg/day**	**DE-71 60 mg/kg/day**
Females:					
Number Evaluated	15	15	15	15	15
Number Died or Sacrificed Moribund	0	0	0	0	0
Initial Body Weight (PND 22) , g, (%)	55.1	0%	-0.5%	+0.9%	+1.1%
Clinical Observations (overt signs of toxicity)	-	-	-	-	-
Age at Vaginal Opening, days [b]	33.0	+1.1 days	+5.8** days	+1.2* days	+0.3 days
Body weight at Vaginal Opening g, (%) [b]	114.4	+0.6%	+19.7%**	+5.3%	+0.3%
Age at first estrus, days [b]	34.6	+0.2 days	+6.4 days	+2.7 days	+0.1 days
Estrous Cycling	-	-	incomplete	-	-
Final Body Weight (PND 42), g, (%)	157.6	0%	-2.7%	+2.4%	+2%
Body weight gain (PND 22-42), g, (%)	102.4	0.1%	-3.8%	+3.3%	+2.5%
Adrenals weight g, (%)[b]	0.0423	-14.4	-19.9	-4.3	-9.7
Kidney weight, g, (%)[b]	1.478	-3.5	-0.9	-1.5	-0.1
Liver weight g, (%)[b]	7.505	+23.2**	+49.6**	+26.6**	+44.2**
Ovaries, g, (%)[b]	0.0932	-16.8**	-19.4**	-7.9	-10.6*
Pituitary weight g, (%)[b]	0.0090	-10.0**	-33.3**	-1.1	-7.8
Thyroid g, (%)[b]	0.0217	-4.6	-3.2	+5.1	+12.9*
Uterus g, (%)[b]	0.355	-6.8	-22.8	-1.4	+1.7
Uterus blotted g, (%)[b]	0.333	-10.5	-23.7	+0.3	+0.6

(For footnotes, see next page)

Set A Continued

Daily Dose	**Corn oil (Set A) 0 mg/kg/day**	**1-Chloro-2-Nitrobenzene 25 mg/kg/day**	**1-Chloro-2-Nitrobenzene 100 mg/kg/day**	**DE-71 30 mg/kg/day**	**DE-71 60 mg/kg/day**
Necropsy/Histopathology: Ovaries	-	-	-	-	-
Necropsy/Histopathology Uterus hypertrophy/hyperplasia (score) [c]	1.1	0.4*	0.2**	0.6	0.8
Necropsy/Histopathology Thyroid epithelial height (score) [c]	2.9	2.9	2.9	3.0	3.3
Necropsy/Histopathology Thyroid colloid area (score) [c]	3.1	2.9	2.9	2.7	2.7*
Hormones: Tetra-iodothyronine (T_4)[b]	5.29	-22.3%**	-28.2%**	-71.5%**	-87.3%**
Thyroid stimulating hormone (TSH)[b]	4.30	+7.7%	+28.8%*	+40.0%**	+53.5%**

a- Control group means (and for treated groups the % difference or difference in days) are shown. Absolute values shown (unadjusted by covariance analysis), unless indicated otherwise by superscript b. Statistical comparisons: ANOVA or ANCOVA (PND 21 body weight as covariate), and body weight blocking factor, as appropriate.

b- Adjusted for PND 21 weight by covariance analysis.

c- The mean Histology score is shown for all groups.

- = No noteworthy findings

* $p<0.05$

** $p<0.01$

Executive Summary- **Report Title: Assessment of Pubertal Development and Thyroid Function in Juvenile Female Rats.** **Test Substances: 1-Chloro-2-Nitrobenzene, DE-71, or Methoxychlor.**

Duration of Dosing: 21-22 days (Postnatal days 22 to 42/43)
Species/Strain: rat, Sprague-Dawley
Initial Age: Postnatal day (PND) 22 (1st dose)
Date of First Dose (PND 22): 01 and 02 April 2005
Day of Necropsy: PND 42/43

Method of Administration: Gavage, once daily
Vehicle/Formulation: Corn oil/ Solution

Study No. 04-4275

GLP Compliance: Yes

	Set B [a]		
Daily Dose	**Corn oil (Set B) 0 mg/kg/day**	**Methoxychlor 12.5 mg/kg/day**	**Methoxychlor 50 mg/kg/day**
Females:			
Number Evaluated	15	15	15
Number Died or Sacrificed Moribund	0	0	0
Initial Body Weight (PND 22) , g, (%)	56.1	-0.7%	-0.5%
Clinical Observations (overt signs of toxicity)	-	-	-
Age at Vaginal Opening, days [b]	33.7	-1.3 days**	-6.2 days**
Body weight at Vaginal Opening g, (%)[b]	120.7	-8.7%**	-32.7%**
Age at first estrus, days [b]	35.6	-1.8 days	-5.1 days
Estrous Cycling	-	-	Irregular
Final Body Weight (PND 42), g, (%)	165.6	-3.7%	-5.9%
Body weight gain (PND 22-42), g, (%)	109.5	-5.3%	-8.6%
Adrenals weight g, (%)[b]	0.0480	-5.6	-9.0
Kidney weight, g, (%)[b]	1.487	-5.0*	-8.0**
Liver weight g, (%)[b]	8.041	-9.6**	-11.5**
Ovaries, g, (%)[b]	0.1048	-15.0	-21.5*
Pituitary weight g, (%)[b]	0.0096	-1.0	-11.5
Thyroid g, (%)[b]	0.0233	-5.6	+1.7
Uterus g, (%)[b]	0.399	+8.5	-3.8
Uterus blotted g, (%)[b]	0.347	-0.3	-4.9

(For footnotes, see next page)

Set B Continued

Daily Dose	Corn oil (Set B) 0 mg/kg/day	Methoxychlor 12.5 mg/kg/day	Methoxychlor 50 mg/kg/day
Necropsy/Histopathology: Ovaries	-	-	-
Necropsy/Histopathology Uterus hypertrophy/hyperplasia (score)[c]	0.7	0.7	0.7
Necropsy/Histopathology Thyroid epithelial height (score)[c]	3.0	2.7	3.1
Necropsy/Histopathology Thyroid colloid area (score)[c]	3.1	3.1	2.9
Hormones: Tetra-iodothyronine (T_4)[b]	4.61	-7.6%	-5.9%
Thyroid stimulating hormone (TSH)[b]	3.52	23.0%	16.8%

a- Control group means (and for treated groups the % difference or difference in days) are shown. Absolute values shown (unadjusted by covariance analysis), unless indicated otherwise by superscript b. Statistical comparisons: ANOVA or ANCOVA (PND 21 body weight as covariate), and body weight blocking factor, as appropriate.

b- Adjusted for PND 21 weight by covariance analysis.

c- The mean Histology score is shown for all groups.

- = No noteworthy findings

* $p<0.05$

** $p<0.01$

TABLE OF CONTENTS

TABLE OF CONTENTS

FIGURES

TABLES

TABLE OF CONTENTS

APPENDICES

1. INTRODUCTION

This study was intended to detect the endocrine disruptive potential of xenobiotics in pubertal female rats, and was part of an inter-laboratory validation project designed to determine whether independent laboratories, using the same study design, arrived at the same conclusions regarding the ability of selected chemicals with known effects to interact with the female endocrine system (that is, to demonstrate the inter-laboratory reproducibility and reliability of the assay). Secondary objectives were to identify any areas of ambiguity in the design description, and to make a qualitative estimate of inter-laboratory variability for the various endpoints.

2. MATERIALS AND METHODS

2.1. STUDY MANAGEMENT

2.1.1. SPONSOR

Batelle
505 King Avenue
Columbus, Ohio 43201

2.1.2. SPONSOR REPRESENTATIVE

Jerry D. Johnson, Ph.D., DABT

2.1.3. TESTING FACILITY

Huntingdon Life Sciences
P.O. Box 2360
Mettlers Road
East Millstone, New Jersey 08875-2360

2.1.4. STUDY DIRECTOR

Edward R. Frizell MD, PhD.

2.2. STUDY DATES

2.2.1. STUDY INITIATION

02 March 2005 (Date Study Director Signed The Protocol)

2.2.2. DATE OF ANIMAL RECEIPT (OECD EXPERIMENTAL START DATE)

25 February 2005

2.2.3. DOSING INITIATION (EPA EXPERIMENTAL START DATE)

31 March 2005 (Set A)
01 and 02 April 2005 (Set B)

2.2.4. DOSING TERMINATION

20 and 21 April 2005 (Set A)
21, 22 and 23 April 2005 (Set B)

2.2.5. TERMINAL SACRIFICES

20 and 21 April 2005 (Set A)
22 and 23 April 2005 (Set B)

2.2.6. EXPERIMENTAL COMPLETION DATE

04 August 2005 (Date of last data collection)

2.2.7. STUDY COMPLETION DATE

30 December 2005 (Date Final Report is signed by the Study Director)

2.3. EXPERIMENTAL DESIGN

	Doses (once daily, by oral gavage)	Dosage (mg/kg)	Conc. (mg/mL)	Set A	Set B	F_1 Females
Group	**Designation[a]**					
1	Test Substance C (1-Chloro-2-nitrobenzene)	25	10	X		15
2	Test Substance B (1-Chloro-2-nitrobenzene)	100	40	X		15
3	Test Substance H (DE-71)	30	12	X		15
4	Test Substance G (DE-71)	60	24	X		15
5	Test Substance F (Corn Oil)	-	0	X		15
6	Test Substance L (Methoxychlor)	12.5	5		X	15
7	Test Substance I (Methoxychlor)	50	20		X	15
8	Test Substance E (Corn Oil)	-	0		X	15

[a]Designation of formulation preparations with letters were implemented in order to ensure that the study was carried out 'blind'.

The litters from 44 time-mated females were culled on postnatal day (PND) 4. For each litter all the female pups were kept, and enough number of males were retained as to allow 10 pups/litter. Each group was derived from 15 litters. F_1 females were utilized for the assessment of pubertal development and thyroid function. F_1 males were not utilized. The F_1 females were weaned at PND 21 and dosed orally once daily, from PND 22 to PND 42/43. In-life endpoints for the F_1 females included body weight, body weight gain, age and body weight at vaginal opening, and vaginal cytology. Necropsies were performed 2 hours post dose on PND 42/43. Blood was collected for hormone analyses by decapitation, and selected tissues were collected, weighed and processed for macroscopic and microscopic pathology.

2.4. JUSTIFICATIONS

2.4.1. ROUTE, FREQUENCY AND DURATION OF ADMINISTRATION

The present design had been shown in pre-validation studies to be effective in detecting effects of test substances on female pubertal development and thyroid function in juvenile/peri-pubertal animals. A regime such as this permitted detection of effects of a test substance on the integrity and performance of the female endocrine system.

2.4.2. DOSE LEVEL SELECTION

The test substances and dosages utilized in this validation were selected by the US EPA.

For each substance, the High dose levels were selected to represent a dose just below the maximum tolerable dose (MTD), while the low dose levels represent approximately 25% of the High dose level for 1-Chloro-2-nitrobenzene and methoxychlor, but 50% for DE-71.

Detailed justification for the dose levels selected, according to each of the test substances is as follows:

1-Chloro-2-nitrobenzene - Study A, undertaken with Sprague-Dawley rats, days 6-15 of gestation (10 days). Animals dosed at 0, 25, 75, or 150 mg/kg/day in corn oil, by gavage. Results: at 150 mg/kg/ day severe maternal toxicity associated with mortality (dosing was suspended prior to scheduled sacrifice). At 75 mg/kg slightly reduced but not significant, body weight gain was observed, along with reduced feed consumption during the first 5 days of treatment (animals recovered), some urinary staining and alopecia was also noted. One animal out of 25 treated in this group died. Study B, same as above at 0 and 100 mg/kg/day in corn oil. Results: at 100 mg/kg/day consisted in slight maternal body weight loss (first 5 days of treatment) accompanied by reduction in food consumption from initiation of treatment through Gestation Day 16

Endocrine disruptor effects: none. Source: OECD documentation, IUCLID dataset, OECD HPV Chemicals Programme.

DE-71 - A study undertaken with Wistar rats. Treatment period: Postnatal Days (PND) 23-53 (for the males) and PND 21-41 (for the females). Animals dosed at 0, 3, 30 and 60 mg/kg/day in corn oil, by gavage. No signs of toxicity were observed at any of the dose levels applied

Endocrine disruptor effects: a) Females: Delayed onset of vaginal opening (VO) (1.8 days) at 60 mg/kg/day only. Decreased tetraiodothyronine (thyroxine, T_4) serum levels at 30 and 60 mg/kg/day. No effect on triiodothyronine (T_3) serum levels at any dose level applied. A non-statistically significant increase in thyroid-stimulating hormone (TSH) at 60 mg/kg/day was also noted. b) Males: Delayed onset of preputial separation (PPS) by 1.7 and 2.1 days in the 30 and 60 mg/kg/day, respectively. Increased weight of anterior pituitary at 30 mg/kg/day only, and decreased weight of ventral prostate and seminal vesicles at 60 mg/kg/day. Lateral prostate, epididymal and testicular weights unaltered at any of the dose levels applied. Decreased serum T_4 at all dose levels, decreased T_3 serum levels at 30 and 60 mg/kg/day. Increased serum levels of TSH in a dose-related manner.

Source: Stoker TE et al., Toxicological Sciences 78: 144-155, 2004.

Methoxychlor - A study undertaken with Sprague-Dawley female rats. Treatment period postnatal days (PND) 22-43. Dose levels: 0, 12.5, 25 and 50 mg/kg/day in corn oil, by gavage. No mortality or test article-related effects on body weight were observed at any of the dose levels applied

Endocrine disruptor effects: dose-related advanced VO onset (by 4, 4.9 and 5.4 days for 12.5, 25 and 50 mg/kg/day, respectively). In addition, Methoxychlor treatment affected age at first estrus, cycle length (↑) and regularity of cycles.

Source: National Toxicology Program, Draft report August 25 2003. Study number RACB 20103.

2.4.3. TEST ANIMAL SELECTION

The rat is accepted by Regulatory Authorities as a surrogate for humans in the detection of effects on reproductive function. It is the preferred rodent species for most aspects of pre-clinical toxicity testing, for practical reasons and in view of the large amount of accumulated background knowledge in the species.

2.4.4. NUMBER OF ANIMALS

The number of animals in this study (24 time mated females) was considered the minimum necessary to implement the present study design (as outlined by the Environmental Protection Agency (EPA)), and to allow for meaningful interpretation of the data, considering individual animal variation. The number of dams ordered (45) was considered appropriate to provide a sufficient number of adequate litters for constructing each F_1 group (allowing for the exclusion of litters that are too small, or that are delivered after Gestation Day 23, and avoiding the presence of litter-mates in the groups). Two dosage groups per test substance were required to indicate a dose relationship in any effects observed, in comparison with a vehicle control group.

2.5. TEST SUBSTANCES

Name (Cas No.)	Lot Number	Purity	Description	Date Received	Expiration Date	Storage
1-Chloro-2-nitrobenzene (88-73-3)	09019MC	99.8%	Yellow crystalline solid	17 Dec 04	01 Nov 10	Room temperature
DE-71 (N/A)	4550OD23D	99.5%	Clear, amber, dense, viscous liquid	15 Dec 04	03 Nov 10	Room temperature
Methoxychlor (72-43-5)	102K1373	95.3%	Faint orange powder	16 Dec 04	13 Oct 09	Room temperature

2.5.1. TEST SUBSTANCE CATEGORY

Positive and presumed-negative endocrine disrupting chemicals.

2.5.2. SUPPLIER

Batelle
1529 West Sequim Bay Rd.
Sequim, WA 98382

2.5.3. ANALYSIS

Information on the identity, purity, composition, batch/lot numbers or other characteristics that define the test substances are on file with the Sponsor. Certificates of Analysis for the test substances are include in this report (Appendix N).

2.5.4. STABILITY

Dose formulation stability and appropriate homogeneity analyses were the responsibility of the Sponsor, and results pertaining to each test substance are reproduced in Appendix N.

2.5.5. ARCHIVAL SAMPLE

A sample of each lot of test substance used during the course of the study will be retained at Testing Facility under the stated storage conditions for the material for a period of 1 year following the issue of the final study report. The samples will be discarded or other arrangements made, as for other archival materials, by agreement with the Sponsor.

2.5.6. DISPOSITION

The unused dosing formulations were discarded by the Testing Facility after completion of daily animal dosing. Following the end of the dosing period, all remaining test substances were returned to the Sponsor on 09 August 2005.

2.6. CONTROL ARTICLE (VEHICLE)

Corn oil (clear, free of sediment, odorless)

2.6.1. SUPPLIER

Battelle
1529 West Sequim Bay Rd.
Sequim, WA 98382

2.6.2. LOT NUMBERS

A0-003, a combination of Lots A0-001 and A0-002 (Lot numbers assigned by Huntingdon Life Sciences)

2.6.3. PURITY

100%

2.6.4. DESCRIPTION

Clear yellow liquid

2.6.5. DATES RECEIVED

16 December 2004 (Lot A0-001)
26 January 2005 (Lot A0-002)

2.6.6. EXPIRATION DATE

28 August 2005

2.6.7. ANALYSIS

Documentation of the identity, purity, composition, or other characteristics that define the control article, and the maintenance of these records, was the responsibility of the Sponsor. A Certificate of Analysis was not available. However, at the Sponsor's request, samples of the corn oil were analyzed for peroxide content, with the specification that this content should not exceed 3 meq/kg. The results of analysis of the 3 samples taken (see Appendix B), ranged from 2.49 to 2.83 meq/kg.

2.6.8. STORAGE

Lot # A0-002 was stored refrigerated (2-8° C) upon receipt. Lot # A0-001 was initially kept at room temperature, then moved to storage in the refrigerator (2-8° C) on 20 January 2005.

2.6.9. ARCHIVAL SAMPLE

A sample of the control article used during the course of the study was retained at Testing Facility under the stated storage conditions for the materials.

2.6.10. DISPOSITION

The unused dosing formulations were discarded by the Testing Facility after completion of daily animal dosing. At the conclusion of the study, the Sponsor will determine the actual disposition, which will be documented in the raw data.

2.7. TEST ANIMALS

2.7.1. SPECIES

Albino rats, Sprague-Dawley strain, Crl: CD® IGS BR

2.7.2. SUPPLIER

Charles River Laboratories
Kingston, New York 12484

2.7.3. NUMBER OF ANIMALS

Ordered:	45 time mated females.
Placed on study:	the litters of 31 females.

Number of female pups placed on study: total 120; 15 F_1 females/group.

2.7.4. AGE AND WEIGHT

The weight range of the time-mated females at mating were 210-272 grams. These time-mated females were approximately 12-15 weeks of age at receipt on Gestation Day (GD) 8 and 10, where GD 0 was the day when vaginal plugs/vaginal plugs + sperm positive smears were detected. Natural delivery was allowed.

The experimental animals were the F_1 generation females (15 pups/group). These pups were 38-69 grams on postnatal day (PND) 21. PND 0 was defined as the day a pup is first seen.

2.7.5. ACCLIMATION PERIOD

F_0 animals were acclimated for a minimum of 5 days at the Testing Facility prior to delivery.

2.8. SELECTION/GROUP ASSIGNMENT

More animals than required for the study were purchased and acclimated. Animals considered suitable for study on the basis of pretest physical examinations, body weight and any other pretest evaluations, were assigned to control or treated groups as follows:

The study was carried out with two sets of animals (A and B). Set A consisted of 5 groups, Set B consisted of 3 groups.

For each set, on PND 21, all the pups were marked with their litter identification number, then all the pups in each litter were individually weighed to the nearest 0.1 gram, and the pups in that litter were sorted by their body weights. The pups assigned to the groups were taken from at least 15 litters (so that litter-mates were not present in any group). The pups were allocated to the experimental groups based on their individual body weights, so as to generate groups with mean body weights that were similar, both in mean value and in variation.

Disposition of all animals not utilized in the study was maintained in the study file.

2.9. ANIMAL IDENTIFICATION

Each time-mated F_0 female was identified with a unique consecutively numbered ear tag upon receipt.

On Postnatal Day 21, each F_1 animal was ear-tagged with an identification number (Postnatal Day 22 for Animal Nos. 651, 751 and 851). In addition, the pups' toe-tattooing (used for identification prior to weaning) served as a back-up system in case the ear-tags were lost.

Appropriate records of identification numbers for each F_0 and corresponding F_1 progeny were kept, and the records were utilized to identify members of the same litter.

Each F_1 animal's cage (post-weaning) was ascribed a cage card, which was color-coded for group identification at Pharmacy and contained the study number and relevant animal number(s) only.

Group color-coding for both Pharmacy and In-Life did not follow standard coding patterns, as the study was to be conducted in a 'blind' fashion. Decoding tables were kept confidential within Huntingdon Life Sciences and were available only to the Study Director, the Alternate Contact, Quality Assurance, the Report Writer, Pharmacy and Analytical Chemists.

2.10. VETERINARY CARE

Animals were monitored by the technical staff for any conditions requiring possible veterinary care.

2.11. HUSBANDRY

2.11.1. FACILITIES MANAGEMENT/ANIMAL HUSBANDRY

Currently acceptable practices of good animal husbandry were followed, e.g. *Guide for the Care and Use of Laboratory Animals;* National Academy Press, 1996. Huntingdon Life Sciences Inc., East Millstone, New Jersey is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

2.11.2. HOUSING

F_0 dams were housed individually in plastic "shoebox" cages (10 inches wide X 17.5 inches long X 8 inches high (with pine bedding).

Following weaning on PND 21, F_1 males continued to be housed in plastic "shoebox" cages (with pine bedding), allowing for nominally 3 animals/cage.

2.11.3. FEED

Feed was Teklad Certified Rodent Diet, No. 2018c (meal) supplied by Harlan Teklad, Madison, WI and was provided *ad libitum* in a glass feeder jar with a stainless steel lid, secured in each cage. Clean jars and fresh food were provided at least weekly.

The diet had a Genistein-equivalent content of Daidzein and Genistein (Aglycone units) that was lower than 300 parts per million (ppm). The feed's batch content of Daidzein and Genistein were provided by the diet's manufacturer, and this information is reproduced in Appendix N.

2.11.4. FEED ANALYSIS

Analytical certification of batches of feed used are maintained on file by the manufacturer and at the Testing Facility, and are reproduced in Appendix N. There were no known contaminants in the feed that were expected to interfere with the objectives of this study.

2.11.5. BEDDING

Pine bedding (Lot No. 4224804 of laboratory-grade, heat-treated, pine shavings, Northeastern Products, Warrensburg, NY) was provided for each dam. Fresh bedding was provided at least twice weekly or as needed throughout the study. Analysis information for the bedding used is reproduced in Appendix N. There were no known contaminants in the bedding that were expected to interfere with the results of this study. Corn cob bedding was not used.

2.11.6. WATER

The animals were provided reverse-osmosis water *ad libitum* (without chlorine supplementation), produced from tap water from the local water system. The reverse-osmosis system was attached to the cage-rack water delivery system.

2.11.7. WATER ANALYSIS

Water analyses are conducted monthly by Elizabethtown Water Company to assure that water meets standards specified under the EPA Federal Safe Drinking Water Act Regulations (40 CFR Part 141). Water analyses, provided by the supplier, are maintained on file at the Testing Facility. In addition, chemical and microbiological analyses conducted by a Sub-contractor, biannually, on water samples collected from representative rooms in this facility. Results are maintained on file. There are no known contaminants that were expected to interfere with the objectives of this study.

The reverse-osmosis water delivered to the animals was not analyzed.

2.11.8. ENVIRONMENTAL CONDITIONS

Light/Dark Cycle

Fourteen/ten-hour light/dark cycle (actual hours 0500 to 1900 hours) controlled via an automatic timer was provided.

Temperature

Temperature was monitored in accordance with Testing Facility SOPs and was maintained within the specified range.

Desired Range:	20 to 24°C
Actual Range:	22 to 24°C
Daily Average Range:	22 to 24°C

Humidity

Humidity was monitored in accordance with HLS SOPs and maintained within the specified range to the maximum extent possible. The brief, transient excursions that occurred outside the specified range were not considered to have affected the integrity of the study.

Desired Range:	40 to 50%
Actual Range:	6 to 68%
Daily Average Range:	44 to 59%

Air Changes

Animal quarters had 10-15 air changes per hour. The actual number of air changes per hour in each animal room is recorded at least twice each year and the Testing Facility retains these records.

2.12. TEST SUBSTANCE AND CONTROL ARTICLE PREPARATION

The dosing formulations were prepared one time only at the beginning of the study. Each dosing formulation was prepared by weighing an accurate amount of the test substance into a 1-L volumetric flask and mixing it with corn oil, the vehicle. Each formulation was distributed into four 250-mL amber glass bottles and the bottles were capped, labeled and stored in a refrigerator.

CORN OIL PREPARATION

Lot numbers A0-001 and A0-002 were combined into a Nalgene carboy and stirred using an overhead mixer for 80 minutes. The top of this container as well as the spigot were wrapped in parafilm and aluminum foil, and the container was nitrogen capped and returned to the refrigerator. This combined corn oil was designated as Lot number A0-003 for utilization in this study.

1-CHLORO-2-NITROBENZENE

The 1-Chloro-2-Nitrobenzene low dose formulation was prepared by warming the test substance in a water bath in a fume hood set at 40° C until it was in a liquid form. Then 10 grams of this test substance was weighed into a 1 L volumetric flask. Approximately one-half the corn oil was added and agitated quickly to dissolve the test substance and then the flask was filled to the mark at a concentration of 10 mg/mL and stirred for 118 minutes.

The 1-Chloro-2-Nitrobenzene high dose formulation was prepared by warming the test substance in an oven set at 40° C until it was in a liquid form. Then 40 grams of this test substance was weighed into a 1 L volumetric flask. Approximately one-half the corn oil was added and agitated quickly to dissolve the test substance and then the flask was

filled to the mark at a concentration of 40 mg/mL and stirred for 118 minutes.

METHOXYCHLOR FORMULATION PREPARATION

The methoxychlor low dose formulation was prepared by accurately weighing 5.26 grams of this test substance (to account for 95% purity) into a tared weigh boat. The methoxychlor was quantitatively transferred from the weigh boat to a 1-liter (L) volumetric flask and corn oil was added to the mark at a concentration of 5 mg/mL. The solution was stirred refrigerated for approximately 24 hours to disperse the test substance into the vehicle. The solution was sonicated to dissolve the test substance (for up to the minimum time required). During the sonication step, the temperature of the solution did not go above ~40°C. When the test substance was dissolved, the flask was removed from the sonicator and the flask was manually agitated to ensure the contents were uniform.

The methoxychlor high dose formulation was prepared by weighing 21.1 grams of this test substance (to account for 95% purity) into a tared weigh boat. The methoxychlor was quantitatively transferred from the weigh boat to a 1-L volumetric flask and corn oil was added to the mark at a concentration of 20 mg/mL. The solution was stirred refrigerated for approximately 24 hours to disperse the test substance into the vehicle. The solution was sonicated to dissolve the test substance (for up to the minimum time required). During the sonication step, the temperature of the solution did not go above ~40°C. When the test substance was dissolved, the flask was removed from the sonicator and the flask was manually agitated to ensure the contents were uniform.

DE-71 FORMULATION PREPARATION

The DE-71 low dose formulation was prepared by first warming the DE-71 to approximately 40 °C in a water bath (DE-71 thickens rapidly as it cools), then weighing 12 g of the DE-71 into a container and adding a portion of the corn oil vehicle to the container. The DE-71 was stirred vigorously with an overhead stirrer for approximately 52 minutes until it went into solution. The solution was transferred to a calibrated 1 Liter volumetric flask. The mixture container was rinsed well with vehicle and the rinse transferred to the 1-Liter flask and diluted to a final volume with vehicle at a concentration of 12 mg/mL.

The DE-71 high dose formulation was prepared by first warming the DE-71 to approximately 40 °C in a water bath, then weighing 24 g of the DE-71 into a container and adding a portion of the corn oil vehicle to the container. DE-71 was stirred vigorously with an overhead stirrer for approximately 52 minutes until it went into solution. The solution was transferred to a calibrated 1 Liter volumetric flask. The mixture container was rinsed well with vehicle and the rinse transferred to the 1 Liter flask and diluted to a final volume with vehicle at a concentration of 24 mg/mL.

2.13. ANALYSIS OF DOSING FORMULATIONS (APPENDIX B)

Analyses to determine homogeneity and concentration of certain test substances under the conditions of this study were performed by the Testing Facility. For any test substance not requiring stability or homogeneity analysis, the Sponsor provided supporting documentation.

2.13.1. HOMOGENEITY

For any test substance that did not require homogeneity analysis, the Sponsor provided documentation indicating that the formulations were a true solution.

2.13.2. STABILITY

Stability analysis supporting documentation was provided by the Sponsor for each test substance.

2.13.3. CONFIRMATION ANALYSIS

Dose confirmation analysis was carried out prior to the start of the dosing period as follows: Two sets (A and B) of duplicate samples (1 mL each) were taken from the middle layer of each formulation concentration. Samples were collected while constantly stirring, following a stirring period of not less than 15 minutes. Duplicate samples (3 mL each, for sets A and B) were also taken from the middle layer of the vehicle solution.

In each case, set A was refrigerated at 2-8° Celsius and analyzed within 24 hours from collection, while set B was frozen at

approximately -70°C or below, and was only analyzed if necessary.

Formulation analysis and acceptance criteria for analytical results were according to the Sponsor's specifications, as given in Appendix B. Once all analytical results were confirmed, the samples were discarded.

2.14. TEST SUBSTANCE AND CONTROL ARTICLE ADMINISTRATION

The dosing formulations were administered once daily at approximately the same time each day (0700 to 0900 hours) on PND 22 to 42/43 inclusive by oral gavage utilizing a metal catheter attached to an appropriately sized syringe. Dosing formulations were maintained on a magnetic stirrer during dosing procedures. Dosage volume (2.5 ml/kg body weight) was calculated for each animal using the body weight taken on that day.

2.15. IN-LIFE EXPERIMENTAL OBSERVATIONS

2.15.1. CLINICAL OBSERVATIONS (F_0)

Observations for mortality and morbidity were made at least twice daily from arrival. Animals in apparently poor health or a moribund condition were identified for further monitoring and possible euthanasia.

2.15.2. BODY WEIGHTS (F_0)

Maternal body weights were recorded on Postnatal Days 17 and 21. This data was not formally reported, and were for welfare monitoring purposes only.

2.15.3. CULLING

On PND 4, the litters were culled to a maximum of 10 pups, preferentially removing male pups, so as to maximize the number of females that were available in each litter for selection at weaning. Only one litter had less than 10 pups following these procedures (it had 6 pups).

2.15.4. CLINICAL OBSERVATIONS (F_1)

2.15.4.1. Observations

Litters were observed twice daily for the number of live and dead pups, and any pup abnormalities. The sex of each pup with abnormalities or found dead was recorded. The presence of dead pups was recorded, and these were removed from the litter as found. The pups in each litter were counted daily until weaning at PND 21.

2.15.4.2. Sex Determination

The sex of each pup was verified on PND 4, 7 and 21.

2.15.4.3. F_1 Viability Observations (Cage Side)

Observations for mortality, morbidity, and signs of severe toxicity were made at least twice daily: prior to dosing during the treatment period, then again late in the workday.

2.15.4.4. Detailed Physical Examination (In The Hand)

Each pup was given a gross physical examination on PND 4, 7 and 21.

F_1 animals were examined closely, weekly from PND 21 through to terminal euthanasia, for any abnormality/sign of toxicity. Examinations included observations of general condition, skin and fur, eyes, nose, oral cavity, abdomen and external genitalia as well as evaluations of respiration. These evaluations were performed prior to dosing.

2.15.5. BODY WEIGHTS (F_1)

Individual body weights of the F1 females were recorded weekly prior to weaning and daily from PND 21 to the end of the study.

For animals necropsied on PND 43, body weight gain was reported as body weight gain only to PND 42.

2.15.6. FEED CONSUMPTION (F_1)

Feed consumption was measured gravimetrically, once weekly for each F_1 animal cage from Postnatal Day 21 onwards. Feed consumption measurements represented the feed consumed by (nominally) three F_1 animals caged together.

2.15.7. VAGINAL OPENING (F_1)

Beginning on PND 22, the F_1 animals were examined daily for vaginal opening. Complete vaginal opening was recorded on the days they were observed. The date of achieved vaginal opening was defined as the day of complete vaginal opening (partial, pin-hole openings or persistent tissue threads were not seen in the study). The body weight of each female achieving vaginal opening was recorded on the day of achievement.

2.15.8. VAGINAL CYTOLOGY/ESTROUS CYCLING (F_1)

Daily vaginal smears were taken from each F_1 female between 0800 and 1435 each day and the stage of estrus was determined, commencing on the day vaginal opening was achieved. These evaluations continued until the end of the study. The vaginal smears were classified as follows: Diestrus: presence of leucocytes; Proestrus: presence of nucleated epithelial cells; Estrus: presence of cornified epithelial cells. Age at first estrus was recorded and animals that exhibited irregular cycles or pseudopregnancies were noted.

The sequence of stage observations enabled detection of any irregularity of the cycle, arrested cycling, or pseudopregnancy.

2.16. POSTMORTEM EVALUATIONS

2.16.1. METHOD OF EUTHANASIA

F_0 Dams and Excess Pups:

F_0 females that weaned litters were euthanized by exposure to carbon dioxide on PND 21, or as soon as convenient thereafter. One female was found not pregnant and was euthanized.

Pups culled on PND 4 were euthanized by intraperitoneal injection of sodium pentobarbitone. Any excess pups not required to constitute the experimental groups were euthanized on PND 21, or as soon as convenient thereafter, by carbon dioxide intoxication.

No postmortem examinations were performed on these animals.

F_1 Females:

F_1 females were sacrificed on PND 42 or 43 by preliminary exposure to CO2 (not to exceed 60 seconds) followed by decapitation; the only method of euthanasia appropriate for this study. The technique reduced the effect of stress on the hormones that were assayed in this study, which were sensitive to stress.

2.16.2. NECROPSY SCHEDULE

Care was taken to ensure that all groups were balanced as to their termination age: for each group approximately half were necropsied on PND 42 and half on PND 43.

On the day of necropsy the animals were dosed between 0700 and 0900 hours. Necropsies began 2 hours post-dosing and were completed by 1300 hours.

2.16.3. TERMINAL BLOOD COLLECTION

At least 2.0 mL of blood was collected from the neck vessels immediately following decapitation. The blood was allowed to clot for at least 30 minutes at room temperature. Once a clot was observed, the samples were centrifuged for 10 minutes at *ca* 3000 rpm. Serum was collected into siliconized microcentrifuge tubes and stored at –20°C or below, for subsequent thyroxine (T_4), and thyroid stimulating hormone (TSH) measurements.

2.16.4. TERMINAL EXAMINATIONS

At necropsy the thyroid (with the attached portion of the trachea), liver, kidneys (as a pair), pituitary and adrenal glands (as a pair), were removed and the weights of these organs (or pair of organs), except the thyroid/trachea, were recorded to the nearest 0.1 mg. 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.

The removal and preparation of the uterine tissues for weight measurement was carried out as follows:

The pubic symphysis was opened. Each ovary and uterine horn was detached from the dorsal abdominal wall. The urinary bladder and ureters was removed from the ventral and lateral side of the uterus and vagina. Fibrous adhesions between the rectum and the vagina were detached until the junction of vaginal orifice and perineal skin was identified. The uterus, vagina and oviducts/ovaries were detached from the body by incising the vaginal wall just above the junction between perineal skin. The excess of fat and connective tissue were trimmed away. Care was taken to remove the mesenteric fat from the uterine horns with small surgical scissors, to avoid damaging the uterus, so that the uterine fluid was retained.

The vagina was removed from the uterus. The ovaries with oviducts were removed and weighed (left and right together, weight B). The uterus, free of mesenteric fat, was removed and the weight was immediately recorded to the nearest 0.1 mg (weight A). The weight of the uterus with luminal fluid (wet weight) was calculated by subtracting B from A. The uterus was placed on a paper towel, slit to allow the luminal fluid contents to leak out, gently blotted dry and reweighed (blotted weight).

The ovaries and uterus were placed in Bouin's fixative for 24 hours, after which they were rinsed and stored in 70% alcohol until embedded in paraffin. These tissues were stained with hematoxylin and eosin (H&E).

The thyroid, with attached trachea, were fixed in 10% buffered formalin for 24 hours. Then the thyroid was dissected from the trachea, blotted and weighed to the nearest 0.1 mg and placed in 70% ethanol, embedded in paraffin, stained with hematoxylin and eosin.

2.16.5. HISTOPATHOLOGY

The preserved tissues were evaluated for pathologic abnormalities and potential treatment-related effects in a "blind" fashion (treatment group location not known during slide evaluation).

Thyroid sections were subjectively evaluated for follicular epithelial height and colloid area using a five point grading scale [1=shortest; 5=tallest/largest (Capen CC and Martin SL, 1989)] and any abnormalities noted. A minimum of two sections per thyroid was evaluated, so that a representative sample of the organ could be examined.

Ovarian histology included the evaluation of follicular development (including presence/absence of tertiary/antral follicles, presence/absence of corpora lutea, changes in corpus luteum development, and changes in the number of both primary and atretic follicles) in addition to any abnormalities/lesions, such as ovarian atrophy.

Uterine histology documented cases of uterine hyper- or hypotrophy as characterized by changes in uterine horn diameter as well as myometrial, stroma, or endometrial gland development.

2.16.6. HORMONAL ASSAYS (APPENDIX M)

Hormonal assays for the measurement of T_4, TSH were undertaken using radio-immunoassay (RIA). These analyses included multiple quality control samples run in duplicate, dispersed within each assay.

Quality control material was obtained from commercial sources (with defined ranges for the appropriate hormones), or from

aliquots of frozen pooled rat sera collected commercially. Quality control values did not fall at the extremes of the standard curve, and they were used to calculate both inter- and intra-assay coefficients of variation (approximately 10% or less).

National Institute of Diabetes and Digestive and Kidney Diseases (NIDDKD) kits were used and Huntingdon Life Sciences prepared calibration curves against NIDDKD antigen standards, but using different concentrations than the kit's supplier.

2.17. STATISTICAL EVALUATIONS

All data (weaning body weights, body weight gain from PND 22 to 42, age and body weight at vaginal opening, body and organ weights at necropsy, serum hormones, and histology) were analyzed by analysis of variance (ANOVA) or analysis of covariance (ANCOVA). The compounds were analyzed in their separate sets (Set A and B).

For each set, the animals were allocated to groups using body weight ranking. This was included as a blocking factor in the analyses of variance and covariance. Owing to necessary exchanges to break litter clashes, the blocking was not perfect.

The software used for all the analyses was SAS 8.2 (SAS Institute 1999).

2.17.1. CONTINUOUS DATA

For continuous parameters, including age and body weight at vaginal opening, weaning weight, overall body weight gain, and serum hormone levels, analysis of variance or covariance was performed as follows:

A two-sided equivalent of Grubbs' test for outliers (Barnett and Lewis 1978) was first performed for each group and parameter separately. Only values significant at the 0.1% level were considered for exclusion. The criteria for the 0.1% level were established by simulation and were 2.82 (n=12), 2.92 (n=13), 3.00 (n=14) and 3.06 (n=15).

Bartlett's test (Bartlett 1937) was applied (Proc GLM, SAS Institute 1999) to determine if the groups had equal variances. If the test was significant at the 1% level, then the data were converted to normal scores using the Blom transformation (Blom 1958). For weaning body weight and body weight gains, analysis of variance (Armitage *et al* 2002) was then applied with body weight rank as a blocking factor. For all other parameters, analysis of covariance (Armitage *et al* 2002) with PND 21 weight as covariate, and body weight blocking, was applied (Proc GLM, SAS Institute 1999). If the group term in any of these analyses was significant at the 5% level, then each treatment group was compared with the control using *t* tests on the least squares means. For age at first estrus, several values were indeterminate, since no estrus was observed. These 'right-censored' values were likely to be treatment related, and were given an arbitrary high numeric value (44 days) and then Blom-transformed analysis was applied.

2.17.2. DISCRETE DATA

The histology data consisted of integer severity scores. Hence the dose groups were compared with the control data for each compound separately using Kruskal-Wallis tests (Kruskal and Wallis 1952, 1953). If these were significant at the 5% level, then each dose was compared separately with the control using exact Wilcoxon rank sum tests (Wilcoxon 1945).

Outliers:

VOP (vaginal opening) and BW

No outliers were detected.

Organ weights

Pituitary, Group 1 (1-Chloro-2-nitrobenzene, dose 25 mg/kg)
Animal 154, wt=0.0221, Grubbs statistic=3.38 (n=15)

Pituitary, Group 6 (Methoxychlor, dose 12.5 mg/kg)
Animal 665, wt=0.0192, Grubbs statistic=3.22 (n=15)

Ovaries, Group 8 (Corn oil)

Animal 859, wt=0.2548, Grubbs statistic=3.53 (n=15)

In all these cases, the analysis was performed on transformed data. This process accommodated the outliers without the need to exclude them.

Hormones

No outliers were detected.

2.18. DATA STORAGE

At the completion of the study, all reports, as well as the original study protocol, raw data, preserved specimens and retained samples produced by the Testing Facility, will be maintained in the Testing Facility's Archives for a period of 1 year after issue of the signed final study report. The Sponsor will determine the subsequent disposition of these materials. All records/data generated by the Sponsor in support of this study will be stored at the Sponsor's facility.

2.19. REGULATORY REFERENCES

2.19.1. TEST GUIDELINES

There are no specific test guidelines for this type of study at present. The design is subject to approval by the relevant Regulatory Agency.

2.19.2. GOOD LABORATORY PRACTICES

This study was conducted in accordance with the most recent versions of the US Environmental Protection Agency Good Laboratory Practice Standards (40 CFR Part 160) and the Organization for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice ENV/MC/CHEM(98)17. This study was performed according to protocol, at the Test Facility according to Huntingdon Life Sciences' Standard Operating Procedures (SOPs).

2.19.3. ANIMAL WELFARE ACT COMPLIANCE

This study complied with all appropriate parts of the Animal Welfare Act Regulations: 9 CFR Parts 1 and 2 Final Rules, Federal Register, Volume 54, No. 168, August 31, 1989, pp. 36112-36163 effective October 30, 1989 and 9 CFR Part 3 Animal Welfare Standards; Final Rule, Federal Register, Volume 56, No. 32, February 15, 1991, pp. 6426-6505 effective March 18, 1991.

2.20. PROTOCOL DEVIATIONS

The following protocol deviations occurred during the study, but were not considered to have compromised study validity or integrity.

1. Maternal Postnatal Day 17 bodyweights were not performed on 26 March 2005 (predose).

2. On-test ear-tag numbers for all pups in all groups were not recorded.

3. Daily vaginal smears for all animals in all groups were not collected at approximately the same time each day as required by the protocol. The actual collection time ranged from 0800 to 1435.

4. Statistics for incidence data were not performed as per protocol as it was not necessary (i.e., only applied to the F_0 females).

5. Quality control samples for hormone analysis were prepared from pooled commercial rat serum rather than from in-house animals with known concentrations or a commercially available kit for r-TSH.

6. Levels of calibration standards (NIDDKD/NHPP'S) used for hormone analysis (r-TSH) were different than those stated in the protocol.

7. Effective 22 March 2005, new methods (not as per protocol) were used to prepare the test substances for dosing formulation according to a document (sent by the Sponsor) that modified the manner in which the test substance formulations were prepared.

8. F_1 animal numbers 651, 751 and 851 were ear-tagged on Postnatal Day 22 rather than on Postnatal Day 21, as noted in the protocol.

9. The ovaries were weighed together with their oviducts, although the protocol implied that the ovaries would be weighed without these ducts.

10. Statistical analyses were not performed according to protocol, but rather in accordance with Sponsor's Statement of Work.

11. IRMA test was not used for the analysis of the hormones, as per protocol.

12. The 5 mg/mL concentration of Methoxychlor was mixed a second time during formulation, and the protocol indicates that the formulation was to be prepared one time only.

13. Due to an oversight, the corn oil analysis for peroxide (at the Sponsor's request) was not amended to the protocol.

14. There is no existing Certificate of Analysis for the lot of control utilized on this study, as two separate lot numbers of control article were combined to create a third lot number.

15. Chloro-2-Nitrobenzene was warmed first in a water bath at 40°C in a fume hood and not warmed first in an oven at 40°C, as specified by the protocol. However, this change was at the Sponsor's request.

16. Both Certificates of Stability for the neat test substances as well as formulations at a volume similar to and/or bracketing the formulation employed on study were not provided by the Sponsor, as per protocol.

17. A signed and dated formulation report was not provided by the Sponsor for stability and homogeneity analyses of the dosing formulations.

3. RESULTS

3.1. LITTER AND F_0 DELIVERY DATA

(Table 1, Appendix A)

Forty-four of the 45 F_0 dams that were used as sources for the experimental animals were pregnant, the average litter size being 13 pups. There were sufficient pups, therefore, to produce groups of 15 female F_1 female pups for both of Sets A and B.

3.2. DOSE FORMULATION ANALYSIS

(Appendix B)

The data indicated that the dosing formulations of 1-Chloro-2-nitrobenzene (25 and 100 mg/kg/day), DE-71 (30 and 60 mg/kg/day), and Methoxychlor (12.5 and 50 mg/kg/day) were prepared to an adequate level of accuracy, with an average deviation from the respective nominal concentrations within ± 10% for all the samples.

The peroxide content of the corn oil, measured after the conclusion of the study, met the acceptance criteria (less than 3 meq/kg). The results for the 3 samples taken ranged from 2.49 to 2.83 meq/kg.

3.3. MORTALITY

(Appendix C)

There were no test substance related deaths during the study.

3.4. CLINICAL OBSERVATIONS

(Table 2 and Appendix D)

There were no overt clinical signs of toxicity in any of the animals during the treatment period.

3.5. BODY WEIGHT

(Figures 1 and 2, Tables 3 and 4 and Appendices E and F)

The group receiving 1-Chloro-2-nitrobenzene at 100 mg/kg/day showed an -8.6% difference in mean body weight vs. the Control after the first dose. This difference increased to –12% on PND 28, and then decreased to -6% by PND 36.

Other differences (-5 to -6%) in mean body weights were observed from PND 38 to PND 42 in the group receiving Methoxychlor at 50 mg/kg/day. In addition an 8.6% deficit in body weight gain (PND 22 to PND 42) was recorded at 50 mg/kg/day Methoxychlor ($p<0.01$).

3.6. FEED CONSUMPTION

(Table 5 and Appendix G)

There were no test substance related adverse effects on feed consumption during the study.

When compared to the Control, a *ca* +10% transitory increase in mean feed consumption was observed for the groups receiving DE-71 (both dose levels) on postnatal day interval 23-29.

3.7. AGE AND BODY WEIGHT AT THE ONSET OF VAGINAL OPENING AND AGE AT FIRST ESTRUS

(Table 6 and Appendices H and J)

1-Chloro-2-nitrobenzene: The mean age (postnatal day) at the onset of vaginal opening (VO) and the age at first estrus were delayed 5.8 and 6.4 days, respectively, at 100 mg/kg/day 1-Chloro-2-nitrobenzene ($p<0.01$ for both parameters). Accordingly, an increase in mean body weight at VO (+19.7% when compared to Control) ($p<0.01$) was also observed at this dose. Treatment with 1-Chloro-2-nitrobenzene is not known to alter the onset of vaginal opening in the current literature.

DE-71: Statistically significant delays in the onset of vaginal opening (1.2 days, $p<0.05$) and in the age at first estrus (2.8 days) were observed at 30 mg/kg/day DE-71, however, no effect was observed at

60 mg/kg/day DE-71. No statistically significant changes in the weight at VO were observed for DE-71 at any of the dose levels applied. These findings were in contrast with those Stoker TE et al, 2004. In that publication a statistically significant (1.8 days) delay in the onset of vaginal opening was observed in Wistar rats treated with DE-71 at 60 mg/kg/day, while none was seen at 30 mg/kg/day. It is probable that these different results could be explained as due to strain differences.

Methoxychlor: The mean age at the onset of vaginal opening and at first estrus were advanced 6.2 and 5.1 days at 50 mg/kg/day Methoxychlor, ($p<0.01$, for both parameters) while the VO and the age at first estrus were advanced 1.3 and 1.8 days at 12.5 mg/kg/day Methoxychlor, ($p<0.05$, for VO only). Consequently, a –8.7 and -32.7% decreases in mean body weight at VO were observed for Methoxychlor at 12.5 and 50 mg/kg/day, respectively ($p<0.01$ for both dose levels). These changes at 50 mg/kg/day Methoxychlor were in agreement with the literature (Laws SC et al, 2000).

3.8. VAGINAL CYTOLOGY/ESTROUS CYCLING

(Table 7 and Appendix I)

The considerable delay in the onset of vaginal opening observed at 1-Chloro-2-nitrobenzene at 100 mg/kg/day precluded proper evaluation of the estrous cycles in that group.

Fifty-three percent of the animals receiving Methoxychlor at 50 mg/kg/day showed irregular cycles, which were characterized by extended estrus. The changes at 50 mg/kg/day Methoxychlor were in agreement with the literature (Laws SC et al, 2000).

No other test substance related effects on the estrous cycle were observed.

3.9. ORGAN WEIGHTS

(Table 9 and Appendix K)

The abbreviations used in this section are as follows: 1-Chloro-2-nitrobenzene 25 mg/kg/day (CNB-L), 1-Chloro-2-nitrobenzene-100 mg/kg/day (CNB-H); DE-71 30 mg/kg/day (DE-71 L), DE-71

60 mg/kg/day (DE-71 H); Methoxychlor 12.5 mg/kg/day (MXC-L), Methoxychlor 50 mg/kg/day (MXC-H); *Control* (*in italics*: differences between control groups, Sets A and B (absolute value)).

Liver: The administration of 1-Chloro-2-nitrobenzene or DE-71 at any of the dose levels applied was associated with increases in liver weights, as shown below:

	CNB-L	CNB-H	DE-71 L	DE-71 H	MXC-L	MXC-H	*Controls*
% difference	+23.2	+49.6	+26.6	+44.2	-9.6	-11.5	*7.1*

Liver (adjusted means): % differences vs. Control

The increases were dose-related and statistically significant ($p<0.01$), and probably reflected hepatic metabolic induction.

Ovaries: Treatment with 1-Chloro-2-nitrobenzene at 25 or 100 mg/kg/day was associated with statistically significant ($p<0.01$) decreases in ovarian weights. Statistically significant decreases ($p<0.05$) were also observed at 60 mg/kg/day DE-71 and at 50 mg/kg/day Methoxychlor (as shown below).

	CNB-L	CNB-H	DE-71 L	DE-71 H	MXC-L	MXC-H	*Controls*
% difference	-16.8	-19.4	-7.9	-10.6	-15.0	-21.5	*12.4*

Ovaries (adjusted means): % differences vs. Control

Regardless of these changes, microscopic examination of the ovaries did not show substantial differences between the groups.

Pituitary: Treatment with 1-Chloro-2-nitrobenzene at 25 and 100 mg/kg/day was associated with statistically significant decreases in the weight of the pituitary gland ($p<0.01$ at both levels).

	CNB-L	CNB-H	DE-71 L	DE-71 H	MXC-L	MXC-H	*Controls*
% difference	-10.0	-33.3	-1.1	-7.8	-1.0	-11.5	*6.7*

Pituitary (adjusted means): % differences vs. Control

Thyroid: Treatment with DE-71 at 60 mg/kg/day was associated with increased thyroid organ weight. This increase was statistically significant ($p<0.05$). Histopathology findings reported that this group had a slightly greater score for epithelial height than the rest.

	CNB-L	CNB-H	DE-71 L	DE-71 H	MXC-L	MXC-H	*Controls*
% difference	-4.6	-3.2	5.1	12.9	-5.6	1.7	*7.4*

Thyroid (adjusted means): % differences vs. Control

Uterus: There were no statistically significant differences in (intact or blotted) uterine weights

	CNB-L	CNB-H	DE-71 L	DE-71 H	MXC-L	MXC-H	*Controls*
% difference	-6.8	-22.8	-1.4	1.7	8.5	-3.8	*12.4*

Intact uterus (adjusted means): % differences vs. Control

	CNB-L	CNB-H	DE-71 L	DE-71 H	MXC-L	MXC-H	*Controls*
% difference	-10.5	-23.7	0.3	0.6	-0.3	-4.9	*4.2*

Blotted uterus (adjusted means): % differences vs. Control

No substantial differences regarding the proportion of any particular stage of the estrous cycle were observed at the time of necropsy. Microscopic examinations of the uteri, however, showed that the normal degree of endometrial hypertrophy/hyperplasia observed in the rest of the groups was less frequently observed in the groups administered 1-chloro-2-nitrobenzene. The substantial delay in the onset of vaginal opening observed at 100 mg/kg/day 1-chloro-2-nitrobenzene along with the results from estrous cycles evaluations (showing only 50% of the rats in this group passing through one estrus) could probably explain the reduced degree of endometrial hypertrophy/hyperplasia.

Kidneys: A statistically significant decrease in renal weight was observed in association with Methoxychlor treatment at both dose levels ($p<0.05$ at 12.5 mg/kg/day, $p<0.01$ at 50 mg/kg/day).

	CNB-L	CNB-H	DE-71 L	DE-71 H	MXC-L	MXC-H	*Controls*
% difference	-3.5	-0.9	-1.5	-0.1	-5.0	-8.0	*0.6*

Kidneys (adjusted means): % differences vs. Control

Adrenals: No statistically significant findings were observed in the weights of the adrenal glands.

	CNB-L	CNB-H	DE-71 L	DE-71 H	MXC-L	MXC-H	*Controls*
% difference	-14.4	-19.9	-4.3	-9.7	-5.6	-9.0	*13.5*

Adrenals (adjusted means): % differences vs. Control

3.10. GROSS AND MICROSCOPIC OBSERVATIONS

(Table 10 and Appendix L)

Ovaries: A comparison of relative numbers of the different stages of follicles and corpora lutea across the groups showed no marked increases or reductions in any of the groups. Group 5 (corn oil, Set A) had slightly fewer tertiary follicles than the other groups. Groups 1 and 2 (1-chloro-2-nitrobenzene 25 mg/kg/day and 100 mg/kg/day respectively) appeared to have fewer recent (basophilic) corpora lutea but the overall number of corpora lutea were not obviously decreased.

Uterus: One rat in each of Group 1 and 2 (1-chloro-2-nitrobenzene at 25 mg/kg/day and 100 mg/kg/day, respectively) showed atrophic endometrium/myometrium, but there was no other evidence of atrophic change. A number of rats in all groups showed endometrial hypertrophy/hyperplasia, characterized by infolding and thickening of the endometrial mucosa. This is a normal appearance of the uterus during the estrous cycle. This was seen least frequently in the groups administered 1-chloro-2-nitrobenzene at 25 mg/kg/day and 100 mg/kg/day ($p<0.05$ and $p<0.01$, respectively).

Thyroid: All thyroids contained areas of small follicles with cuboidal to columnar epithelium as well as areas with larger follicles lined by squamous to cuboidal epithelium. The assigned grade for epithelial follicular height was based on the relative proportion of the two types of follicles within the thyroid. Comparison of the mean group severity scores showed that Group 4 (DE-71 60 mg/kg/day) had a slightly greater score for epithelial height than the other groups (although not statistically significant), and that the score for the colloid area was statistically significantly lower, ($p<0.05$). This finding is in agreement with Stoker TE et al., 2004.

All other findings in the tissues examined occurred at a similar incidence and/or severity across the groups and were considered to be within normal limits for this age of rat.

3.11. HORMONE DATA

(Table 11 and Appendix M)

The abbreviations used in this section are as follows: 1-Chloro-2-nitrobenzene 25 mg/kg/day (CNB-L), 1-Chloro-2-nitrobenzene-100 mg/kg/day (CNB-H); DE-71 30 mg/kg/day (DE-71 L), DE-71 60 mg/kg/day (DE-71 H); Methoxychlor 12.5 mg/kg/day (MXC-L), Methoxychlor 50 mg/kg/day (MXC-H); *Controls* (*in italics*: differences between control groups, Sets A and B).

T4	CNB-L	CNB-H	DE-71 L	DE-71 H	MXC-L	MXC-H	*Controls*
% difference	-22.3	-28.2	-71.5	-87.3	-7.6	-5.9	*-12.9*
TSH	CNB-L	CNB-H	DE-71 L	DE-71 H	MXC-L	MXC-H	*Controls*
% difference	7.7	28.8	40.0	53.5	23.0	16.8	*-18.1*

Hormone values: % differences vs. Control

DE-71: The mean serum levels of Tetraiodothyronine (T_4) were significantly reduced in the groups receiving 30 and 60 mg/kg/day DE-7 (–71.5 and -87.3%, when compared to Control, respectively)($p<0.01$). Consistent with these findings, increases in Thyroid Stimulating Hormone (TSH) levels (+40 and +53.5%) were observed at 30 and 60 mg/kg/day DE-71, respectively ($p<0.01$). Similar findings in T_4 and TSH levels have been reported after the administration of DE-71 at 30 and 60 mg/kg/day to female Wistar rats (Stoker TE et al, 2004).

1-Chloro-2-nitrobenzene: Marginal decreases in T_4 levels were observed at 25 and 100 mg/kg/day 1-Chloro-2-nitrobenzene (-22.3 and –28.2% when compared to control values, respectively ($p<0.01$)). The decrease in T_4 observed at the High dose was consistent with a marginal increase in serum TSH level (28.8%, $p<0.05$).

Methoxychlor treatment at any of the dose levels applied did not alter T_4 or TSH serum levels.

4. CONCLUSIONS

The administration of the test substances at any of the dose levels applied was well tolerated by the F_1 females.

The administration of 1 Chloro-2-nitrobenzene at 25 or 100 mg/kg/day was associated with statistically significant increases in absolute liver weight, decreased weights of the ovaries and pituitary gland, and decreased T_4 serum

levels. Treatment with 1 Chloro-2-nitrobenzene at 100 mg/kg/day was also associated with increased TSH levels, increased body weight at the onset of vaginal opening (VO), a 5.8-days delay in VO and a 6.4 days delay in the age at first estrus.

Treatment with DE-71 at 30 or 60 mg/kg/day was associated with statistically significant increases in absolute liver weight, decreased T_4 and increased TSH serum levels. A statistically significant decrease in the weight of the ovaries was observed at 60 mg/kg/day DE-71. There were no treatment-related effects on the onset of vaginal opening, or on estrous cycles evaluations.

The administration of Methoxychlor at 12.5 or 50 mg/kg/day was associated with statistically significant decreases in absolute kidney weights, as well as with advanced age at the onset of vaginal opening (1.2-days and 6.2-days at 12.5 and 50 mg/kg/day, respectively). Methoxychlor at 50 mg/kg/day was associated with decreased ovarian weight and irregular estrus cycles characterized by extended estrus.

CALCULATIONS

General Notes:

Individual animal data values presented in this report may be rounded. Unrounded individual animal data values are used to calculate the reported mean and standard deviation values. Therefore, use of the reported individual values to reproduce means, standard deviations and/or to perform any subsequent calculations may produce minor discrepancies between the calculated values and those presented in this report.

Feed Consumption:

total grams of feed presented - amount of feed remaining ÷ no. days/#animals per cage = grams/animal/day

Female Mating Index:

no. of females with confirmed mating (sperm and/or vaginal plug) plus no. of pregnant females without evidence of mating (no sperm or vaginal plug)/no. of females placed with males

Female Fertility Index:

no. of females pregnant/no. of females confirmed mating or pregnancy for females without evidence of mating

Gestation Index:

no. of females with liveborn/no. of females with confirmed pregnancy

Viability Index:

no. of pups alive Day 4 precull/no. of liveborn pups

Live Birth Index:

total no. of liveborn pups/total no. of pups born

REFERENCES

ARMITAGE, P., BERRY, G. and MATTHEWS, J.N.S. (2002) *Statistical Methods in Medical Research, 4th edition.* Blackwell Science, Oxford, UK.

BARNETT, V. and LEWIS, T. (1978) *Outliers in Statistical Data, 1st edition.* Wiley, New York.

BARTLETT, M.S. (1937) Properties of sufficiency and statistical tests. *Proceedings of the Royal Society, Series A.* 160: 268-282.

BLOM, G. (1958) *Statistical Estimates and Transformed Beta Variables.* John Wiley and Sons, New York.

CAPEN, C.C. and MARTIN, S.L. (1989) The effects of xenobiotics on the structure and function of thyroid follicular and C-cells. *Toxicol. Pathol.* 17(2): 266-93.

DUNLAP, W.P. and DUFFY, J.A. (1975). Fortran IV Functions for Calculating Exact Probabilities Associated with Z, Chi-Square, T and F Values. *Behav. Res. Methods and Instrumentations* 7: 59-60.

DUNLAP, W.P., MARX, M.S. and AGAMY, G.G. (1981). Fortran IV functions for calculating probabilities associated with Dunnett's test. *Behav. Res. Methods and Instrumentation* 13: 363-366.

KRUSKAL, W.H. and WALLIS, W.A. (1952) Use of Ranks in One-Criterion Variance Analysis. *Journal of the American Statistical Association* 47: 583-621.

KRUSKAL, W.H. and WALLIS, W.A. (1953) Errata for Kruskal-Wallis (1952). *Journal of the American Statistical Association* 48: 907-911.

LAWS, S.C., CAREY, S.A., FERRELL, J.M., et al. (2000) Estrogenic activity of Octylphenol, Nonylphenol, Bisphenol A and Methoxychlor in rats. *Toxicol Sci* 54: 154-167.

SAS INSTITUTE (1999) *SAS OnlineDoc® Version Eight.* SAS Institute Inc., Cary, NC.

SEIGEL, S. (1956) *Nonparametric Statistics for the Behavioral Sciences.* McGraw-Hill, New York.

REFERENCES

STOKER, T.E., LAWS, S.C., CROFTON, K.M., et al. (2004) Assessment of DE-71, a Commercial Polybrominated Diphenyl Ether (PBDE) Mixture, in the EDSP Male and Female Pubertal Protocols. *Toxicol Sci* 78: 144-155.

WILCOXON, F. (1945) Individual comparisons by ranking methods. *Biometrics Bulletin* 1: 80-83.