STUDY NO. 04-4276
SPONSOR STUDY NO. WA 4-15, Task 4

ASSESSMENT OF PUBERTAL DEVELOPMENT AND THYROID FUNCTION IN JUVENILE MALE RATS

Final Report

Submitted to: Batelle
505 King Avenue
Columbus, Ohio 43201

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

Date: 22 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 Dibutyl Phthalate, 1-Chloro-2-nitrobenzene, and Vinclozolin at a dose volume that covered the lowest dosing formulation concentration. Stability and homogeneity results were provided for the high-dose concentration and volume of Vinclozolin used on study.

2. The protocol did not identify the name and contact information of the Priciple 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).

Elizabeth Horsley 22 Dec 05

Elizabeth T. M. Horsley, 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 P).

Elizabeth Horsley — 22 Dec 05

Elizabeth T. M. Horsley, Ph.D. — Date
Study Director

SCIENTIFIC REVIEW

The following Scientist has reviewed and approved this report.

K. P. Hazelden — 22 Dec 05

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

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 #2)	12-14 Jan 05	14 Jan 05
GLP Protocol Review (Draft #4)	20 Jan 05	20 Jan 05
Litter Checks And Training Records	17 Feb 05	1 Mar 05
Dose Preparation and Equipment Records	3 Mar 05	4 Mar 05
Dose Administration	7 Mar 05	8 Mar 05
Preputial Separation Evaluations	17 Mar 05	17 Mar 05
Terminal Blood Collection, Necropsy and Training Records	5 Apr 05	5 Apr 05
Draft Report and Data	17-20 & 31 May 05	31 May 05
Excel Transfer Files	17 & 20 Jun 05	21 Jun 05
Hormone Analysis Procedure, Appendix and Data	13, 19 & 20 Jul 05	20 Jul 05
Dose Confirmation Report and Data	8-11 Aug 05	12 Aug 05
Group 10 TSH Repeat Analysis Data	12, 15 Aug 05	15 Aug 05
Final Report & Study Data	27-31 Aug 05	31 Aug 05
Executive Summary Table & Report	12 & 13 Dec 05	13 Dec 05
Hormone Analysis Sub-report	13 & 15 Dec 05	15 Dec 05

Fran Jannone

Fran Jannone, B.A., RQAP-GLP
Quality Assurance Group Leader

22 Dec 05

Date

SUMMARY

This study was intended to detect the endocrine disruptive potential of xenobiotics in pubertal male 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 at least 27 time-mated Sprague-Dawley females were culled on Postnatal Day (PND) 4 to a maximum litter size of 10 pups. F_1 females were not utilized.

Fourteen F_1 males per dose group (Set A) were weaned at PND 21 and dosed orally once daily, from PND 23 to 52/53 (inclusive) with 30 or 100 mg/kg/day of Vinclozolin, and 30 or 60 mg/kg/day of DE-71. Fifteen F_1 males per dose group (Set B) were also weaned at PND 21 and dosed orally once daily, from PND 23 to 52/53 (inclusive) with 25 or 100 mg/kg/day of 1-Chloro-2-Nitrobenzene, and 500 or 1000 mg/kg/day of Dibutyl Phthalate. Each Set (14 males/Set A and 15 males/Set B) received a 0 mg/kg/day corn oil control. Designation of formulation preparations with letters were implemented in order to ensure that the study was carried out 'blind'.

The following parameters were evaluated: clinical signs of toxicity, body weights, food consumption and pubertal development. Necropsies were performed 2 hours post dose on PND 52/53. Blood was collected for hormone analyses (thyroid function and testosterone levels), and selected tissues were collected, weighed and processed for macroscopic and microscopic pathology.

There were no clinical signs of toxicity or effects on feed consumption during the study. There was a slight decrease in body weight gain from PND 23-52/53 associated with DE-71 and Vinclozolin.

Endocrine disruptive effects of Dibutyl Phthalate included a decrease in size of the testis and epididymis, which correlated with a substantial decrease in testicular weight for males treated with 500 and 1000 mg/kg of Dibutyl Phthalate, respectively. The decrease in testicular size was in agreement with a previous study (Rocca and Pepperl, 2000). The epididymides, seminal vesicles and levator ani plus bulbocavernosus complex weights were decreased in males receiving both 500 mg/kg and 1000 mg/kg of Dibutyl Phthalate. Severe seminiferous tubular atrophy was found at 1000 mg/kg of Dibutyl Phthalate, and in most cases all seminiferous tubules were totally depleted of germ cells. At 500 mg/kg of Dibutyl Phthalate there was loss of germ cells ranging in severity from minimal to severe. T_4 and TSH levels were significantly reduced in males receiving 500 mg/kg and 1000 mg/kg. Testosterone levels were significantly reduced in males receiving 1000 mg/kg of Dibutyl Phthalate. A slight delay in the onset of preputial separation occurred in males receiving 1000 mg/kg of Dibutyl Phthalate. Liver weight was slightly elevated at 500 and 1000 mg/kg of Dibutyl Phthalate.

An increase in liver weight was observed for 1-Chloro-2-Nitrobenzene treated animals, which was probably attributable to microsomal hepatic enzyme induction associated with metabolism. An increase in kidney weight was found in males receiving both 25 and 100 mg/kg of 1-Chloro-2-Nitrobenzene. Seminal vesicle, prostate (ventral) and levator ani plus bulbocavernosus complex weights were decreased in males receiving 100 mg/kg of 1-Chloro-2-Nitrobenzene. Several histopathological findings were associated with this compound, such as slight spermatid retention and an increase in tubular vacuolation, but these findings were considered minimal and not associated with endocrine disruption. A slight delay in the onset of preputial separation occurred in males dosed with 100 mg/kg of 1-Chloro-2-Nitrobenzene.

An increase in liver weight was found in males receiving DE-71, which may be attributable to direct enzyme induction of one or all of the individual components contained in this test substance. Levator ani plus bulbocavernosus complex weights were decreased in males receiving 30 and 60 mg/kg of DE-71. An increase in testicular weight was found in males receiving 30 mg/kg DE-71 which was also found at 60 mg/kg but to a lesser degree. T_4 levels were substantially reduced in males treated with DE-71, which was accompanied by an increase in TSH levels. This decrease in T_4 and increase in TSH was an expected finding, based on a previous endocrine disruptor screening assay (Stoker et al., 2004). Testosterone levels were also decreased with this test substance, however the biological relevance is uncertain as Testosterone levels were highly variable. Histopathological findings included an increase in the height of follicular cells.

Vinclozolin delayed preputial separation in a dose related manner, which was in agreement with a previous NTP study (NTP study RACB 20103). A dose dependent decrease occurred in weights of the epididymides and levator ani plus bulbocavernosus complex. A decrease in seminal vesicle weight occurred in males dosed with 100 mg/kg Vinclozolin. A slight increase in testicular weight was found in males dosed with 30 and 100 mg/kg Vinclozolin. An increase in Testosterone was found at 100 mg/kg Vinclozolin. A dose dependent decrease in T_4 was also found with this test substance.

Executive Summary-

Report Title: Assessment of Pubertal development and Thyroid Function in Juvenile Male Rats

Test substances: DE-71, 1-Chloro-2-Nitrobenzene Vinclozolin, Dibutyl Phthalate

Duration of Dosing: Postnatal days (PND) 23-53
Species/Strain: Rat, Sprague-Dawley
Initial Age: PND 23 (1st dose)
Date of First Dose: 6 March 2005
Day of Necropsy: PND 52-53

Study No. 04-4276

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

GLP Compliance: Yes

Set A

Daily Dose (mg/kg/day)	**0 (Corn oil)**	**Vinclozolin 30 mg/kg**	**Vinclozolin 100 mg/kg**	**DE-71 30 mg/kg**	**DE-71 60 mg/kg**
Males:					
No. Evaluated	14	14	14	14	14
No. Died or Sacrificed Moribund	1	0	0	0	0
Initial Body Weight (PND 23), g, (%)[a]	63.1	-1%	-4%	-1%	0%
Clinical Observations:	-	-	-	-	-
Age at preputial separation[b]	43.9	+3.3**	+6.4**	+0.2	+0.8
Body weight at preputial separation g, (%)[a]	237.6	+6%*	+15%**	-5%	0
Final Body Weight (PND 52-23) g, (%)[a]	313.3	-7%	-9%	-8%	-6%
Body Weight gain (PND 52-23) g, (%)[a]	250.5	-9%**	-11%**	-9%**	-7%**
Liver weight g, (%)[a]	15.294	-5%	-2%	+11%**	+35%**
Kidney weight, g, (%)[a]	2.584	-10%	-10%	-7%*	-5%
Pituitary weight g, (%)[a]	0.0094	-3%	+2%	+33%	-2%
Thyroid g, (%)[a]	0.0270	+1%	-6%	-1%	-12%
Testis weight, (Left) g, (%)[a]	1.415	+12%*	+6%*	+9%**	+4%*
Testis weight, (Right) g, (%)[a]	1.403	+7%*	+7%*	+14%**	+5%**
Epididymides weight, (%)[a]	0.545	-13%**	-16%**	-1%	-4%
Levator ani plus bulbocavernosus g, (%)[a]	0.746	-20%**	-25%**	-14%**	-18%**
Seminal vesicles weight, g, (%)[a]	0.733	-12%	-37%**	-5%	-3%
Dorsolateral prostate weight g, (%)[a]	0.128	-26%	-18%	-5%	-16%

Daily Dose (mg/kg/day)	0 (Corn oil)	Vinclozolin 30 mg/kg	Vinclozolin 100 mg/kg	DE-71 30 mg/kg	DE-71 60 mg/kg
Ventral prostate weight g, (%)[a]	0.286	-16%	-19%	-3%	-7%
Adrenals weight g, (%)[a]	0.047	+3%	+3%	-2%	+4%
Hormones: Tetra-iodothyronine (T_4)[a]	5.93	-31%**	-51%**	-78%**	-86%**
Thyroid stimulating hormone (TSH)[a]	4.47	+40%	+17%	+39%*	+100%**
Testosterone[a]	221.72	+44%	+67%**	-49%*	-47%
Necropsy/Histopathology: (scores)					
Thyroid epithelial height	3.2	3.3	3.1	3.6	4.2**
Thryoid colloid area	3.7	3.7	3.9	3.6	2.5**

- = No noteworthy findings

a- Control group mean shown, percent differences for treated groups.
b- Control group mean shown, differences treated groups vs control expressed in days (Treated mean – Control mean).

* $p<0.05$
** $p<0.01$

Executive Summary- **Report Title: Assessment of Pubertal development and Thyroid Function in Juvenile Male Rats** **Test Substances: DE-71, 1-Chloro-2-Nitrobenzene Vinclozolin, Dibutyl Phthalate**

Duration of Dosing: Postnatal days (PND) 23-53
Species/Strain: Rat, Sprague-Dawley
Initial Age: PND 23 (1st dose)
Date of First Dose: 8 March 2005
Day of Necropsy: PND 52-53

Study No. 04-4276

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

GLP Compliance: Yes

Set B

Daily Dose (mg/kg/day)	**0 (Corn oil)**	**1-Chloro-2-Nitrobenzene 25 mg/kg/day**	**1-Chloro-2-Nitrobenzene 100 mg/kg/day**	**Dibutyl Phthalate 500 mg/kg/day**	**Dibutyl Phthalate 1000 mg/kg/day**
Males:					
No. Evaluated	15	15	15	15	15
No. Died or Sacrificed Moribund	0	0	0	0	0
Initial Body Weight (PND 23), g (%)[a]	61.6	+0.6%	+1.0%	+0.8%	+0.6%
Clinical Observations:	-	-	-	-	-
Age at preputial separation[b]	43.3	+0.9	+2.8**	+1.6	+2.7**
Body weight at preputial separation g, (%)[a]	211.2	+7%	+8%	+5%	+8%
Final Body Weight, (PND 52, all animals), g, (%)[a]	278.3	+5%	+4%	+2%	-1%
Body Weight gain (PND 52-23), g, (%)[a]	216.6	+6%*	-1%	+2%	-1%**
Liver weight g, (%)[a]	12.909	+41%**	+70%**	+10%*	+19%**
Kidney weight, g, (%)[a]	2.236	+10%**	+10%**	+6%*	+5%
Pituitary weight g, (%)[a]	0.0090	-10%	-10%	+2%	+10%
Thyroid g, (%)[a]	0.0231	+13%	-3%	+6%	+2%
Testis weight, (Left) g, (%)[a]	1.437	+7%	-6%	-46%**	-74%**
Testis weight, (Right) g, (%)[a]	1.446	+1%	-6%	-45%**	-74%**
Epididymides weight, (%)[a]	0.516	0%	-5%	-10%*	-22%**
Levator ani plus bulbocavernosus g, (%)[a]	0.649	0%	-18%**	-20%**	-24%**
Seminal vesicles weight, g, (%)[a]	0.664	-3%	-17%*	-15%*	-27%**
Dorsolateral prostate weight g, (%)[a]	0.098	+30%	-2%	+1%	-1%

Daily Dose (mg/kg/day)		**0 (Corn oil)**	**1-Chloro-2-Nitrobenzene 25 mg/kg/day**	**1-Chloro-2-Nitrobenzene 100 mg/kg/day**	**Dibutyl Phthalate 500 mg/kg/day**	**Dibutyl Phthalate 1000 mg/kg/day**
Ventral prostate weight g, (%)[a]		0.291	-6%	-29%**	-17%	-17%
Adrenals weight g, (%)[a]		0.0464	+2%	-8%	-6%	-7%
Hormones: Tetra-iodothyronine (T_4)[a]		5.31	+14%*	-7%	-24%**	-25%**
Thyroid stimulating hormone (TSH) [a]		9.16	-23%	-27%*	-42%**	-54%**
Testosterone [a]		157.71	+60%	+14%	-28%	-56%**
Necropsy/Histopathology: (scores)						
Testes:	Partially/totally atrophic tubules	0	0	0	3.6**	4.9**
	Elongate spermatid degeneration/depletion	0	0.1	1.9**	1.7**	0.3
	Germ cell exfoliation	0	0.1	0.9**	0	0
	Spermatid retention	0	0	1.1**	0.3	0
	Tubular vacuoles	0	0.1	0.8**	0.2	0
Epididymides:	Caput sperm content	2.9	2.9	1.7**	0.6**	0**
	Caudal sperm content	1.1	1.4	0.7	0.1**	0**
	Caput: increased lumenal sloughed germ cells/debris	0	0.2	1.8**	1.1**	0.3
	Cauda: increased lumenal sloughed germ cells/debris	0	0	0	1.7**	2.6**
	Caudal ductal contraction	1.5	1.8	1.7	2.6**	3.8**

- = No noteworthy findings

a- Control group mean shown, percent differences for treated groups.
b- Control group mean shown, differences treated groups vs control expressed in days (Treated mean – Control mean).

* $p<0.05$
** $p<0.01$

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APPENDICES

1. INTRODUCTION

This study was intended to detect the endocrine disruptive potential of xenobiotics in pubertal male 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 male 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

Elizabeth T. M. Horsley, Ph.D.

2.2. STUDY DATES

2.2.1. STUDY INITIATION

02 February 2005 (Date Study Director Signed The Protocol)

2.2.2. GESTATION DAY 0

21 January 2005 (Set A)
23 January 2005 (Set B)

2.2.3. DATE OF ANIMAL RECEIPT (OECD EXPERIMENTAL START DATE/ FIRST DATE OF DATA COLLECTION)

31 January 2005

2.2.4. POSTNATAL DAY (PND) 0

11-12 February 2005 (Set A)
13-14 February 2005 (Set B)

2.2.5. DOSING INITIATION (EPA EXPERIMENTAL START DATE) - PND 23

06-07 March 2005 (Set A)
08-09 March 2005 (Set B)

2.2.6. DOSING TERMINATION - PND 52/53

05-06 April 2005 (Set A)
07-08 April 2005 (Set B)

2.2.7. TERMINAL SACRIFICES – PND 52/53

05-06 April 2005 (Set A)
07-08 April 2005 (Set B)

2.2.8. EXPERIMENTAL COMPLETION DATE

29 April 2005 (Date of last data collection)

2.2.9. STUDY COMPLETION DATE

22 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 Males (Litters)
Group	**Designation[a]**					
1	Test Substance E (Corn Oil)	-	0	X		14 (3)
2	Test Substance K (Vinclozolin)	100	40	X		14 (3)
3	Test Substance J (Vinclozolin)	30	12	X		14 (3)
4	Test Substance H (DE-71)	30	12	X		14 (3)
5	Test Substance G (DE-71)	60	24	X		14 (3)
6	Test Substance C (1-Chloro-2-nitrobenzene)	25	10		X	15 (3)
7	Test Substance B (1-Chloro-2-nitrobenzene)	100	40		X	15 (3)
8	Test Substance D (Dibutyl Phthalate)	500	200		X	15 (3)
9	Test Substance A (Dibutyl Phthalate)	1000	400		X	15 (3)
10	Test Substance F (Corn Oil)	-	0		X	15 (3)

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

The litters from 32 time-mated females were culled on Postnatal Day (PND) 4 to a maximum litter size of 10 pups, preferentially removing females. The F_1 males were utilized for the assessment of pubertal development and thyroid function. F_1 females were not utilized. F_1 males were weaned at PND 21 and dosed orally once daily, from PND 23 to 53/54. Necropsies were performed 2 hours post dose on PND 53/54. Blood was collected for hormone analyses, 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

This design had been shown in pre-validation studies to be effective in detecting effects of test substances on male 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 male endocrine system, including: gonadal function, potential pubertal delay, target organ effects and thyroid function.

2.4.2. DOSE LEVEL SELECTION

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

Detailed justification for the dose levels selected, according to each of the test substances, are 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 (the 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 there was 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 tetra-iodothyronine (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 at 30 and 60 mg/kg/day, respectively. Increased weight of anterior pituitary at 30 mg/kg/day only, and increased weight of ventral prostate and seminal vesicles at 30 and 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.

Vinclozolin - A study undertaken with Sprague-Dawley male rats. Treatment period PND 23-54. Dose levels: 0, 10, 30 and 100 mg/kg/day in corn oil. No mortality or test article-related effects on body weight were observed at any of the dose levels applied.

Endocrine disruptor effects: dose-related delayed PPS onset (by 1.5, 2.8 and 6.6 days for 10, 30 and 100 mg/kg/day, respectively).

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

Dibutyl Phthalate (DBP) - A pubertal study was undertaken with Sprague-Dawley (SD) and Long-Evans (LE) rats using a single dose level of 1000 mg/kg/day, run as two separate blocks.

In Block 1 there were no unscheduled deaths and no treatment-related clinical signs during the study. PND 53 mean body weights were lower than control males for both SD and LE rats. In Block 2 there was one death, which was an accidental death, and there were no adverse clinical observations noted. PND 53 mean body weights were lower than controls for LE rats only.

Endocrine disruptor effects: In Block 1 there was a small decrease in size of the testes (33 % of males) and seminal vesicles (17 % of males) treated with Dibutyl Phthalate. Liver weights increased in both SD (absolute) and LE (relative to body weight); kidney weights increased in LE but not SD males. Ventral prostate and epididymides weights decreased in LE rats only. Levator ani plus bulbocavernosus complex weight decreased in LE males. Dibutyl Phthalate treatment resulted in a small, but significant decrease in both T_4 and TSH levels in LE rats. In Block 2 testicular size was decreased (55 % of males). Levator ani plus bulbocavernosus complex, seminal vesicle and prostate weights were decreased in LE rats. Mean age to complete preputial separation was significantly higher than in controls for LE rats, but not SD rats treated with DBP. Decreased serum T_4 levels in LE rats treated with DBP.

Source: Rocca MS, Pepperl S. (2000) Assessment of pubertal development and thyroid function in juvenile male rats. TherImmune Research Corporation, Study Numbers 1143-100 and 1143-102. Performed for the US Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Research Triangle Park, NC.

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 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 (32 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 (36) was considered appropriate to provide a sufficient number of adequate litters for constructing each F_1 group (allowing for the exclusion of litters that were too small, or that delivered after Gestation Day 23). Fifteen F_1 males were needed to form each treatment group (considered a minimum number). 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/Batch 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
Dibutyl Phthalate (84-74-2)	00323PU	99.9%	Colorless liquid	16 Dec 04	11 Oct 10	Room temperature
Vinclozolin (50471-44-8)	329-72-B	99.5%	White powder	15 Dec 04	Oct 07	Room temperature

2.5.1. TEST SUBSTANCE CATEGORY

'Positive' and '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 reproduced in this report (Appendix M).

2.5.4. STABILITY

Stability analyses (dose formulation stability) pertaining to each of the test substances were provided by the Sponsor and are included in Appendix M.

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 animal dosing. Following the end of the dosing period, all remaining test substances (not used for additional studies) 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 (Combination of Lot A0-001 and A0-002: Lot # 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 test article, and the maintenance of these records, was the responsibility of the Sponsor. A Certificate of Analysis was not available.

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 animal dosing. Any remaining test articles were returned to the Sponsor on 09 August 2005.

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 and Received	36 time-mated females
Placed on Study:	litters of 32 females
	145 male pups (14/Set A and 15/Set B)

2.7.4. AGE AND WEIGHT

The approximate weight range at mating of the time-mated females was expected to be 210-270 grams. The time-mated F_0 females were 12-14 weeks of age at receipt on Gestation Day (GD) 8-10, where GD 0 was the day when vaginal plugs were detected. Natural delivery was allowed. Any litters that did not deliver by Gestation Day 23 were excluded.

The experimental animals were the F_1 generation males (14 pups/Set A and 15 pups/Set B). These pups were 42-62 grams on Postnatal Day (PND) 21. PND 0 was defined as the day a pup was 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:

On PND 21, all the pups were marked with their litter identification number, then all the pups from all the litters were individually weighed to the nearest 0.1 gram, and their body weights were ranked. Pups were randomly allocated to the experimental groups, starting with those with body weights closest to the overall mean body weight, so as to generate groups with mean body weights that were similar, both in mean value and in variation. Some Set A animals that were litter-mates were found to be in the same treatment groups. However, no litter-mates were in the same treatment group for Set B animals.

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 identified using only ear tags. This was a unique form of identification which could be used to trace the maternal dam. Tail tattoo was not performed.

Appropriate records of identification numbers for each F_0 and corresponding F_1 progeny 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 (animal room staff) did not follow standard coding patterns, as the study was 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. Miscellaneous, non-test article-related veterinarian evaluations for individual animals were reviewed by the study director and are documented in the study file.

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), with up to 3 animals/cage[1] until the end of the study.

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) and deemed acceptable by the Sponsor. The feed's batch content of Daidzein and Genistein were provided by the diet's manufacturer.

2.11.4. FEED ANALYSIS

Analytical certification of batches of feed as used on study are maintained on file by the manufacturer and at the Testing Facility and can be found in Appendix M. 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 Nos. 4224304, 4224806 and 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 for a representative batch of bedding as used on study provided by the supplier, is maintained on file at the Testing Facility and can be found in Appendix M. There are 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 Subcontractor, 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 actually delivered to the animals was not analyzed.

2.11.8. ENVIRONMENTAL CONDITIONS

Light/Dark Cycle

A fourteen hour light/dark cycle controlled by an automatic timer was provided. A standard light cycle was lights on at 6:00 a.m. and lights out at 6:00 p.m.

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 23°C

Humidity

Humidity was monitored in accordance with HLS SOPs and maintained within the specified range to the maximum extent possible. Excursions outside the specified range were not considered to have affected the integrity of the study.

Desired Range:	40 to 60%
Actual Range (including all transients):	1 to 68%
Daily Average Range:	42 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 on one occasion only, at the beginning of the study. Each formulation was distributed into four 250-mL amber glass bottles and the bottles were tightly 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 at least 1 hour. 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.

DIBUTYL PHTHALATE

The Dibutyl Phthalate low dose formulation was prepared by weighing 200 grams of this test substance into a 1 L volumetric flask and adding corn oil to the mark at a concentration of 200 mg/mL and stirred for 88 minutes. The mixture was agitated to dissolve the test substance.

The Dibutyl Phthalate high dose formulation was prepared by weighing 400 grams of this test substance into a 1 L volumetric flask and adding corn oil to the mark at a concentration of 400 mg/mL and stirred for 81 minutes. The mixture was agitated to dissolve the test substance.

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 were 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 68 minutes.

The 1-Chloro-2-Nitrobenzene high dose formulation was prepared by warming the test substance in a water bath in a fume hood set at 40°, until it was in a liquid form. Then 40 grams of the test substance were 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 64 minutes.

VINCLOZOLIN FORMULATION PREPARATION

The Vinclozolin low dose formulation was accurately weighed (12.0 grams) into a 1 L (calibrated) volumetric flask and corn oil was added to the mark at a concentration of 12 mg/mL. The solution was sonicated and mixed to dissolve the test substance (for approximately 3 hours). Caution was used to ensure that sonication did not overly heat the formulation. The flask was only warm to the touch.

The Vinclozolin high dose formulation was prepared by weighing 40.0 grams of this test substance into a volumetric flask and corn oil was added to the mark at a concentration of 40 mg/mL. The solution was sonicated and stirred intermittently for approximately 21 hours to dissolve the test substance. Caution was used to ensure that sonication did not overly heat the formulation and the flask was only warm to the touch.

DE-71 FORMULATION PREPARATION

DE-71 was stored at room temperature prior to use.

The 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) until it liquefied, then the bottle was sealed and agitated for approximately 2 minutes. Twelve grams of the DE-71 was weighed into a container and a portion of the corn oil vehicle was added to the container. The DE-71 was stirred 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. Then the final contents were stirred vigorously with an overhead stirrer for approximately 41 minutes.

The DE-71 high dose was prepared by first warming the DE-71 to approximately 40 °C in a water bath, then weighing 24 grams of the DE-71 into a container and adding a portion of the corn oil vehicle to the container. DE-71 was stirred 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. Then the final contents were stirred vigorously with an overhead stirrer for approximately 41 minutes.

2.13. ANALYSIS OF DOSING FORMULATIONS

Analyses to determine concentration of certain test substances under the conditions of this study were performed by the Testing Facility (see Appendix A). A pre-start chemistry document for the preparation and analyses of the test substances performed by the Sponsor is also provided in Appendix A. For any test substance not requiring stability or homogeneity analysis, the Sponsor provided supporting documentation (see Appendix M).

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 documentation for dose formulations 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 for Groups 1-3 [Corn Oil, Vinclozolin (100 mg/kg/day) and Vinclozolin (30 mg/kg/day)] and Groups 6-10 [1-Chloro-2-nitrobenzene (25 and 100 mg/kg/day), Dibutyl Pthalate (500 and 1000 mg/kg/day), and Corn Oil, respectively] were prepared on 03-March 2005 and the samples analyzed on 04 March 2005. Repeat sampling was performed on 07 March for Groups 4, 5 7, 8 and 9 [DE-71 (30 and 60 mg/kg/day), 1-Chloro-2-nitrobenzene (100 mg/kg/day), and Dibutyl Phthalate (500 and 1000 mg/kg/day), respectively]. Group 5 [DE-71 (60 mg/kg/day)] was re-sampled on 10 March 2005.

Samples were collected while constantly stirring, following a stirring period of not less than 15 minutes. In each case, set A was refrigerated at 2-8° C and analyzed within 24 hours from collection. Set B was retained frozen at approximately -70°C for possible analysis, but was discarded upon report finalization.

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 23 to 52/53 inclusive by oral gavage utilizing a suitably sized 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 most recent body weight available (the weight taken before dosing 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.

2.15.2. BODY WEIGHTS (F_0)

Maternal body weights were recorded on PND 17. These weights were not formally reported, and were collected for welfare monitoring purposes only.

2.15.3. CULLING

On PND 4, the litters were culled to a maximum of 10 pups with the exception of one litter. Female pups were preferentially removed so as to maximize the number of males that were available in each litter for selection at weaning.

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. Offspring in apparently poor health were identified for further monitoring and possible euthanasia.

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. BODYWEIGHTS (F_1)

Individual body weights of the F_1 males were recorded weekly prior to weaning and then daily from Postnatal Day 21. The animals were also weighed on the day of necropsy (PND 52 or

53). For those necropsied on PND 53, body weight gain was reported to both PND 52 and 53.

2.15.6. FEED CONSUMPTION (F_1)

Feed consumption was measured gravimetrically, once weekly for each F_1 animal cage from PND 23/24 onwards and more often than once weekly from Postnatal Day 23 onward for Groups 1-5 and from Postnatal Days 23/24 onward for Groups 6-10. Feed consumption measurements represented the feed consumed by at least two animals (and up to 3 or 4) F_1 animals caged together.

2.15.7. PREPUTIAL SEPARATION

Beginning on Postnatal Day 30, males were examined daily for preputial separation. The appearance of partial and complete preputial separation, or a persistent thread of tissue between the glans and prepuce, was recorded when each condition was observed. In addition, the body weight at complete preputial separation was recorded.

2.16. SCHEDULED TERMINAL EXAMINATIONS

2.16.1. METHOD OF EUTHANASIA

F_0 Dams and Excess Pups:

Females that failed to deliver a litter by GD 23 were euthanized by exposure to carbon dioxide on that day, or on GD 24.

F_0 females that weaned litters were euthanized by exposure to carbon dioxide on PND 21-23, or as soon as convenient thereafter.

Pups delivered on GD 23 had clinical observations and bodyweights taken up to Lactation Day 21.

Pups culled on PND 4 were euthanized by intraperitoneal injection of sodium pentobarbital. Any excess pups not required to constitute the experimental groups were euthanized on PND 21-23, or as soon as convenient thereafter, by exposure to

carbon monoxide (Set B). Dams 2-18 were euthanized by intraperitoneal injection of sodium pentobarbital. No postmortem examination was performed for these animals.

F_1 Males

F_1 males were sacrificed on PND 52 or 53 by preliminary exposure to CO_2 (did not exceed 60 seconds) followed by decapitation*. *Note:* *Decapitation was 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 are sensitive to stress.

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.

Groups were not balanced equally, as three of fifteen animals were sacrificed on Day 52 and twelve of fifteen animals were sacrificed on Day 53.

General terminal examinations, blood collection (from the severed neck) and specific terminal examinations were undertaken for these animals.

2.17. UNSCHEDULED TERMINATIONS

Any animal that exhibited signs of severe toxicity, or found in a moribund condition, was euthanized by exposure to carbon dioxide and a necropsy was performed.

2.18. TERMINAL BLOOD COLLECTION IN F_1 MALES

At least 2.5 mL of blood was collected from the neck 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), testosterone and thyroid stimulating hormone (TSH) measurements.

2.19. F_1 TERMINAL EXAMINATIONS

At necropsy, the testes, epididymides, ventral prostate, dorsolateral prostate, seminal vesicle (with coagulating glands and fluid), levator ani plus bulbocavernosus muscles, thyroid (with attached portion of the trachea), liver, kidneys, pituitary, and adrenals were removed and the weights of each except the thyroid/trachea recorded to the nearest 0.1 mg. The kidneys, adrenals and epididymides were weighed as pairs. Left and right testes were weighed individually.

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

The right testis and epididymis were placed in Bouin's fixative for 24 hours, after which they were rinsed and stored in 70% ethanol.

The thyroid, with attached trachea, was fixed in 10% neutral buffered formalin for 24 hours. The thyroid was then dissected from the trachea, blotted and weighed to the nearest 0.1 mg and stored in 70% ethanol.

No other tissues were preserved.

2.20. HISTOPATHOLOGY

The preserved tissues were embedded in paraffin, stained with hematoxylin and eosin (H&E), and evaluated for pathologic abnormalities and potential treatment-related effects in a "blind" fashion (treatment group location not known during 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 were noted. A minimum of two sections per thyroid were evaluated. Sufficient sections were examined to allow a representative sample of thyroid to be evaluated.

Besides detection of gross lesions such as atrophy or tumors, testicular histopathological examination of the right testis was conducted in order to identify treatment-related effects such as retained spermatids, missing germ cell layers or types, multinucleated giant cells, or sloughing of spermatogenic cells into the lumen.

Examination of the intact right epididymis included the caput, corpus, and cauda, accomplished by evaluation of a longitudinal section, and was conducted in order to identify such lesions as sperm granulomas, leukocytic infiltration (inflammation), aberrant cell types within the lumen, or the absence of clear cells in the cauda epididymal epithelium.

2.21. HORMONAL ASSAYS

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

Quality control material was prepared from pooled commercial rat serum rather than from in-house animals with known concentrations or a commercially available kit for r-TSH.

Huntingdon Life Sciences prepared calibration curves against National Institute of Diabetes and Digestive and Kidney Diseases (NIDDKD) antigen standards, using the same concentrations as the kit's supplier.

2.22. STATISTICAL EVALUATIONS

All data (weaning body weights, body weight gain from PND 23 to 52/53, age and body weight at preputial separation, body and organ weights at necropsy, serum hormones and histology) were analyzed by analysis of variance or analysis of covariance (ANCOVA) as described below.

For each set, the animals were allocated to groups by bodyweight rank. This was included as a blocking factor in the analyses of variance and covariance. Note that because of exchanges to break litter clashes, the blocking was not perfect.

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

2.22.1. CONTINUOUS DATA

For continuous parameters, including age and body weight at PPS, weaning weight, overall body weight gain and hormones,

analysis of variance or analysis of 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 bodyweight and bodyweight gains, analysis of variance was then applied with bodyweight rank as a blocking factor. For all other parameters, analysis of covariance (Armitage *et al* 2002) with PND21 as the covariate, and bodyweight rank 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.

2.22.2. 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).

2.22.3. OUTLIERS

PPS (preputial separation) and BW

Body weight gain (PND23 to PND52), Group 1 (control)
Animal 114, BWgain=302.7, Grubbs statistic=3.00 (n=13)

Body weight gain (PND23 to PND53), Group 1 (control)
Animal 114, BWgain=314.3, Grubbs statistic=3.07 (n=13)

This one animal had been getting relatively heavier than the others in the group all the way through the study. Thus the values are not isolated outliers and should therefore be retained. To confirm, excluding them from the analysis did not alter the pattern of the results.

Organ weights

Pituitary, Group 4 (DE-71, dose 30 mg/kg)
Animal 410, wt=0.052, Grubbs statistic=3.46 (n=14)

Testis (Left), Group 4 (DE-71, dose 30 mg/kg)
Animal 411, wt=2.121, Grubbs statistic=3.02 (n=14)

Testis (Right), Group 4 (DE-71, dose 30 mg/kg)
Animal 402, wt=3.044, Grubbs statistic=3.37 (n=14)

Prostate gland (dorsolateral), Group 6 (1-Chloro-2NB, dose 25 mg/kg)
Animal 610, wt=0.342, Grubbs statistic=3.19 (n=15)

In all these cases, the analysis was performed on transformed data. This analysis will have accommodated the outliers (i.e. reduced their influence) without the need to exclude them.

Hormones

No outliers were detected.

2.23. 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 and the statistical analysis report generated by Huntington Life Sciences (UK division) 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. All analytical samples will be discarded after results are confirmed.

2.24. REGULATORY REFERENCES

2.24.1. TEST GUIDELINES

There are no specific test guidelines for this type of study at present. The design was approved by the relevant Regulatory Agency.

2.24.2. 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) with the exception of Hormone analysis.

2.24.3. 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.

2.25. PROTOCOL DEVIATIONS

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

1. All pups were identified using only ear tags. This was a unique form of identification that could be used to trace the maternal dam. Tail tattoo was not performed.

2. 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.

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

4. 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.

5. Recording of feed consumption initiated on Postnatal Day 23 or 24 rather than on PND 21, as required by protocol.

6. Dam # 30 delivered pups on Gestation Day 23, but was not sacrificed by CO_2 exposure on that day or as convenient thereafter, as specified by the protocol. In addition, data (e.g., pup observations, body weights, etc.) was collected and included in the report.

7. Body weight gains from PND 21 to 53 were supposed to be analyzed statistically, according to the protocol. However, body weight gains were actually analyzed from PND 23 onward.

8. Females used on study were actually 12-14 weeks of age, thereby some females were one week younger than the protocol requirement of 13 weeks.

9. There were as many as 4 pups per cage (Pup # 104-107) when the protocol specified for at least two pups, but ideally three F_1 animals per cage.

10. 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.

11. The protocol specified that littermates be equally distributed between treatment groups, however the Environmental Protection Agency relaxed the requirement that no littermates be

included in the same treatment group for this particular study since it was preferable to the alternative, which would have been to have fewer animals per group in Set A.

12. Statistics for incidence data were not performed as per protocol as these only applied to untreated females.

13. Animal Nos. 606-609, 706-709, 806-809, 906-909 and 1006-1009 (All Set B) were sacrificed on Day 52 in error. Also, groups were not balanced as to their termination age (approximately half divided over a two day period), as required by the protocol.

14. 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.

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

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

3. RESULTS

(Each Chemical in Set A was compared with the Group 1 Control; Each Chemical in Set B was compared with the Group 10 Control).

3.1. DOSE FORMULATION ANALYSIS

(Appendix A)

The data indicated that the dosing formulations of 1-Chloro-2-Nitrobenzene, Vinclozolin, Dibutyl Phthalate and DE-71 were prepared to an acceptable level of accuracy, with an average deviation within ± 10% of the nominal concentration for all the samples.

Lipid hydroperoxide content was 2.7 mEq/kg which was within the acceptable range (<3 mEq/kg).

3.2. LITTER AND DELIVERY DATA

(Table 1 and Appendix B)

Of the 36 dams that were ordered for the study, 3 were non-pregnant and 1 was sacrificed. The average litter size from these dams was 12 pups. There were insufficient pups to create 15 animals per group for Set A, so groups of 14 were constructed.

3.3. MORTALITY

(Appendix C)

There were no test substance related deaths during the study.

Animal 108 (Group 1, corn oil) was found dead on study day 37 after 14 days of dosing, but no pathologic findings were observed at necropsy in this animal.

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

(Tables 3, 4 and Appendices E, F)

Slight but statistically significant decreases in mean body weight gains were observed over PND 23-52 with the chemicals DE-71 (9% decrease at 30 mg/kg and 7% decrease at 60 mg/kg) and Vinclozolin (9% decrease at 30 mg/kg and 11% decrease at 100 mg/kg).

3.6. FEED CONSUMPTION

(Table 5 and Appendix G)

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

3.7. POSTWEANING DEVELOPMENTAL LANDMARKS

(Table 6 and Appendix H)

A dose-related delay in preputial separation was observed in males dosed with 30 and 100 mg/kg of Vinclozolin respectively, amounting to 3.3 and 6.4 days respectively.

Marginal delays in preputial separation occurred with Dibutyl Phthalate at 1000 mg/kg (2.7 days) and with 1-Chloro-2-Nitrobenzene at 100 mg/kg (2.8 days).

3.8. ORGAN WEIGHTS

(Table 7 and Appendix I)

Liver weight (absolute) was increased by 11% and 35% in animals dosed with 30 and 60 mg/kg DE-71, by 41% and 70% in animals dosed with 25 mg/kg and 100 mg/kg of 1-Chloro-2-Nitrobenzene, and by 10% and 19% in animals dosed with 500 and 1000 mg/kg of Dibutyl Phthalate, respectively. This increase in liver weight was most likely the result of hepatic microsomal induction resulting from the metabolism of these test substances.

Testicular weight was decreased by approximately 45% in males treated with 500 mg/kg of Dibutyl Phthalate and by 74% in males treated with 1000

mg/kg of Dibutyl Phthalate. Epididymides, seminal vesicles, and levator ani plus bulbocavernosus complex weights were decreased by 10%, 15% and 20% respectively for males receiving 500 mg/kg of Dibutyl Phthalate and by 22%, 27% and 24% respectively for males receiving 1000 mg/kg of Dibutyl Phthalate.

Epididymides and levator ani plus bulbocavernosus complex weights were decreased by 13% and 20% respectively for males receiving 30 mg/kg of Vinclozolin and by 16% and 25% respectively in males receiving 100 mg/kg of Vinclozolin. Seminal vesicle weights were decreased by 37% in males receiving 100 mg/kg of Vinclozolin. Testicular weight was decreased by approximately 10% in males treated with 30 mg/kg of Vinclozolin and by 6% in males treated with 100 mg/kg of Vinclozolin.

Levator ani plus bulbocavernosus complex weights were decreased by 14% in males treated with 30 mg/kg of DE-71 and by 18% in males treated with 60 mg/kg of DE-71. Testicular weight was increased by approximately 12% in males treated with 30 mg/kg of DE-71 the increase was less pronounced at 60 mg/kg of DE-71 (5% increase in testicular weight) but was statistically significant.

Kidney weights were increased by 10% in males treated with 25 mg/kg of 1-Chloro-2-nitrobenzene and by 10% in males treated with 100 mg/kg of 1-Chloro-2-nitrobenzene. Seminal vesicles, levator ani plus bulbocavernosus complex and prostate (ventral) weights were decreased by 17%, 18% and 29% respectively for males receiving 100 mg/kg of 1-Chloro-2-nitrobenzene.

Other statistically significant minimal changes in organ weights were attributed to biologic variation due to the lack of dose dependency.

3.9. MACROSCOPIC NECROPSY

(Table 8 and Appendix J)

The right testis and epididymis was decreased in size in 50% of males dosed with 500 mg/kg of dibutyl phthalate; the right testis and epididymis was decreased in all males dosed with 1000 mg/kg of dibutyl phthalate.

There were no other macroscopic findings.

3.10. HISTOPATHOLOGY

(Table 9 and Appendix J)

All males receiving 1000 mg/kg of Dibutyl Phthalate had marked or severe tubular atrophy. In most cases all seminiferous tubules were totally depleted of germ cells and were lined only by Sertoli cells. This finding correlated with the small testes and decreased testicular weight in this group. Males showed a significant reduction in the amount of caput and caudal sperm and an increase in the degree of ductal contraction in the cauda epididymis. All males showed aspermia throughout the epididymis, which is consistent with the severe tubular atrophy observed in the testis.

All males receiving 500 mg/kg of Dibutyl Phthalate showed partial or total loss of germ cells, ranging in severity from minimal to severe. In addition, many of the lesser affected males (8/15) had degeneration and/or depletion of the elongating spermatid population, which was sometimes associated with loss of round spermatids. Spermatid retention and increased tubular vacuolation was also present in a few (3/15) males. There was a significant reduction in the amount of caput and caudal sperm and an increase in the degree of ductal contraction in the cauda epididymis. There were also increased numbers of sloughed testicular germ cells and cell debris in the ductal lumens of the caput and cauda epididymis, reflecting the degeneration and loss of germ cells from the seminiferous tubules.

Males receiving 100 mg/kg of 1-Chloro-2-nitrobenzene showed minimal to slight spermatid retention (10/15), an increase in tubular vacuolation (8/15), and in addition there was degeneration and/or depletion of the elongating spermatid population (9/15). There was a significant reduction in the amount of caput and caudal sperm, and increased numbers of sloughed testicular germ cells and cell debris in the ductal lumens of the caput and cauda epididymis, reflecting the degeneration and loss of germ cells from the seminiferous tubules.

Males receiving 60 mg/kg of DE-71 had an increase in the height of the follicular cells and a decrease in the colloid area of the thyroid.

3.11. HORMONE DATA

(Table 10 and Appendix K)

Tetra-iodothyronine (T_4) levels were reduced by 78% and 86% in males receiving 30 and 60 mg/kg of DE-71 respectively. Consistent with these results were increases in TSH levels of 39% and 100% in these dose groups, respectively. Testosterone levels were decreased by 49% in males receiving 30 of DE-71.

T_4 levels were reduced by 24% and 25% in males receiving 500 and 1000 mg/kg of Dibutyl Phthalate, respectively. Testosterone levels were reduced by 56% in animals receiving 1000 mg/kg of Dibutyl Phthalate. There was a dose dependent decrease in TSH levels also, (42% in males receiving 500 mg/kg of Dibutyl Phthalate and 54% in males receiving 1000 mg/kg of Dibutyl Phthalate) however, the biological relevance of this is uncertain due to the relatively high TSH levels in control males.

Tetraiodothyronine (T_4) levels were reduced by 31% and 51% in males receiving 30 and 100 mg/kg of Vinclozolin, respectively. Testosterone levels were increased by 67% in males receiving 100 mg/kg of Vinclozolin.

4. CONCLUSION

There were no clinical signs of toxicity or effects on feed consumption during the study. There was a slight decrease in body weight gain from PND 23-52 associated with DE-71 and Vinclozolin.

Endocrine disruptive effects of Dibutyl Phthalate included a decrease in size of the testis and epididymis, which correlated with a substantial decrease in testicular weight for males treated with 500 and 1000 mg/kg of Dibutyl Phthalate, respectively. The decrease in testicular size was in agreement with a previous study (Rocca and Pepperl, 2000). The epididymides, seminal vesicles and levator ani plus bulbocavernosus complex weights were decreased in males receiving both 500 mg/kg and 1000 mg/kg of Dibutyl Phthalate. Severe seminiferous tubular atrophy was found at 1000 mg/kg of Dibutyl Phthalate, and in most cases all seminiferous tubules were totally depleted of germ cells. At 500 mg/kg of Dibutyl Phthalate there was loss of germ cells ranging in severity from minimal to severe. T_4 and TSH levels were significantly reduced in males receiving 500 mg/kg and 1000 mg/kg. Testosterone levels were significantly reduced in males receiving 1000 mg/kg of Dibutyl Phthalate. A slight delay in preputial separation occurred in

males receiving 1000 mg/kg of Dibutyl Phthalate. Liver weight was slightly elevated at 500 and 1000 mg/kg of Dibutyl Phthalate.

An increase in liver weight was observed for 1-Chloro-2-Nitrobenzene treated animals, which was probably attributable to microsomal hepatic enzyme induction associated with metabolism. An increase in kidney weight was found in males receiving both 25 and 100 mg/kg of 1-Chloro-2-Nitrobenzene. Seminal vesicle, prostate (ventral) and levator ani plus bulbocavernosus complex weights were decreased in males receiving 100 mg/kg of 1-Chloro-2-Nitrobenzene. Several histopathological findings were associated with this compound, such as slight spermatid retention and an increase in tubular vacuolation, but these findings were considered minimal and not associated with endocrine disruption. A slight delay in preputial separation occurred in males dosed with 100 mg/kg of 1-Chloro-2-Nitrobenzene.

An increase in liver weight was found in males receiving DE-71, which may be attributable to direct enzyme induction of one or all of the individual components contained in this test substance. Levator ani plus bulbocavernosus complex weights were decreased in males receiving 30 and 60 mg/kg of DE-71. An increase in testicular weight was found in males receiving 30 mg/kg DE-71 which was also found at 60 mg/kg but to a lesser degree. T_4 levels were substantially reduced in males treated with DE-71, which was accompanied by an increase in TSH levels. This decrease in T_4 and increase in TSH was an expected finding, based on a previous endocrine disruptor screening assay (Stoker et al., 2004). Testosterone levels were also decreased with this test substance, however the biological relevance is uncertain as Testosterone levels were highly variable.

Vinclozolin delayed preputial separation in a dose related manner, which was in agreement with a previous NTP study (NTP study RACB 20103). A dose dependent decrease occurred in weights of the epididymides and levator ani plus bulbocavernosus complex. A decrease in seminal vesicle weight occurred in males dosed with 100 mg/kg Vinclozolin. A slight increase in Testicular weight was found in males dosed with 30 and 100 mg/kg Vinclozolin. An increase in Testosterone was found at 100 mg/kg Vinclozolin. A dose dependent decrease in T_4 was also found with this test substance.

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

Lactation Index:

no. of pups alive Day 21/no. of pups Day 4 postcull

Live Birth Index:

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

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