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UNAUDITED DRAFT REPORT

Pubertal Toxicity Study of Vinclozolin, Flutamide and Phenobarbital in Male Sprague Dawley Rats and Methoxychlor, Ethinyl Estradiol and Phenobarbital in Female Sprague Dawley Rats when Administered in Corn Oil by Oral Gavage

Sponsor

National Toxicology Program (NTP)
National Institute of Environmental Health Sciences (NIEHS)
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Sponsor Study Numbers

Pubertal Vinclozolin Study: RACB 20104
Pubertal Flutamide Study: RACB 20105
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Testing Facility

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TherImmune Study Number: 7244-600

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Study Completion Date

[To be added at finalization]

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COMPLIANCE STATEMENT

Pubertal Toxicity Study of Vinclozolin, Flutamide and Phenobarbital in Male Sprague Dawley Rats and Methoxychlor, Ethinyl Estradiol and Phenobarbital in Female Sprague Dawley Rats when Administered in Corn Oil by Oral Gavage

This study was conducted in compliance with U.S. Food and Drug Administration (FDA) Good Laboratory Practice (GLP) Regulations for Nonclinical Laboratory Studies as set forth in Title 21 of the U.S. Code of Federal Regulations Part 58.

Portions of the study performed by subcontractors were performed according to the protocol and GLP compliance was verified by their Quality Assurance Units (QAU).

The protocol, amendment, and deviations are presented in Appendix 17. There were no deviations from the aforementioned regulations that affected the quality or integrity of the study or the interpretation of the results in the report.

Study Director:

Meredith S. Rocca, PhD, DABT

Date

QUALITY ASSURANCE STATEMENT

Pubertal Toxicity Study of Vinclozolin, Flutamide and Phenobarbital in Male Sprague Dawley Rats and Methoxychlor, Ethinyl Estradiol and Phenobarbital in Female Sprague Dawley Rats when Administered in Corn Oil by Oral Gavage

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SIGNATURE PAGE

Pubertal Toxicity Study of Vinclozolin, Flutamide and Phenobarbital in Male Sprague Dawley Rats and Methoxychlor, Ethinyl Estradiol and Phenobarbital in Female Sprague Dawley Rats when Administered in Corn Oil by Oral Gavage

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SUMMARY

Pubertal Toxicity Study of Vinclozolin, Flutamide and Phenobarbital in Male Sprague Dawley Rats and Methoxychlor, Ethinyl Estradiol and Phenobarbital in Female Sprague Dawley Rats when Administered in Corn Oil by Oral Gavage

The purpose of this study was to examine the sensitivity of the pubertal assay to an estrogen (Methoxychlor), anti-androgen (Vinclozolin and Flutamide), and a thyroid-active agent (Phenobarbital) in intact juvenile/peripubertal male and female rats. Fifty-five time-mated female (F₀) Sprague Dawley Rats were received from Charles River Laboratories and allowed to litter at TherImmune. After weaning on Postnatal Day (PND) 21, 135 F₁ males and 135 F₁ females were assigned to male and female cohort studies, respectively. The F₁ males were dosed with corn oil (0 mg/kg), or the test articles: Phenobarbital (25, 50, and 100 mg/kg/day), Vinclozolin (10, 30, and 100 mg/kg/day), or Flutamide (25 and 50 mg/kg/day), once daily via oral gavage from PND 23-53/54. The F₁ females were dosed with corn oil (0 mg/kg), or the test articles: Ethinyl Estradiol (0.0025 and 0.0050 mg/kg/day), Methoxychlor (12.5, 25, and 50 mg/kg/day), or Phenobarbital (50 and 100 mg/kg/day), once daily via oral gavage from PND 22-42/43.

Parameters evaluated include mortality and clinical observations, body weights, day of preputial separation, day of vaginal opening, estrous cyclicity, gross pathology, organ weights and histopathology.

The following parameters were affected by treatment:

Males

Test Article	Concentration (mg/kg/day)	Body Weight	Age of Preputial Separation	Organ Weights	Histology
Phenobarbital	25	-	-	↑ liver	-
	50	-	-	↑ liver	-
	100	↓	↑	↑ liver ↓pituitary	-
Vinclozolin	10	-	↑	-	-
	30	-	↑	↓repro. organs ↑ testis,	-
	100	-	↑	↓repro. organs ↑ testis, ↑ adrenal	-
Flutamide	25	-	↑	↓repro. organs ↑ testis, ↑ liver ↓ kidneys	-
	50	-	↑	↓repro. organs ↑ testis, ↑ liver ↓ kidneys	testis lesions

- = no effect; ↑ = higher than control means; ↓ = lower than control means
repro. = reproductive

Females

Test Article	Concentration (mg/kg/day)	Age at Vaginal Opening	Age at First Estrus	Irregular Estrous Cycles	Organ Weights
Phenobarbital	25	-	-	-	↑ liver ↑ adrenal
	50	-	-	-	↑ liver ↑ adrenal
	100	↑	-	-	↑ liver ↑ adrenal ↑ thyroid
Methoxychlor	12.5	↓	↓	-	-
	25	↓	↓	X	-
	50	↓	↓	X	↓ liver ↓ ovary ↓ pituitary
Ethinyl Estradiol	0.0025	-	-	-	↑ adrenal
	0.005	↓	↓	X	↑ adrenal

- = no effect; ↑ = higher than control means; ↓ = lower than control means; X =present
repro. = reproductive

The lowest observable effect level (LOEL) for an endocrine effect was 25 mg/kg/day for Flutamide, 10 mg/kg/day for Vinclozolin, 100 mg/kg/day for Phenobarbital, 12.5 mg/kg/day for Methoxychlor, and 0.005 mg/kg/day for Ethinyl Estradiol.

STUDY PERSONNEL AND TEST SITES

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STUDY TIMETABLE

Study Initiation Date:	August 13, 2002
Experimental Start Date: [Gestation Day 1 Body weights]	October 30, 2002
Receipt of Animals:	November 7, 2002
Randomization of F ₁ Animals:	December 12 and 13, 2002
First Day of Dosing:	
F ₁ Females:	December 13, 2002
F ₁ Males:	December 14, 2002
Last Day of Dosing:	
F ₁ Females:	January 3, 2003
F ₁ Males:	January 14, 2003
Necropsy:	
F ₁ Females:	January 2 and 3, 2003
F ₁ Males:	January 13 and 14, 2003
Experimental Completion Date: [Histology Analysis]	Month Day, Year

INTRODUCTION

Proposed Investigations/Rationale for Dose Selection

The purpose of this study was to obtain data on proposed procedures and to evaluate the lowest observable effect level of Vinclozolin, Methoxychlor, Flutamide, Ethinyl Estradiol, and Phenobarbital on pubertal development in the intact juvenile/peripubertal male and female rats. Puberty is a time of rapid interactive endocrine and morphological changes, and numerous pharmaceutical and environmental agents have been shown to alter the timing of pubertal development in mammals. This study was designed to detect agents that have antithyroid, estrogenic, androgenic, or antiandrogenic activity, or alter puberty by changing follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin, growth hormone (GH), or hypothalamic function (Stoker *et al.*, 2000 and Goldman *et al.*, 2000). The Sprague Dawley rat was selected as the test system due to its established quality as a breeder and the availability of historical control data for reference.

Vinclozolin, 3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-oxazolone-2,4-dione, is a fungicide used on fruits, vegetables, turfgrass, and ornamental plants (U.S. EPA, 1998). *In vivo*, Vinclozolin inhibits androgen receptor (AR)-dependent gene expression (Kelce *et al.*, 1997) and produces a spectrum of anatomical defects. Administration of Vinclozolin (400 mg/kg) to rats on Gestation Day (GD) 14 through PND 3 resulted in effects similar to those caused by Flutamide, a well-known AR antagonist. These effects included reduced anogenital distance (AGD); persistent nipples; cleft phallus; hypospadias; reduced weights of the ventral prostate, seminal vesicles, and epididymis; and, reduced sperm count (Gray *et al.*, 1994; 1999a). Exposing weanling male rats to the antiandrogenic pesticides p,p'-DDE or Vinclozolin delayed pubertal development as indicated by delayed preputial separation and increased body weight at puberty. In contrast, to delays associated with exposure to estrogenic substances, antiandrogens do not inhibit food consumption or retard growth (Anderson *et al.*, 1995b).

Flutamide (4'-nitro-3'-trifluoromethyl-isobutyranilide) is a potent non-steroidal androgen-receptor antagonist that has been used therapeutically to treat androgen-dependent prostate cancer (Delaere and Van Thillo, 1991; Murphy *et al.*, 1991) and as a tool to study male reproductive development. Studies in rats have demonstrated that pre- or postnatal Flutamide (6.25 to 50 mg/kg) exposure alters androgen-dependent reproductive development (Imperato-McGinley *et al.*, 1992; Kassim *et al.*, 1997) and has been shown to produce decreased reproductive organ weights, feminization of male external genitalia, altered androgen-dependent testicular descent, and retention of nipples when male offspring are exposed *in utero* (Imperato-McGinley *et al.*, 1992).

Methoxychlor has been used for nearly 50 years for insect and larval control. Its advantage over DDT is that the Methoxychlor is more readily metabolized and excreted by mammalian systems (Kapoor *et al.*, 1970) and is therefore less likely to undergo bioconcentration than is DDT. This metabolism also yields mono- and bis-hydroxy metabolites of Methoxychlor (Bulger *et al.*, 1978), which helps explain both the uterotrophic effects noted earlier for Methoxychlor (Tullner, 1961) and the observations that Methoxychlor *in vivo* reduced the uterine uptake of radiolabeled estradiol (Welch *et al.*, 1969). Treatment of rats with Methoxychlor at 5, 50, or 150 mg/kg for the week before and the week after birth until PND 7 resulted in unchanged anogenital distance, accelerated vaginal opening, and delayed prepuce separation; at 50 and 150 mg/kg, it disrupted adult estrous cyclicity and reduced epididymal sperm counts and testis weights.

As cited in Goodman and Gilman's Pharmacological Basis of Therapeutics (1996), estrogens are among the most commonly prescribed drugs in the United States. The two major uses are as components of combination oral contraceptives and hormone replacement therapy. The pharmacological considerations for estrogen use in oral contraceptives, as opposed to postmenopausal hormone replacement, are substantially different, primarily because of the doses used. Historically, conjugated estrogens have been the most common agents for postmenopausal use, and 625 µg/kg/day is effective in most women. In contrast, most combination oral contraceptives in current use employ 20 to 35 µg/day of Ethinyl Estradiol. Conjugated estrogens

and Ethinyl Estradiol differ widely in their oral potencies; for example, a dose of 625 µg of conjugated estrogens generally is considered equivalent to 5 to 10 µg of Ethinyl Estradiol.

Several authors have demonstrated estrogenic responses to Ethinyl Estradiol in rodents. Laws *et al.* (2000) showed that *in vivo* studies indicated the 3-day uterotrophic assay in prepubertal rats was best for detecting estrogenic activity when compared with all other models, based upon the dose-response data for Ethinyl Estradiol (0.01-0.1 mg/kg, oral), 4-tert-octylphenol (50-200 mg/kg, oral), and 4-nonylphenol (25-100 mg/kg, oral). Although oral doses of Ethinyl Estradiol (0.01 mg/kg) induced a significant increase in uterine weight in the prepubertal rat, this dose was ineffective for stimulating a similar response in ovariectomized adult rats. The age of vaginal opening was advanced following oral exposure from postnatal days 21-35 to Ethinyl Estradiol (0.01 mg/kg). Ethinyl Estradiol advanced the day of vaginal opening by 6.0 ± 0.18 days (30.6 days in control vs. 24.6 days in treated groups). In addition, the number of 4-5 day estrous cycles was reduced during a 25-day exposure to Ethinyl Estradiol (0.01 mg/kg). Advanced vaginal opening was also demonstrated by Odum *et al.* (1997) using doses of 2-400 µg/kg/day, and Singh and Kamboj (1980) using doses of 5 µg/kg/day for 5 days. Singh and Kamboj (1980) also showed an advance in the appearance of cornified vaginal cells.

Phenobarbital is a commonly prescribed antiepileptic agent whose hepatotoxicity and effects on the thyroid have been established. Endpoints of reproductive and developmental toxicity have not been as well explored, although some data are available. Gupta *et al.* (1980) evaluated the reproductive and developmental toxicity of Phenobarbital in male rats. Males were exposed *in utero* from GD 12-19 (40 mg/kg/day administered to the dam). Treatment-related effects included reduced anogenital distance; delayed testes descent; decreased seminal vesicles weight; and reduced fertility. Both serum testosterone and luteinizing hormone were decreased in the exposed males. However, the age at onset of puberty (i.e., age at preputial separation) was not affected by Phenobarbital exposure. In a similar study (Gupta and Yaffe, 1981), female rats were exposed to Phenobarbital *in utero* (40 mg/kg/day administered to the dam) or immediately following birth (20 mg/kg/day on PND 1-8). Phenobarbital treatment resulted in delayed vaginal

opening (34.6 ± 1.2 days in the control vs. 37.5 ± 1.2 days in the treated group); disruptions in the estrous cycle (only 40% of treated rats displayed normal estrous cyclicity, compared to 91% of control rats); and impaired fertility (50% of treated rats were fertile, and 100% of the control rats were fertile). These effects were observed during critical periods for neuroendocrine development: GD 17-20 and PND 1-8 (Gupta and Yaffe, 1981).

Based on the above-cited literature, the hypotheses of the current study were:

1. Ethinyl Estradiol and Methoxychlor administration to juvenile female rats will result in advanced vaginal opening, advanced first estrous and onset of estrous cycles, and/or persistent vaginal estrus
2. Administration of Vinclozolin and Flutamide to juvenile male rats will result in delayed preputial separation, decreased reproductive organ weights, altered external genitalia, and/or retention of nipples
3. Phenobarbital treatment will result in delayed vaginal opening and irregular estrous cyclicity in juvenile female rats, reduced reproductive organ weights, and possibly delayed preputial separation in juvenile males. Phenobarbital treatment may also cause increased thyroid weights and follicular cell hyperplasia, particularly in the males.

METHODS AND MATERIALS

TEST AND CONTROL ARTICLES

Neat Materials

The neat test and control articles used in this study are described in Text Table 1A and 1B.

Text Table 1A: Neat Test and Control Articles

Name	Lot No.	Supplier	Purity	Date Received
Corn Oil	N/A	TherImmune	100%	NA
Vinclozolin	102996	Battelle	100%	07/12/2002
Flutamide	109H0952	Battelle	100%	07/12/2002
Methoxychlor	124F0575	Research Triangle Institute	95%	07/12/2002
Ethinyl Estradiol	45H0716	Midwest Research Institute	99.7%	07/13/2002
Phenobarbital	Q10645	Midwest Research Institute	100%	07/18/2002

Information on the methods of synthesis and stability, data on the composition, and other characteristics that define the test articles are on file with the Sponsor. Test article analyses are presented in Appendix 1.

Text Table 1B: Neat Test and Control Articles- Storage Condition

Name	Lot No.	Storage Condition
Corn Oil	N/A	Room temperature
Vinclozolin	102996	Room temperature protected from light
Flutamide	109H0952	Room temperature protected from light
Methoxychlor	124F0575	Sealed under nitrogen at room temperature and protected from light
Ethinyl Estradiol	45H0716	Sealed under nitrogen at room temperature and protected from light
Phenobarbital	Q10645	Room temperature in a cool dry place away from heat

Two 5 g reserve samples of each bulk test article were taken prior to use on the study, and stored at -20°C for possible future re-analysis. One 5 g sample of each bulk test article was taken within 30 days of the receipt, and sent on dry ice to the appropriate NTP subcontractor for purity and stability testing. Samples of the bulk test articles and their formulations were sent for analysis to the following NTP subcontractors: Flutamide and Vinclozolin were sent to Battelle, Columbus, Ohio, Ethinyl Estradiol and Phenobarbital were sent to MRI, Kansas City, Missouri, and Methoxychlor was sent to RTI International, Research Triangle Park, North Carolina.

Prior to the start of the study, the dose-formulation stability study for each test article was conducted by the Sponsor. The Vinclozolin dose formulation (2.0 mg/mL in corn oil) was stable for 42 days at 25°C , 5°C , and -20°C . The Methoxychlor dose formulation (1.82 mg/mL in corn oil) was stable for 30 days under refrigerated conditions (2 to 5°C) and for 23 days under ambient (23 to 28°C) conditions. The Flutamide dose formulation (10 mg/mL in corn oil) was stable for 42 days at 5°C or -20°C , with -20°C being preferable. The Ethinyl Estradiol dose formulation (1.0 $\mu\text{g}/\text{mL}$ in corn oil) was stable for up to 14 days under refrigerated conditions (approximately 5°C). The Phenobarbital dose formulation (2.5 mg/mL in corn oil) was stable for up to 14 days (refrigerated at approximately 5°C). All formulations were stored in sealed amber glass bottles and protected from light. Under conditions that simulate animal dosing (room temperature, exposed to air and light), the dosage formulations showed no appreciable loss.

Dose Formulations

Dose formulations of Vinclozolin, Methoxychlor, Ethinyl Estradiol, Flutamide, and Phenobarbital were prepared every ten days. For each dose level, an appropriate amount of test article was accurately weighed into a pre-calibrated beaker using a stainless steel spatula. A sufficient quantity of corn oil was added to achieve the desired final volume and formulation was mixed using a magnetic stir bar for at least 15 minutes and sonicated, if necessary, to ensure complete dissolution. A table of test articles, concentrations, dose levels and group numbers is given in Text Tables 3A and 3B. Formulations were dispensed in daily aliquots that were stored

in amber glass bottles with Teflon®-coated lids, and kept protected from light in a refrigerator set to maintain 2-8 °C until used for dosing. Formulations were stirred prior to and during dosing. Formulation procedures are presented in Appendix 3.

Since this was conducted as a “blind” study, the vials containing the daily aliquots were not labeled explicitly with the test article name and concentration. Instead, the group number, mix number and a one-letter code were used to uniquely identify each vial. This prevented the animal room technicians from knowing the identity of the dosing compound. Group identification was assigned separately for males and females.

Dosage Analysis

Every time a new mix was prepared, three 35 mL archival samples from each dose level of each test article were collected and stored at TherImmune in amber glass vials with Teflon®-coated lids, protected from light in a refrigerator at 2-8°C. One set of samples was forwarded on ice packs to the NTP subcontractors for dose concentration analysis at the following intervals: initial, middle, and final formulations (Mix 1, 2, and 4). Samples of the corn oil were also forwarded for analysis. Additionally, 35 mL homogeneity samples were collected from the top, middle, and bottom of the low and high dose formulations for each test article from mix 1. Samples were stored in a refrigerator at 2-8°C in glass vials with Teflon®-coated lids, protected from light and forwarded to the NTP subcontractors on ice packs for analysis. Analytical methods are described in the dose formulation concentration analysis reports presented in Appendix 2.

TEST ANIMALS AND HUSBANDRY

F₀ Animals

Fifty-five time-mated female (F₀) Sprague Dawley Rats (CrI: CD® (SD) IGS BR) were received (11 to 12 weeks old) from Charles River Laboratories, Raleigh, North Carolina, on November 7, 2002 (GD 9). The animals were received with unique tail identification numbers. For the

purpose of this study, the day of mating was considered as GD 0, and the day of littering was considered as PND 0. For all animals, the day of positive mating was October 30, 2002 (GD 0). Throughout the study, the F₀ dams were identified by tail mark and cage label, and housed one per cage. GD 0 body weights (between 200 and 225 g) were recorded and provided by the supplier. Animals were acclimated to laboratory conditions for two weeks and released from quarantine by a staff veterinarian. During the acclimation period, one dam was sent to Taconic Anmed (Rockville, Maryland) for serological testing. The results of these tests are presented in Appendix 14. F₀ dams were never exposed to the test articles.

F₁ Animals

The litters (F₁ animals) delivered on November 21, 2002 were selected for the juvenile male and female cohort studies. On PND 4, the selected litters were culled to 10 pups/litter, using computer-generated random numbers. The targeted litter size after culling was 5 males and 5 females. When less than 5 pups/sex were available in a litter, additional pups from the opposite sex (same litter) were selected to achieve a litter size of 10 pups. Culled pups were euthanized and discarded without necropsy on PND 4. Throughout the lactation period, the pups were not individually identified. At weaning on PND 21, 135 males and 135 females were selected for the study, identified individually by tail tattoo and cage label, and housed 3 per cage (sexes separated) for the remainder of the study. Animals not selected on PND 21 were euthanized and discarded without necropsy on PND 24.

TherImmune's Institutional Animal Care and Use Committee (IACUC) approved this protocol and found it to be in accordance with provisions of the USDA Animal Welfare Act, the PHS Policy on Humane Care and Use of Laboratory Animals and the US Interagency Research Animal Committee Principles for the Utilization and Care of Research Animals.

Husbandry

Animal husbandry was provided as described in Text Table 2.

Feed and water were provided *ad libitum*, unless otherwise noted. The feed was analyzed by the vendor for concentrations of nutrients, heavy metals, aflatoxins, nitrosamines, chlorinated hydrocarbons, organophosphates, PCBs, nitrates, nitrites, BHA, BHT, total bacterial plates, genistein, daidzein, glycite, metabolizable energy content, coliforms, *E coli* and *Salmonella*. Results of this analysis are presented in Appendix 15. A 71g sample of feed was collected from the same lot number used in the study and stored at room temperature at TherImmune, for possible future analysis. Water and bedding analyses/certification requirements are on file at TherImmune. No contaminants were known to be present in the diet, water, or bedding at levels that might have interfered with achieving the objectives of the study.

Environmental controls were set to maintain animal room conditions as shown in Text Table 2. Actual temperature and relative humidity in the animal room were monitored continuously by a computerized system and manually recorded at least once daily. All environmental parameters were maintained within the protocol requirements except for deviations detailed in Appendix 16. While humidity was frequently outside the protocol specified range (40-50%) it was within the acceptable range (30-70%) specified in the *Guide for the Care and Use of Laboratory Animals* (National Academic Press, 1996) and did not influence the health of the animals and/or the outcome of the study.

Text Table 2: Husbandry

Feed	Purina Certified 5002, Lot no. 082802 3B
Water^a	Filtered tap water via an automatic watering system and/or water bottles
Bedding^b	Sani-Chip® Certified Hardwood Bedding, autoclaved prior to use
Caging	Polycarbonate cages, 19" x 10½" x 8"
Racks	Stainless steel racks
Animals Per Cage	One (F ₀ dams), 3 (F ₁ animals)
Temperature Range	Set to maintain 22 ± 2 °C or 68 to 75.2 °F
Humidity Range	Set to maintain 40 to 50%
Light Cycle^c	14-hour light/10-hour dark
Air Changes	Minimum of 10 air changes per hour

a = The water was routinely analyzed for contaminants and specific microbes.

b = The bedding was analyzed by the manufacturer for acceptable levels of heavy metals, aflatoxins, bacteria, yeasts, molds, and organophosphates prior to certification.

c = Light cycles were interrupted when necessary for study related events.

EXPERIMENTAL DESIGN

Group Assignment and Doses

Animals were assigned to groups as shown in Text Table 3A and 3B.

Text Table 3A: Study Design, F₁ Males

Group	Treatment	Test Article Dosage (mg/kg/day)	Test Article Concentration (mg/mL)	Males	
				N	Animal Numbers
1	Corn Oil	0	0	15	9026-9040
2	Phenobarbital	25	10	15	9041-9055
3	Phenobarbital	50	20	15	9056-9070
4	Phenobarbital	100	40	15	9071-9085
5	Vinclozolin	10	4	15	9086-9100
6	Vinclozolin	30	12	15	9101-9115
7	Vinclozolin	100	40	15	9116-9130
8	Flutamide	25	10	15	9131-9145
9	Flutamide	50	20	15	9146-9160

N = number of animals per group

Text Table 3B: Study Design, F₁ Females

Group	Treatment	Test Article Dosage (mg/kg/day)	Test Article Concentration (mg/mL)	Females	
				N	Animal Numbers
1	Corn Oil	0	0	15	9161-9175
2	Ethinyl Estradiol	0.0025	0.001	15	9176-9190
3	Ethinyl Estradiol	0.005	0.002	15	9191-9205
4	Methoxychlor	12.5	5	15	9206-9220
5	Methoxychlor	25	10	15	9221-9235
6	Methoxychlor	50	20	15	9236-9250
7	Phenobarbital	25	10	15	9251-9265
8	Phenobarbital	50	20	15	9266-9280
9	Phenobarbital	100	40	15	9281-9295

N = number of animals per group

Animals were accepted into the randomization pool based on PND 21 body weights and physical examinations. The following procedure was performed separately for males and females: after

collecting PND 21 body weights, the weanlings were assigned to 15 different blocks (9 animals/block), ranging from the heaviest to the lightest (block number 1 being the heaviest). Using random card draw, the weanlings were then assigned to study groups, such that each group received exactly one pup from each of the 15 weight blocks. At randomization the mean body weight for each group was not statistically different from the control mean. During the randomization process, each study animal was assigned a unique number and identified by tail tattoo and cage label. Randomization was performed on PND 21 for females and PND 22 for males.

Dose Administration

Animals were given the appropriate dose formulation as shown in Text Table 4.

Text Table 4: Dose Administration

Route of Administration	Oral Gavage
Days Dosed	Males: SD 23 through termination (PND 53-54) Females: SD 22 through termination (PND 42-43)
Dose Volume	2.5 mL/kg/day, based on most recent body weight
Equipment	1-2 inches, stainless steel, 18 gauge, gavage needle with a 2.25 mm ball, and 1 cc syringe
Test Article Conditions	Formulations were removed from the refrigerator at least 30 min prior to dosing. Formulations were stirred continuously during dosing.

Dosing was performed each day between 7:00 and 10:00 AM, with one exception: female dosing ended at 10:37 AM on PND 42.

OBSERVATIONS

F₀ dams were observed twice daily for signs of mortality and general health. Body weights were collected at littering and on PND 21.

In addition, on PND 0, 4, 7, 14 and 21, the litters were observed for the following: number of live pups, number of dead pups, number of males, total body weights of males, number of females, and total body weights of females. Individual pup body weights were collected on PND 4, 7, 14 and 21, although the pups were not uniquely identified until PND 21.

Following randomization, F₁ animals were observed as shown in Text Table 5.

Cage-side observations included observation for mortality, moribundity, general health and signs of toxicity. Clinical observations included evaluation of skin and fur characteristics, eye and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, and somatomotor and behavior patterns.

Text Table 5: F₁ Animal Observations

Procedure	Frequency of Testing	
	F ₁ Males	F ₁ Females
Cage-side Observations	≥ 2 Daily	≥ 2 Daily
Clinical Observations	At randomization, on PND 23 and weekly thereafter	At randomization, on PND 22 and weekly thereafter
Body Weights	At randomization, on PND 23 and daily thereafter	At randomization, on PND 22 and daily thereafter
Preputial Separation	Daily, starting on PND 23 until complete separation or termination, whichever came first	NA
Vaginal Opening	NA	Daily, starting on PND 22 until complete opening
Estrous Cyclicity	NA	Starting on the day of vaginal opening, daily until termination

Note: A partial preputial separation was recorded, however, the day of complete separation was the endpoint used in the analysis for the age of preputial separation.

TERMINATION, NECROPSY AND HISTOPATHOLOGY

Termination

F₀ dams that did not litter, and the dams that delivered on November 20, 2002 and their litters, were euthanized and discarded without necropsy. Remaining F₀ females were terminated without necropsy on PND 21. F₁ pups culled on PND 4 were terminated without necropsy. F₀ dams were euthanized by carbon dioxide inhalation and the pups were euthanized by sodium pentobarbital injection. F₁ animals not selected for study on PND 21 were terminated by carbon dioxide inhalation on PND 24 and discarded without necropsy. Treated F₁ males and females were terminated on PND 53-54 or PND 42-43, respectively, by carbon dioxide inhalation and exsanguination.

Necropsy

Scheduled necropsies were conducted under the supervision of a veterinary pathologist. A gross necropsy, which included examination of the external surface of the body, all orifices, the cranial, thoracic, and abdominal cavities, and their contents, was performed. One 100 mg/kg/day Flutamide-treated male was found dead on PND 24; a gross necropsy was performed and protocol specified tissues were collected, but organs weights were not collected.

For all F₁ males that survived to the terminal necropsy, the following organs were weighed as soon as possible after dissection: adrenal glands (paired), epididymides (right and paired weights), kidneys (paired), levator ani plus bulbocavernosus muscles, liver, pituitary, seminal vesicles plus coagulating glands (with and without fluid), testes (paired), thyroid and parathyroid (post-fixation), ventral and dorsal-lateral prostate (separately). After weighing, the following tissues were placed in Bouin's fixative and transferred to 70% ethanol within 24-48 hours: adrenal glands (paired), epididymides (paired), kidneys (paired), liver, pituitary, testes (paired), gross lesions. Thyroid and parathyroid (with attached portion of the trachea) were fixed in 10% neutral buffered formalin, then the attached portion of trachea was removed and the thyroid/parathyroid weights were collected.

For all F₁ females, the following organs were weighed as soon as possible after dissection: adrenal glands (paired), kidneys (paired), liver, ovaries (paired), pituitary, thyroid and parathyroid (post-fixation, as described for males), uterus and cervix (with and without luminal fluid). Small tissues, as well as tissues that contained fluid, were weighed immediately to prevent partial drying prior to weighing. After weighing, the following tissues were placed in Bouin's fixative and transferred to 70% ethanol within 24-48 hours: adrenal glands (paired), kidneys (paired), liver, ovaries (paired), pituitary, uterus and cervix, gross lesions.

Histopathology

Preserved tissues were transferred to PAI, Frederick, Maryland. The following tissues were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined microscopically by the study pathologist: thyroid, testes, epididymides, and gross lesions from all males, as well as the ovaries, uterus, cervix, thyroid and gross lesions from all females. The methods are described in the Pathology Report, presented in Appendix 16.

STATISTICAL ANALYSES

Data from the treated F₁ animals were analyzed by Analytical Sciences Inc. (Durham, North Carolina).

All endpoints (age and weight at vaginal opening/preputial separation, body weights, estrous cycle length, and organ weights at necropsy) with the exception of a few related to vaginal cytology (number of cycling females and number of females with regular cycles) were analyzed using ANOVA or ANCOVA to determine if there was a dose effect. For endpoints that were analyzed with an ANOVA, Levene's test was used to assess whether the assumption of equal variance across dose groups was tenable. For those endpoints where the Levene's test was rejected at the 0.1 level, no p-values for the overall ANOVA are reported.

Organ weights were analyzed using ANCOVA with terminal body weight as the covariate. In fitting the ANCOVA models, appropriate statistical tests were carried to ascertain the validity of

a constant slope for each endpoint. The hypothesis of constant slope was rejected only in a handful of cases (ovaries, uterus and cervix without fluid for ethinyl estradiol and pituitary for vinclozolin). However, caution should be used in interpreting the results from the ANCOVA model given that the covariate (terminal body weight) could be impacted by the dose treatment.

Animals in each treated group were compared with animals in the control group using appropriate multiple comparison procedures. The Dunnett's (1955) test was used for all endpoints that were analyzed with an ANCOVA model or an ANOVA models in which the assumption of homogeneity of variance was not rejected. When the Levene's test was rejected at the 0.1 level, Dunn's test (nonparametric) for multiple comparisons with a control was used. For number of cycling females and number of females with regular cycles, multiple comparisons with the control were performed using an unadjusted chi-square test.

RECORD RETENTION

All original study records, including all original data sheets, and original final report will be archived by the TherImmune. Preserved tissues, blocks, slides, and paper data associated with histological processing will be maintained at the Archive facility at PAI. Copies of the final report and computer printouts generated in the statistical analysis will be forwarded to the contracting agency, the N.I.E.H.S., Research Triangle Park, North Carolina. Study data generated by the Sponsor will be archived by the Sponsor. Data generated by analytical subcontractors will be archived by the subcontractors.

RESULTS

DOSE FORMULATION RESULTS

Bulk Test Article

The results of bulk test article analyses are presented in Appendix 1 and summarized in Text Table 6.

Text Table 6: Bulk Test Article Analyses

Test Article	Average Purity (%)
Vinclozolin	100.1
Flutamide	99.3
Phenobarbital	99.4
Methoxychlor	99.2
Ethinyl Estradiol	98.8

FORMULATION ANALYSES

The results of formulation analyses are presented in Appendix 2.

All analyses of Flutamide and Methoxychlor formulations were within $\pm 10\%$ of the target concentrations. Out of specification results are presented in Text Table 7.

Text Table 7: Formulation Analyses

Test Article	Target Concentration (mg/mL)	Mix 1	Mix 2	Mix 3	Mix 4
Vinclozolin	4	126.6%	-	-	-
	12	113.2	-	-	-
Phenobarbital	10	-	-	87.0%	-
	20	-	82.8%	-	-
	40	-	-	80.3	77.2
Ethinyl Estradiol	0.001	< LOQ	350.0%	-	183.8
	0.002	45.0%	332.5%	111.2%	112.1%

LOQ = Limit Of Quantification; - = Within Specifications or Analysis Not Required.

The high and low concentrations of all dose formulations were homogeneous with the exception of Ethinyl Estradiol. Ethinyl estradiol analysis was a problem throughout the study. Results varied from below the limit of quantification to 350% of target. As the same mixing procedure was used each time and the target concentrations were very close to the limits of detection of assay, in spite of the variable results we believe the test article was properly formulated.

F₀ GENERATION RESULTS

Of the 55 F₀ females received for this study, 53 of them had viable litters, one was used for serological testing, and one did not litter. Littering took place over a two-day period, as follows: 18 litters were delivered on November 20, 2002 and 35 litters were delivered on November 21, 2002. The dam that did not litter plus the dams that delivered on November 20, 2002 and their litters were terminated on PND 5. Dams littering on November 21, 2002 were allowed to raise their litters to PND 21. On PND 4, these litters were culled to 10 pups/litter, with an equal number of males and females wherever possible. On PND 21, 135 male and 135 female weanlings were selected for the F₁ generation study. F₀ dams were terminated on December 12, 2002. Data recorded during the lactation period are presented in Appendices 4-6.

F₁ GENERATION RESULTS

All findings described in this report as "increased" or "decreased" were statistically significant as compared to the control group.

MORTALITY AND CLINICAL OBSERVATIONS

Data are presented in Table 1 and Appendix 7.

Treatment with Vinclozolin, Flutamide, Methoxychlor or Ethinyl Estradiol had no effect on mortality or clinical observations.

One 100 mg/kg/day Phenobarbital-treated male was found dead on PND 24 (the second day of treatment). Treatment with Phenobarbital affected clinical observations in the 100 mg/kg/day

dose group males and females for the first few days of treatment. Minimal to severe ataxia was recorded between PND 22-23 to PND 26 in all males and females, prostration was noted in 3 males and 8 females on PND 23-24, and languid behavior was noted in one male on PND 24.

BODY WEIGHT AND BODY WEIGHT GAINS

Data are presented in Tables 2 and 3 and in Appendix 8.

Males

Male body weight data are presented in Figures 1-3.

Treatment with 100 mg/kg/day Phenobarbital affected male body weights. Although males gained weight daily, body weight gains were lower than controls resulting in significantly lower mean body weights on SD 32, 34, 37, 38, and 40-54 (11.3% lower than control on PND 54).

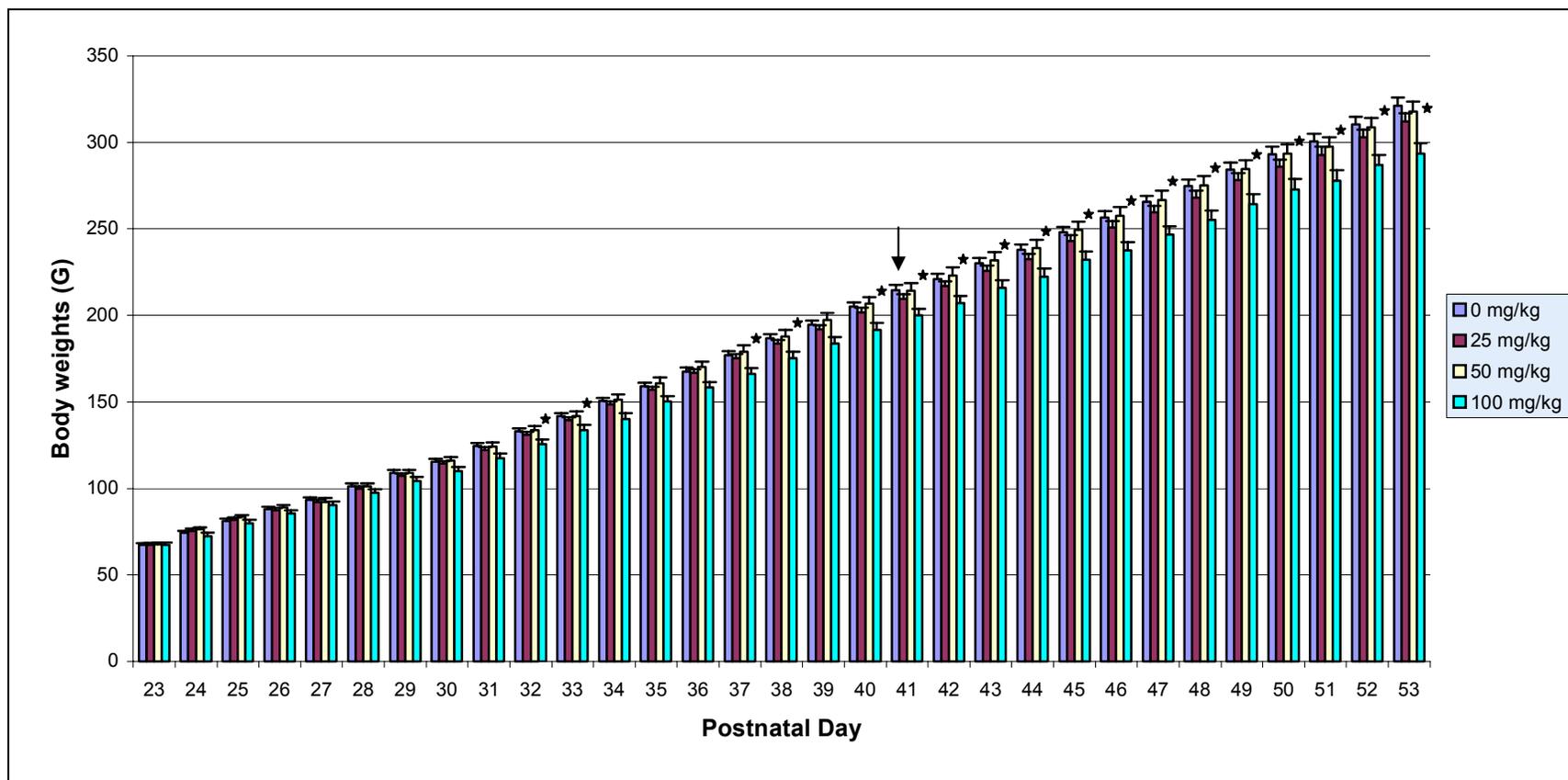
Treatment with Vinclozolin, Flutamide, 25 mg/kg/day Phenobarbital, or 50 mg/kg/day Phenobarbital had no effect on mean body weight. There were significant differences in mean body weight gain among groups, but not consistent enough in magnitude or direction to affect mean body weights and not considered to be test-article related.

Females

Female body weight data are presented in Figures 4-6.

Treatment with Ethinyl Estradiol, Methoxychlor, or Phenobarbital had no effect on female body weights. No consistent or meaningful changes were observed in the body weight gains of the treated females as compared to the control females.

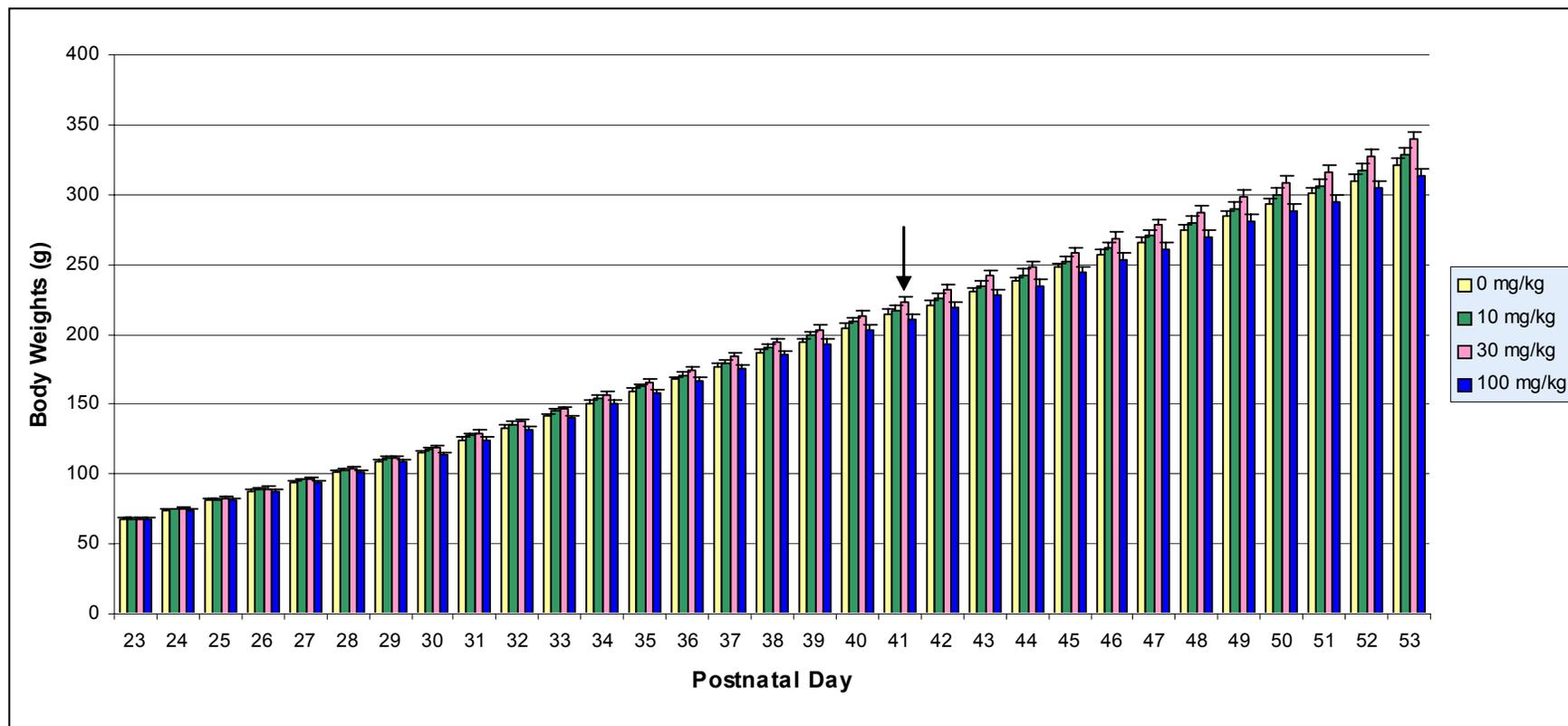
Figure 1: Phenobarbital – Male Body Weights



↓ = Mean age of control animals at preputial separation.

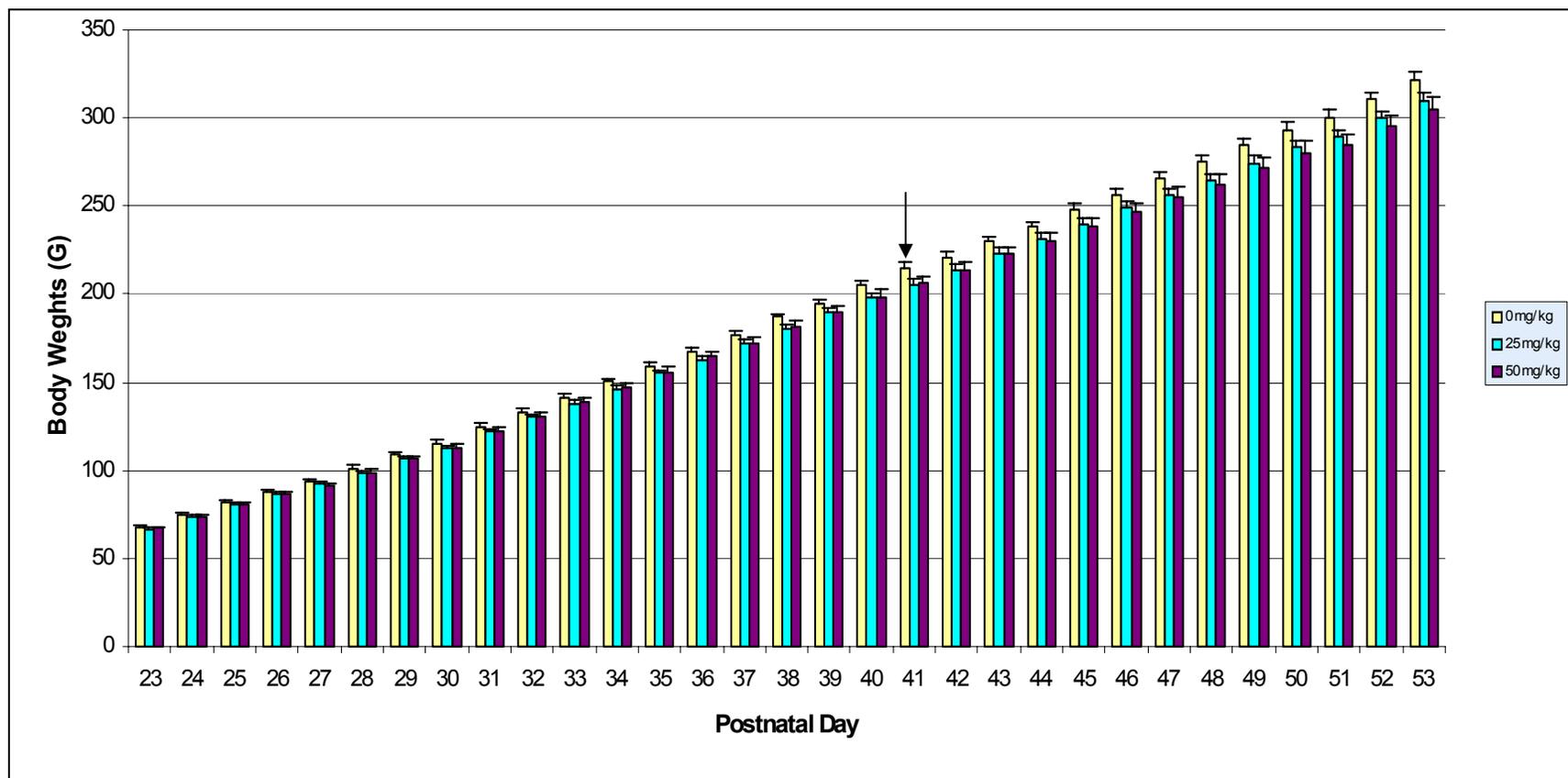
* Statistical Significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) using Dunnett's Test

Figure 2: Vinclozolin – Male Body Weights



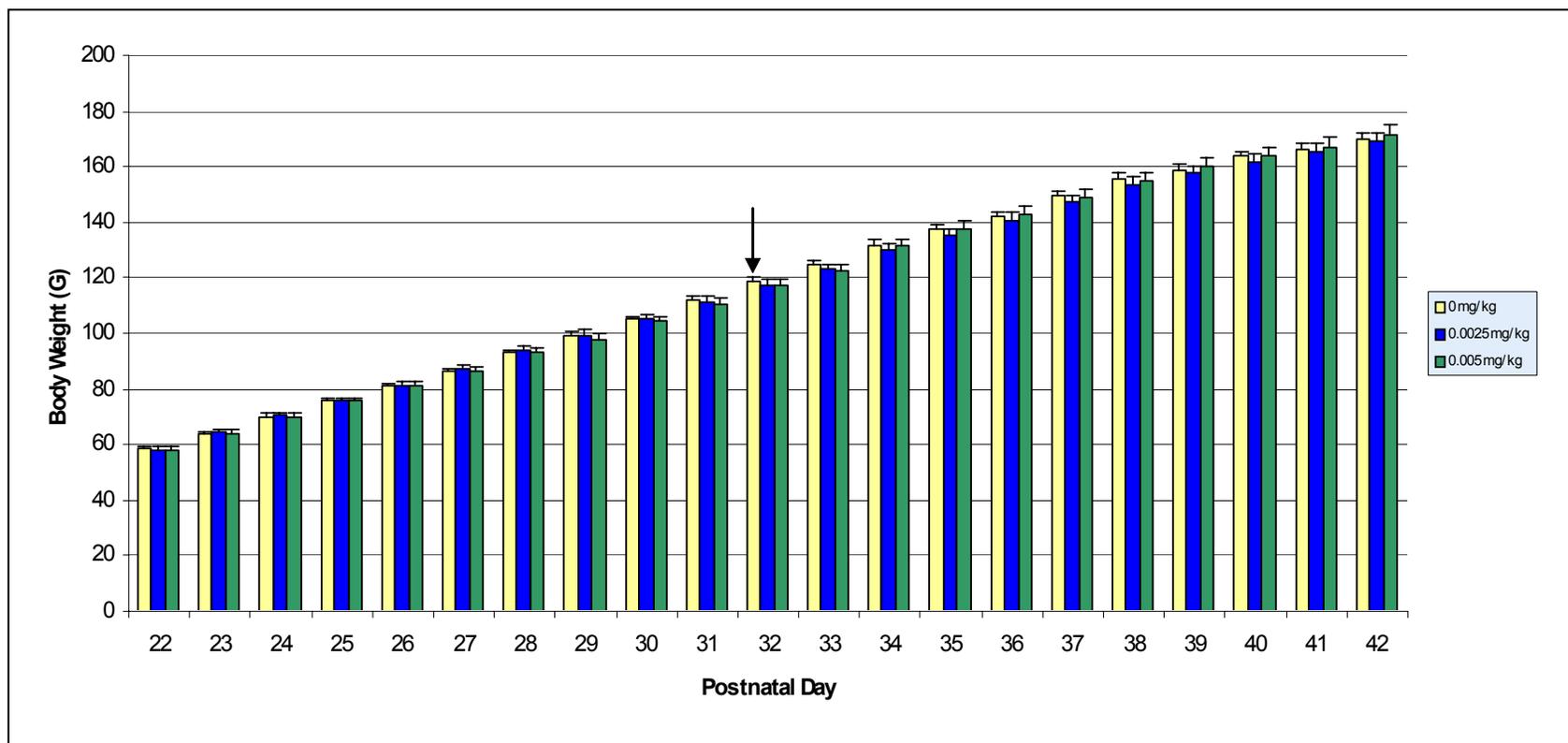
↓ = Mean age of control animals at preputial separation.

Figure 3: Flutamide – Male Body Weights



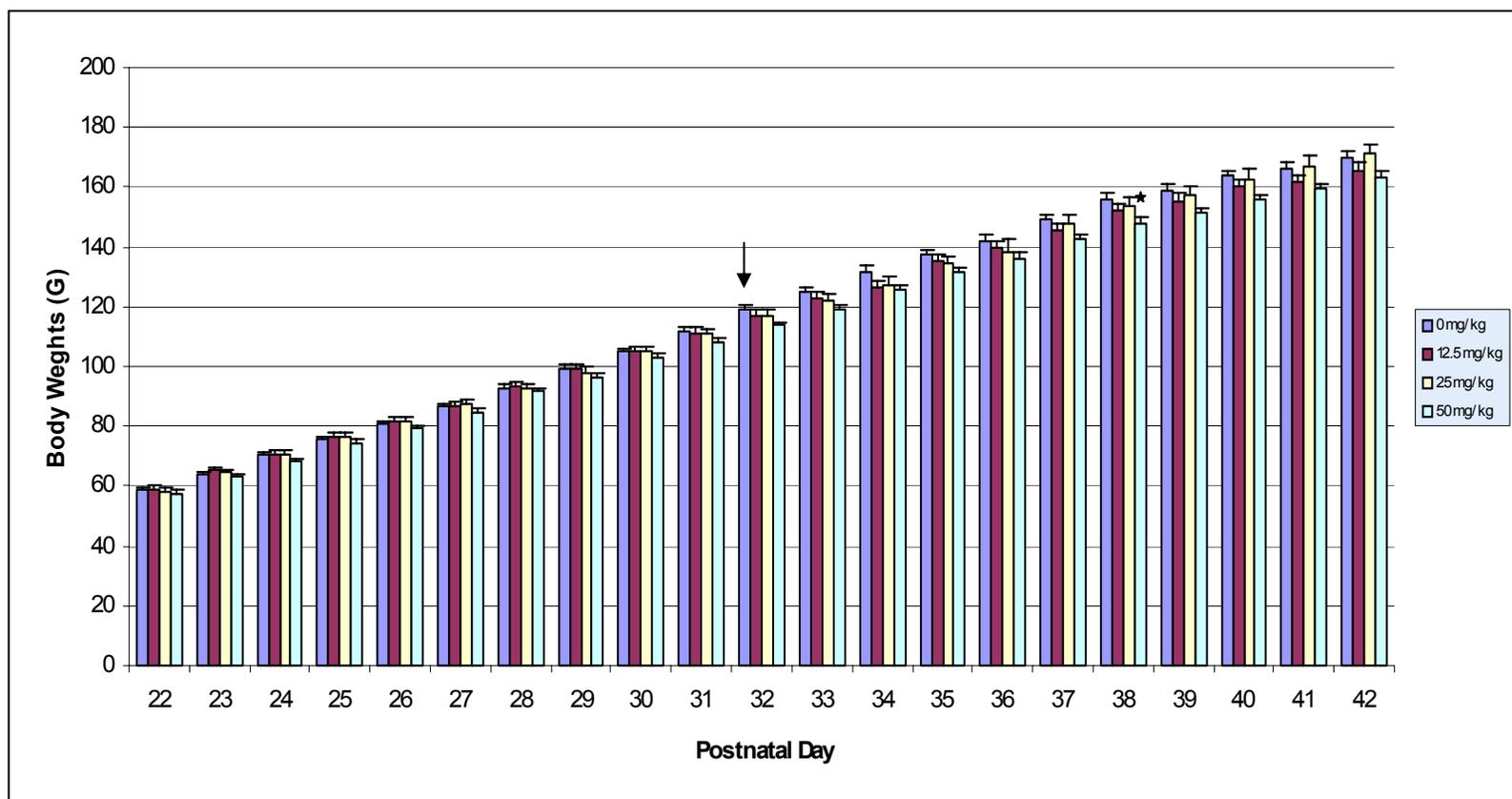
↓ = Mean age of control animals at preputial separation.

Figure 4: Ethinyl Estradiol – Female Body Weights



↓ = Mean age of control animals at preputial separation.

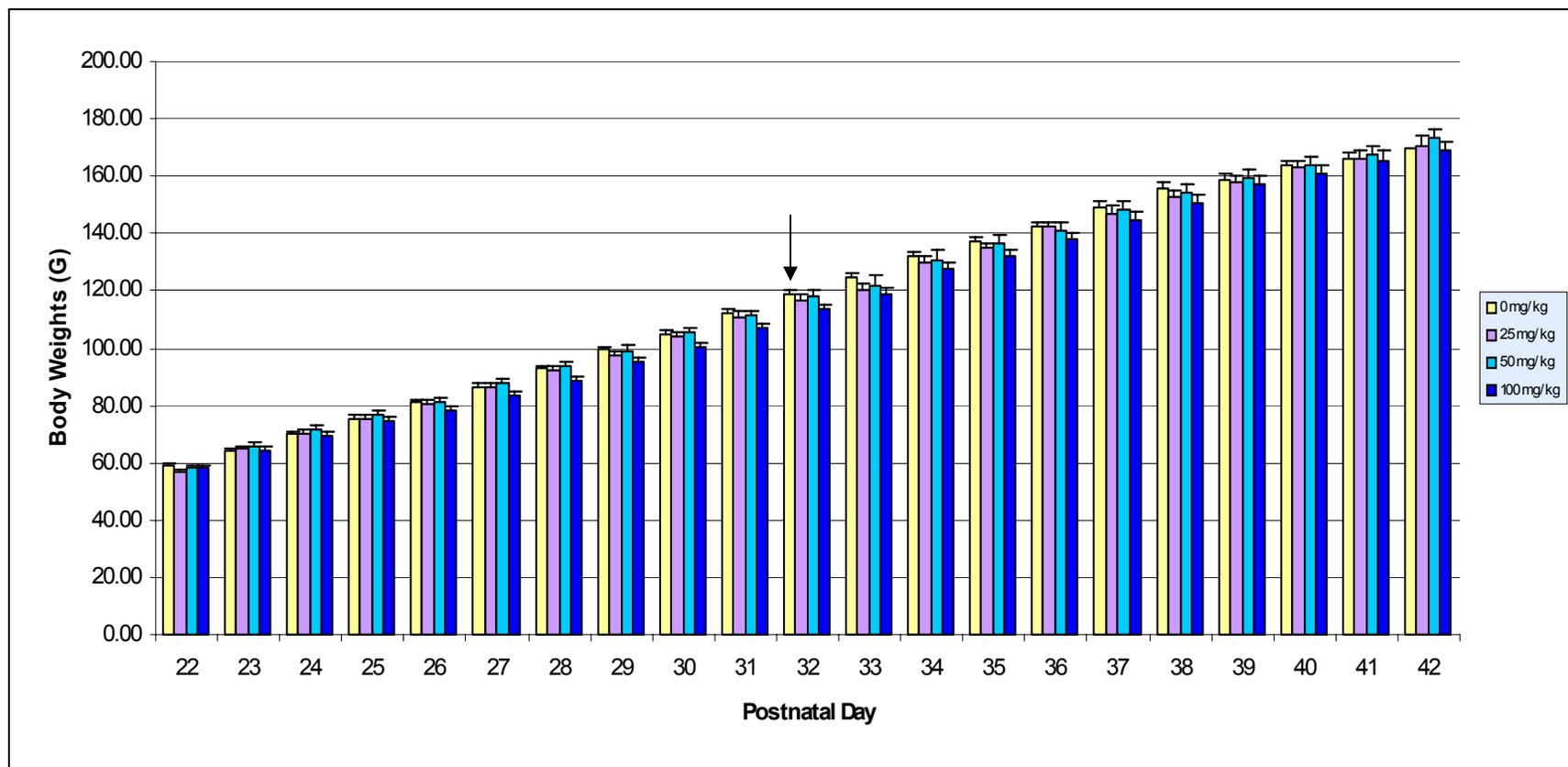
Figure 5: Methoxychlor – Female Body Weights



↓ = Mean age of control animals at preputial separation.

★ Statistical Significance for comparison of dosed groups to control (* = $P < 0.05$, ** = $P < 0.01$) using Dunnett's Test

Figure 6: Phenobarbital – Female Body Weights



↓ = Mean age of control animals at preputial separation.

PREPUTIAL SEPARATION

Data are presented in Table 4 and Appendix 9.

The mean day and weight at preputial separation (PPS) were PND 41.1 and 213.63 g, respectively, in control males. For statistical purposes, males that did not exhibit PPS by the time of termination were assigned PND 54 as the day of PPS.

Age at PPS was affected by treatment with all three test articles. Phenobarbital treatment showed the least effect, with only the highest dose, 100 mg/kg/day, being significantly different than control (PND 43.6). The age of PPS increased with increasing dose of Vinclozolin - PND 42.6, 43.9, and 47.7 for 10, 30, and 100 mg/kg/day males, respectively. Flutamide treatment had a dramatic effect on PPS, with only six 50 mg/kg/day and one 100 mg/kg/day males achieving PPS prior to termination on PND 54. Mean body weight at PPS was increased in the Vinclozolin and Flutamide-treated males in direct relationship to their increased age. Comparison of body weight gains and pubertal development in males is given in Text Table 8.

Text Table 8: Comparison of Body Weight Gain and Pubertal Development in Males

Parameter	Phenobarbital (mg/kg/day)			
	0	25	50	100
Age at preputial separation (days)	41.1 ± 0.38	41.5 ± 0.36	41.1 ± 0.39	**43.6 (14) ± 0.54 ^a
Body weight at preputial separation (g)	213.63 ± 4.522	213.44 ± 3.609	215.78 ± 3.748	219.81 (14) ± 4.903
Body weight at PND 23 (g)	67.41 ± 0.995	67.32 ± 1.151	67.84 ± 0.998	67.53 ± 1.116
Body weight at PND 53 (g)	320.99 ± 5.006	312.19 ± 4.734	317.86 ± 5.656	**293.56 (14) ± 5.790 ^a
Body weight as % of control at PND 53 (%)	.	97.26	99.02	91.45
Body weight gain PND 23-53 (g)	253.6 ± 4.799	245.3 ± 5.106	243.1 ± 4.295	238.2 ± 6.191

Mean ± SE (n = 15 unless specified in parenthesis by the mean)

^aSignificant treatment effect by ANOVA and statistical significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

Parameter	Vinclozolin (mg/kg/day)			
	0	10	30	100
Age at preputial separation (days)	41.1 ± 0.376	* 42.6 ± 0.434 ^a	**43.9 ± 0.336 ^a	**47.7 ± 0.319 ^a
Body weight at preputial separation (g)	213.63 ± 4.522	231.29 ± 5.120	**248.59 ± 4.902 ^a	**267.83 ± 6.046 ^a
Body weight at PND 23 (g)	67.41 ± 0.995	67.65 ± 0.812	68.08 ± 1.132	67.91 ± 1.020
Body weight at PND 53 (g)	320.99 ± 5.006	328.37 ± 5.673	339.25 ± 5.247	313.17 ± 5.850
Body weight as % of control at PND 53 (%)	.	102.30	105.69	97.56
Body weight gain PND 23-53 (g)	253.6 ± 4.799	244.9 ± 4.374	250.0 ± 5.208	226.2 ± 4.904

Mean ± SE (n = 15 unless specified in parenthesis by the mean)

^aSignificant treatment effect by ANOVA and statistical significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

Text Table 8: Comparison of Body Weight Gain and Pubertal Development in Males (continued)

Parameter	Flutamide (mg/kg/day)		
	0	25	50
Age at preputial separation (days)	41.1 ± 0.38	**53.3 (6) ± 0.33 ^a	**54.0 (1) ± 0.0 ^a
Body weight at preputial separation (g)	213.63 ± 4.522	**318.57 (6) ± 8.237 ^a	**311.00 (1) ± 0.0 ^a
Body weight at PND 23 (g)	67.41 ± 0.995	66.93 ± 0.850	67.06 ± 1.054
Body weight at PND 53 (g)	320.99 ± 5.006	310.03 ± 4.332	305.21 ± 6.811
Body weight as % of control at PND 53 (%)	.	96.59	95.08
Body weight gain PND 23-53 (g)	253.6 ± 4.799	260.7 ± 5.543	271.2 ± 5.020

Mean ± SE (n = 15 unless specified in parenthesis by the mean)

^aSignificant treatment effect by ANOVA and statistical significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

VAGINAL OPENING

Data are presented in Table 5 and Appendix 10.

The mean day and weight at vaginal opening (VO) were PND 31.9 and 117.58 g, respectively, in control females. All three test articles affected the age at VO. Ethinyl Estradiol advanced the age at VO to PND 28.4 at 0.005 mg/kg/day. Methoxychlor advanced the age at VO in a dose-related fashion to PND 27.9, 27.0 and 26.5 in 12.5, 25, and 50 mg/kg/day females, respectively. The highest dose of Phenobarbital, 100 mg/kg/day, delayed VO to PND 34.5. Body weight at VO was decreased in Ethinyl Estradiol and Methoxychlor-treated females in relationship to their decreased age, but not statistically different in Phenobarbital-treated females. Comparison of body weight gains and pubertal development in females is given in Text Table 9.

Text Table 9: Comparison of Body Weight Gain and Pubertal Development in Females

Parameter	Methoxychlor (mg/kg/day)			
	0	12.5	25	50
Age at vaginal opening (days)	31.9 ± 0.322	**27.9 ± 0.215 ^a	**27.0 ± 0.195 ^a	**26.5 ± 0.13 ^a
Body weight at vaginal opening (g)	117.58 ± 2.482	**92.41 ± 2.255 ^a	**87.48 ± 2.214 ^a	**82.02 ± 1.217 ^a
Body weight at PND 22 (g)	58.87 ± 0.755	58.97 ± 1.003	58.32 ± 1.151	57.47 ± 0.998
Body weight at PND 42 (g)	169.83 ± 2.002	165.79 ± 2.330	171.03 ± 3.441	163.54 ± 1.823
Body weight as % of control at PND 42 (%)	.	97.62	100.71	96.30
Body weight gain PND 22-42 (g)	111.0 ± 1.769	110.8 ± 2.355	113.5 ± 3.080	106.8 ± 1.717

Mean ± SE (n = 15 unless specified in parenthesis by the mean)

^aSignificant treatment effect by ANOVA and statistical significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

Parameter	Ethinyl Estradiol (mg/kg/day)		
	0	0.0025	0.005
Age at vaginal opening (days)	31.9 ± 0.32	31.1 ± 0.63	**28.4 ± 0.22 ^a
Body weight at vaginal opening (g)	117.58 ± 2.482	112.05 ± 3.967	**95.20 ± 1.797 ^a
Body weight at PND 22 (g)	58.87 ± 0.755	58.17 ± 0.884	58.03 ± 0.999
Body weight at PND 42 (g)	169.83 ± 2.002	169.01 ± 2.808	171.55 ± 3.728
Body weight as % of control at PND 42 (%)	.	99.52	101.01
Body weight gain PND 22-42 (g)	111.0 ± 1.769	112.7 ± 2.576	106.1 ± 1.500

Mean ± SE (n = 15 unless specified in parenthesis by the mean)

^aSignificant treatment effect by ANOVA and statistical significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

Text Table 9: Comparison of Body Weight Gain and Pubertal Development in Females (continued)

Parameter	Phenobarbital (mg/kg/day)			
	0	25	50	100
Age at vaginal opening (days)	31.9 ± 0.32	33.1 ± 0.42	32.8 ± 0.53	**34.5 ± 0.67 ^a
Body weight at vaginal opening (g)	117.58 ± 2.482	122.63 ± 3.311	120.67 ± 4.548	128.04 ± 5.107
Body weight at PND 22 (g)	58.87 ± 0.755	56.70 ± 0.769	57.96 ± 1.070	58.14 ± 0.966
Body weight at PND 42 (g)	169.83 ± 2.002	170.39 ± 3.703	173.11 ± 3.357	169.15 ± 3.128
Body weight as % of control at PND 42 (%)	.	100.33	101.93	99.60
Body weight gain PND 22-42 (g)	111.0 ± 1.769	113.7 ± 3.234	115.1 ± 2.725	111.0 ± 2.786

Mean ± SE (n = 15 unless specified in parenthesis by the mean)

^aSignificant treatment effect by ANOVA and statistical significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

ESTROUS CYCLICITY

Data are presented in Table 6 and Appendix 11.

The mean age at first estrus, cycle length, percentage cycling, and percentage cycling normally are shown in Text Table 10. Treatment with Ethinyl Estradiol and Methoxychlor affected age at first estrus, cycle length, and/or the percentage of regular cycles. Although 100 mg/kg/day Phenobarbital-treatment appears to affect the percentage of females cycling, this is due to 3 females (9287, 9290, 9292) with unclear cycles. If these females are excluded from evaluation, 92% of females in this group were cycling.

Text Table 10: Estrous Cyclicity Data

Test Article	Dosage (mg/kg/day)	Age at First Estrus (PND)	Cycle Length (days)	Cycling (%)	Regular Cycles (%)
Vehicle	0	33.4	4.9	100	80
Ethinyl Estradiol	0.0025	32.5	5.1	93	67
	0.005	28.7*	5.1	100	20**
Methoxychlor	12.5	30.9*	5.2	100	80
	25	30.7*	6.2*	100	27**
	50	28.6*	5.9*	100	20**
Phenobarbital	25	35.6	4.9	100	80
	50	34.0	5.0	93	80
	100	34.5	5.3	73*	67

* = statistically significant $p \leq .05$, ** = statistically significant $p \leq .01$

GROSS PATHOLOGY

Data are presented in Table 7 and Appendix 12.

Males

Treatment with Flutamide produced size reductions in the seminal vesicles, coagulating glands, ventral prostate, dorsolateral prostate, epididymides, and/or levator ani and bulbocavernosus muscles and an increase in testis size. The incidence of these findings was confined to or slightly higher in the 50 mg/kg/day males.

Treatment with Vinclozolin resulted in a higher incidence of kidney dilation: 6/15 males in the 100 mg/kg/day group, 4/15 males in the 30 mg/kg/day group and 3/15 males in the 10 mg/kg/day group.

Treatment with Phenobarbital had no effect on gross pathology.

Females

There were no gross observations in females that were considered to be related to treatment.

ORGAN WEIGHTS

Data are presented in Table 8 and in Appendix 13.

Males

Body weight and organ weights at necropsy are presented in Text Table 11.

Phenobarbital treatment resulted in a dose-related higher liver weights at all doses and lower pituitary weight at 100 mg/kg/day. Observed differences in kidney and ventral prostate weights at 100 mg/kg/day are most likely due to lower body weight and delayed age of sexual maturation and not a direct test-article effect.

Vinclozolin treatment resulted in lower weights of seminal vesicles, prostate, and levator ani plus bulbocavernosus muscles, and increased adrenal weight at 100 mg/kg/day. Testis weight was higher than control for 30 and 100 mg/kg/day males.

Flutamide treatment also resulted in lower weights of testosterone-sensitive tissues including seminal vesicles, prostate, epididymides, and levator ani plus bulbocavernosus muscles, while testis weights were higher at 25 and 50 mg/kg/day. Liver weights were higher and kidney weights were lower at 25 and 50 mg/kg/day. Adrenal weight was higher than control in 50 mg/kg/day Flutamide-treated males.

Text Table 11: Body Weight and Organ Weights at Necropsy - Males (continued)

Parameter	Phenobarbital (mg/kg/day)			
	0	25	50	100
Terminal Body Weight (g)	324.18 ± 5.027	315.81 ± 4.780	322.71 ± 5.789	**298.27 (14) ± 5.153 ^b
Adrenal Glands, Paired (g)	0.04927 ± .002135	0.05323 ± .002243	0.04787 ± .002981	0.05256 (14) ± .002905
Adrenal Glands, Paired (g) (ANCOVA mean) ^a	0.04893 ± .002643	0.05322 ± .002578	0.04759 ± .002623	0.05323 (14) ± .002911
Dorsolateral Prostate (g)	0.26575 ± .011858	0.22301 ± .014107	0.25227 ± .021030	0.21192 (13) ± .013477
Dorsolateral Prostate (g) (ANCOVA mean) ^a	0.26272 ± .015833	0.22295 ± .015451	0.24976 ± .015714	0.21839 (13) ± .018162
Epididymides, Paired (g)	0.52313 ± .016801	0.52643 ± .023714	0.53801 ± .020924	0.50856 (14) ± .025601
Epididymides, Paired (g) (ANCOVA mean) ^a	0.52205 ± .022445	0.52639 ± .021894	0.53711 ± .022276	0.51073 (14) ± .024719
Kidneys, Paired (g)	3.11392 ± .048012	2.90428 ± .061123	3.06683 ± .065461	**2.81193 (14) ± .083957 ^b
Kidneys, Paired (g) (ANCOVA mean) ^a	3.04396 ± .051112	2.90201 ± .049856	3.00874 ± .050725	2.95157 (14) ± .056290
Levator Ani Plus Bulbocavernosus Muscles (g)	0.59862 ± .035631	0.63485 ± .043294	0.58468 ± .043846	0.58501 (14) ± .028037
Levator Ani Plus Bulbocavernosus Muscles (g) (ANCOVA mean) ^a	0.59001 ± .039217	0.63457 ± .038254	0.57753 ± .038920	0.60219 (14) ± .043190
Liver (g)	16.61980 ± .414274	18.13560 ± .496956	**19.59220 ± .585405 ^b	**19.8170 (14) ± .677245 ^b
Liver (g) (ANCOVA mean) ^a	15.91920 ± .358643	**18.1128 ± .349834 ^c	**19.0105 ± .355929 ^c	**21.21540 (14) ± .394979 ^c
Pituitary (g)	0.01041 ± .000365	0.00955 ± .000461	0.01003 ± .000418	* 0.00873 (14) ± .000331 ^b
Pituitary (g) (ANCOVA mean) ^a	0.01042 ± .000409	0.00955 ± .000399	0.01003 ± .000406	* 0.00871 (14) ± .000451 ^c
Right Epididymis (g)	0.25189 ± .007466	0.25883 ± .014121	0.26270 ± .013686	0.25520 (14) ± .015511
Right Epididymis (g) (ANCOVA mean) ^a	0.25228 ± .013316	0.25884 ± .012989	0.26303 ± .013216	0.25441 (14) ± .014665

Mean ± SE (n = 15 unless specified in parenthesis by the mean)

^aMean ± SE has been adjusted for body weight at necropsy using ANCOVA.

^bSignificant treatment effect by ANOVA and statistical significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

^cSignificant treatment effect by ANCOVA and statistical significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

Text Table 11: Body Weight and Organ Weights at Necropsy - Males (continued)

Parameter	Phenobarbital (mg/kg/day)			
	0	25	50	100
Seminal Vesicles and Coagulating glands, with fluid(g)	0.69805 ± .045150	0.77617 ± .035129	0.68632 ± .059840	0.58574 (14) ± .038317
Seminal Vesicles and Coagulating glands, with fluid (g) (ANCOVA mean) ^a	0.68813 ± .046656	0.77584 ± .045510	0.67809 ± .046303	0.60553 (14) ± .051383
Seminal Vesicles and Coagulating glands, without fluid (g)	0.41088 ± .025802	0.45304 ± .023274	0.39272 ± .027148	0.36340 (14) ± .029119
Seminal Vesicles and Coagulating glands, without fluid (g) (ANCOVA mean) ^a	0.40358 ± .026642	0.45280 ± .025988	0.38666 ± .026440	0.37796 (14) ± .029341
Testes, Paired (Grams)	2.70352 ± .041039	2.74459 ± .034174	2.81499 ± .060105	2.65386 (14) ± .072035
Testes, Paired (g) (ANCOVA mean) ^a	2.65840 ± .047018	2.74313 ± .045863	2.77752 ± .046662	2.74393 (14) ± .051782
Thyroid/Parathyroid, Post Fixation (grams)	0.02165 ± .001256	0.02045 ± .000869	0.02189 ± .000794	0.02323 (14) ± .001309
Thyroid/Parathyroid, Post Fixation (g) (ANCOVA mean) ^a	0.02160 ± .001101	0.02045 ± .001074	0.02185 ± .001092	0.02332 (14) ± .001212
Ventral Prostate (Grams)	0.23843 ± .013510	0.19983 ± .013573	0.21057 ± .013437	**0.17921 (14) ± .011430 ^b
Ventral Prostate (g) (ANCOVA mean) ^a	0.23116 ± .012642	0.19959 ± .012332	0.20454 ± .012547	0.19372 (14) ± .013923

Mean ± SE (n = 15 unless specified in parenthesis by the mean)

^aMean ± SE has been adjusted for body weight at necropsy using ANCOVA.

^bSignificant treatment effect by ANOVA and statistical significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

^cSignificant treatment effect by ANCOVA and statistical significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

Text Table 11: Body Weight and Organ Weights at Necropsy - Males

Parameter	Vinclozolin (mg/kg/day)			
	0	10	30	100
Terminal Body Weight (g)	324.18 ± 5.027	332.56 ± 6.112	* 343.99 ± 5.750 ^b	317.08 ± 5.450
Adrenal Glands, Paired (g)	0.04927 ± .002135	0.05312 ± .002528	0.05177 ± .002297	0.05627 ± .001981
Adrenal Glands, Paired (g) (ANCOVA mean) ^a	0.04986 ± .002191	0.05277 ± .002180	0.05014 ± .002301	* 0.05766 ± .002267 ^c
Dorsolateral Prostate (g)	0.26575 ± .011858	0.24932 ± .014290	0.24642 ± .008573	**0.18895 ± .007196 ^b
Dorsolateral Prostate (g) (ANCOVA mean) ^a	0.26652 ± .010994	0.24886 ± .010937	0.24429 ± .011545	**0.19076 ± .011373 ^c
Epididymides, Paired (g)	0.52313 ± .016801	0.58333 ± .020179	0.51642 ± .023740	0.45851 ± .015936
Epididymides, Paired (g) (ANCOVA mean) ^a	0.52685 ± .019328	0.58114 ± .019230	0.50618 ± .020299	0.46723 ± .019996
Kidneys, Paired (g)	3.11392 ± .048012	3.02505 ± .069358	3.14487 ± .055835	2.95730 ± .042097
Kidneys, Paired (g) (ANCOVA mean) ^a	3.14712 ± .042640	3.00548 ± .042423	3.05332 ± .044780	3.03522 ± .044113
Levator Ani Plus Bulbocavernosus Muscles (g)	0.59862 ± .035631	0.62068 ± .032690	0.62008 ± .022220	**0.45897 ± .037204 ^b
Levator Ani Plus Bulbocavernosus Muscles (g) (ANCOVA mean) ^a	0.59770 ± .033003	0.62122 ± .032835	0.62260 ± .034659	* 0.45682 ± .034143 ^c
Liver (g)	16.61980 ± .414274	17.08590 ± .523925	18.07300 ± .457382	16.38420 ± .538874
Liver (g) (ANCOVA mean) ^a	17.00750 ± .263041	16.85730 ± .261701	17.00400 ± .276244	17.29410 ± .272125
Pituitary (g)	0.01041 ± .000365	0.01016 ± .000410	0.01021 ± .000290	0.00947 ± .000414
Pituitary (g) (ANCOVA mean) ^a	0.01047 ± .000374	0.01012 ± .000372	0.01004 ± .000393	0.00962 ± .000387
Right Epididymis (g)	0.25189 ± .007466	0.28631 ± .011063	0.25524 ± .012479	0.22400 ± .010342
Right Epididymis (g) (ANCOVA mean) ^a	0.25358 ± .010519	0.28532 ± .010465	0.25058 ± .011047	0.22797 ± .010882

Mean ± SE (n = 15 unless specified in parenthesis by the mean)

^aMean ± SE has been adjusted for body weight at necropsy using ANCOVA.

^bSignificant treatment effect by ANOVA and statistical significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

^cSignificant treatment effect by ANCOVA and statistical significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

Text Table 11: Body Weight and Organ Weights at Necropsy - Males (continued)

Parameter	Vinclozolin (mg/kg/day)			
	0	10	30	100
Seminal Vesicles and Coagulating glands, with fluid(g)	0.69805 ± .045150	0.67235 ± .040422	0.62576 ± .029288	**0.45636 ± .026981 ^b
Seminal Vesicles and Coagulating glands, with fluid (g) (ANCOVA mean) ^a	0.70918 ± .034857	0.66579 ± .034680	0.59506 ± .036607	**0.48249 ± .036061 ^c
Seminal Vesicles and Coagulating glands, without fluid (g)	0.41088 ± .025802	0.40970 ± .020047	0.37417 ± .012722	**0.29803 ± .014735 ^b
Seminal Vesicles and Coagulating glands, without fluid (g) (ANCOVA mean) ^a	0.41642 ± .018390	0.40643 ± .018296	0.35888 ± .019313	**0.31104 ± .019025 ^c
Testes, Paired (g)	2.70352 ± .041039	2.81745 ± .031426	**3.00937 ± .037069 ^b	**2.97171 ± .048928 ^b
Testes, Paired (g) (ANCOVA mean) ^a	2.71865 ± .037394	2.80853 ± .037204	**2.96767 ± .039271 ^c	**3.00721 ± .038686 ^c
Thyroid/Parathyroid, Post Fixation (g)	0.02165 ± .001256	0.01923 ± .000828	0.02189 ± .000845	0.02101 ± .000854
Thyroid/Parathyroid, Post Fixation (g) (ANCOVA mean) ^a	0.02159 ± .000977	0.01926 ± .000972	0.02206 ± .001026	0.02087 ± .001011
Ventral Prostate (g)	0.23843 ± .013510	0.20364 ± .016725	0.21021 ± .013379	* 0.18338 ± .014043 ^b
Ventral Prostate (g) (ANCOVA mean) ^a	0.24041 ± .014567	0.20247 ± .014493	0.20474 ± .015299	* 0.18803 ± .015070 ^c

Mean ± SE (n = 15 unless specified in parenthesis by the mean)

^aMean ± SE has been adjusted for body weight at necropsy using ANCOVA.

^bSignificant treatment effect by ANOVA and statistical significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

^cSignificant treatment effect by ANCOVA and statistical significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

Text Table 11: Body Weight and Organ Weights at Necropsy - Males (continued)

Parameter	Flutamide (mg/kg/day)		
	0	25	50
Terminal Body Weight (g)	324.18 ± 5.027	313.49 ± 4.523	308.69 ± 6.675
Adrenal Glands, Paired (g)	0.04927 ± .002135	0.051800 ± .001562	**0.05916 ± .002285 ^b
Adrenal Glands, Paired (g) (ANCOVA mean) ^a	0.04922 ± .002103	0.05181 ± .002046	**0.05919 ± .002079 ^c
Dorsolateral Prostate (g)	0.26575 ± .011858	**0.12383 ± .007379 ^b	**0.10189 ± .007076 ^b
Dorsolateral Prostate (g) (ANCOVA mean) ^a	0.26297 ± .009243	**0.12445 ± .008990 ^c	**0.10404 ± .009138 ^c
Epididymides, Paired (g)	0.52313 ± .016801	**0.34428 ± .016145 ^b	**0.35045 ± .016320 ^b
Epididymides, Paired (g) (ANCOVA mean) ^a	0.52239 ± .017110	**0.34445 ± .016642 ^c	**0.35102 ± .016915 ^c
Kidneys, Paired (g)	3.11392 ± .048012	**2.71213 ± .052210 ^b	**2.76443 ± .074129 ^b
Kidneys, Paired (g) (ANCOVA mean) ^a	3.05519 ± .048267	**2.72534 ± .046947 ^c	**2.80995 ± .047716 ^c
Levator Ani Plus Bulbocavernosus Muscles (g)	0.59862 ± .035631	**0.38779 ± .018250 ^b	**0.38493 ± .023846 ^b
Levator Ani Plus Bulbocavernosus Muscles (g) (ANCOVA mean) ^a	0.60114 ± .027988	**0.38722 ± .027223 ^c	**0.38297 ± .027669 ^c
Liver (g)	16.61980 ± .414274	16.733900 ± .495116	17.33660 ± .457025
Liver (g) (ANCOVA mean) ^a	16.01600 ± .264420	* 16.86970 ± .257192 ^c	**17.8046 ± .261405 ^c
Pituitary (g)	0.01041 ± .000365	0.011030 ± .000544	0.01101 ± .000498
Pituitary (g) (ANCOVA mean) ^a	0.01036 ± .000494	0.01104 ± .000480	0.01105 ± .000488
Right Epididymis (g)	0.25189 ± .007466	**0.16875 ± .009692 ^b	**0.16479 ± .010204 ^b
Right Epididymis (g) (ANCOVA mean) ^a	0.24974 ± .009481	**0.16924 ± .009222 ^c	**0.16646 ± .009373 ^c

Mean ± SE (n = 15 unless specified in parenthesis by the mean)

^aMean ± SE has been adjusted for body weight at necropsy using ANCOVA.

^bSignificant treatment effect by ANOVA and statistical significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

^cSignificant treatment effect by ANCOVA and statistical significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

Text Table 11: Body Weight and Organ Weights at Necropsy - Males (continued)

Parameter	Flutamide (mg/kg/day)		
	0	25	50
Seminal Vesicles and Coagulating glands, with fluid (g)	0.69800 ± .045150	* 0.17400 ± .017629 ^d	* 0.13667 ± .011083 ^d
Seminal Vesicles and Coagulating glands, with fluid (g) (ANCOVA mean) ^a	0.69253 ± .029697	**0.17607 ± .028885 ^c	**0.13969 ± .029358 ^c
Seminal Vesicles and Coagulating glands, without fluid (g)	0.41088 ± .025802	**0.14489 ± .014215 ^b	**0.11647 ± .009418 ^b
Seminal Vesicles and Coagulating glands, without fluid (g) (ANCOVA mean) ^a	0.40815 ± .018522	**0.14551 ± .018016 ^c	**0.11859 ± .018311 ^c
Testes, Paired (g)	2.70267 ± .041039	* 2.99933 ± .053884 ^d	* 3.49533 ± .130636 ^d
Testes, Paired (g) (ANCOVA mean) ^a	2.67219 ± .086126	* 3.00533 ± .083772 ^c	**3.51951 ± .085144 ^c
Thyroid/Parathyroid, Post Fixation (g)	0.02165 ± .001256	0.021150 ± .001077	0.02037 ± .001162
Thyroid/Parathyroid, Post Fixation (g) (ANCOVA mean) ^a	0.02138 ± .001204	0.02121 ± .001171	0.02058 ± .001190
Ventral Prostate (g)	0.23843 ± .013510	**0.10616 ± .006672 ^b	**0.08347 ± .009508 ^b
Ventral Prostate (g) (ANCOVA mean) ^a	0.23502 ± .010484	**0.10693 ± .010198 ^c	**0.08612 ± .010365 ^c

Mean ± SE (n = 15 unless specified in parenthesis by the mean)

^aMean ± SE has been adjusted for body weight at necropsy using ANCOVA.

^bSignificant treatment effect by ANOVA and statistical significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

^cSignificant treatment effect by ANCOVA and statistical significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

^dSignificant treatment effect by ANOVA and statistical significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunn's Test.

Dunn's Test was used for pair-wise comparisons because HOV test failed.

Females

Body weight and organ weights at necropsy are presented in Text Table 12.

The only significant effect of Ethinyl Estradiol treatment was mean adrenal gland weight that was higher than control means at both 0.0025 and 0.005 mg/kg/day.

Affects of Methoxychlor on adrenal gland and kidney weights were not dose related and not considered to be a treatment effect. Liver, ovary, and pituitary weights were lower than control in 50 mg/kg/day treated females.

Phenobarbital treatment resulted in a dose-related higher liver weights and higher adrenal gland weights at 25, 50 and 100 mg/kg/day. Thyroid weight was higher than control in 100 mg/kg/day females.

Text Table 12: Body Weight and Organ Weights at Necropsy - Females (continued)

Parameter	Ethinyl Estradiol (mg/kg/day)		
	0	0.0025	0.005
Endocrine Status^d			
Diestrus	7	5	4
Proestrus	1	2	2
Estrus	5	7	5
Metestrus	1	1	4
Not cycling	0	1	0
Terminal Body Weight (g)	171.10 ± 1.953	169.91 ± 2.541	173.24 ± 3.358
Adrenal Glands, Paired (g)	0.04136 ± 0.001746	**0.0501 ± 0.002059 ^b	**0.0487 ± 0.001271 ^b
Adrenal Glands, Paired (g) (ANCOVA mean)^a	0.04133 ± .001728	**0.04999 ± .001734 ^c	**0.04887 ± .001737 ^c
Kidneys, Paired (g)	1.72900 ± 0.028952	1.71570 ± 0.035149	1.69916 ± 0.029806
Kidneys, Paired (g) (ANCOVA mean)^a	1.73114 ± .026057	1.72582 ± .026151	1.68691 ± .026196
Liver (g)	8.66695 ± 0.146905	8.41119 ± 0.162899	8.75587 ± 0.158135
Liver (g) (ANCOVA mean)^a	8.68006 ± .111601	8.47325 ± .112000	8.68070 ± .112194
Ovaries, Paired (g)	0.09299 ± 0.004155	0.09435 ± 0.004259	0.09869 ± 0.004581
Ovaries, Paired (g) (ANCOVA mean)^a	0.09297 ± .004386	0.09427 ± .004402	0.09879 ± .004410
Pituitary (g)	0.00861 ± 0.000529	0.00816 ± 0.000564	0.00934 ± 0.000453
Pituitary (g) (ANCOVA mean)^a	0.00863 ± .000495	0.00826 ± .000497	0.00922 ± .000498

Mean ± SE (n = 15 unless specified in parenthesis by the mean)

^aMean ± SE has been adjusted for body weight at necropsy using ANCOVA.

^bSignificant treatment effect by ANOVA and Statistical Significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

^cSignificant treatment effect by ANCOVA and Statistical Significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

^dNumber of females in each stage of the estrous cycle at necropsy, as characterized by vaginal cytology.

Text Table 12: Body Weight and Organ Weights at Necropsy - Females (continued)

Parameter	Ethinyl Estradiol (mg/kg/day)		
	0	0.0025	0.005
Thyroid/Parathyroid, Post Fixation (g)	0.01649 ± 0.001048	0.01687 ± 0.000759	0.01679 ± 0.001072
Thyroid/Parathyroid, Post Fixation (g) (ANCOVA mean) ^a	0.01653 ± .000942	0.01703 ± .000946	0.01660 ± .000947
Uterus and Cervix without Fluid (g)	0.35533 ± 0.027628	0.38714 ± 0.023646	0.36026 ± 0.020087
Uterus and Cervix without Fluid (g) (ANCOVA mean) ^a	0.35545 ± .024258	0.38771 ± .024345	0.35956 ± .024387
Uterus and Cervix, with Fluid (g)	0.41943 ± 0.049172	0.43567 ± 0.037224	0.39721 ± 0.033864
Uterus and Cervix, with Fluid (g) (ANCOVA mean) ^a	0.41953 ± .041110	0.43617 ± .041258	0.39660 ± .041329

Mean ± SE (n = 15 unless specified in parenthesis by the mean)

^aMean ± SE has been adjusted for body weight at necropsy using ANCOVA.

^bSignificant treatment effect by ANOVA and Statistical Significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

^cSignificant treatment effect by ANCOVA and Statistical Significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

^dNumber of females in each stage of the estrous cycle at necropsy, as characterized by vaginal cytology.

Text Table 12: Body Weight and Organ Weights at Necropsy - Females

Parameter	Methoxychlor (mg/kg/day)			
	0	12.5	25	50
Endocrine Status^d				
Diestrus	7	5	11	4
Proestrus	1	1	0	4
Estrus	5	5	3	2
Metestrus	1	4	1	5
Not cycling	0	0	0	0
Terminal Body Weight (g)	171.10 ± 1.953	167.85 ± 2.126	172.53 ± 3.009	165.59 ± 1.761
Adrenal Glands, Paired (g)	0.04136 ± 0.001746	* 0.04865 ± 0.001334 ^b	0.04627 ± 0.002196	0.04476 ± 0.002252
Adrenal Glands, Paired (g) (ANCOVA mean)^a	0.04167 ± .001907	* 0.04841 ± .001903 ^c	0.04683 ± .001931	0.04413 ± .001941
Kidneys, Paired (g)	1.72900 ± 0.028952	1.66866 ± 0.029096	1.66640 ± 0.033034	1.66618 ± 0.032442
Kidneys, Paired (g) (ANCOVA mean)^a	1.71116 ± .022037	1.68244 ± .021985	* 1.63461 ± .022312 ^c	1.70203 ± .022421
Liver (g)	8.66695 ± 0.146905	8.12279 ± 0.218546	8.39611 ± 0.248415	* 7.89805 ± 0.168019 ^b
Liver (g) (ANCOVA mean)^a	8.53798 ± .121895	8.22243 ± .121610	8.16621 ± .123419	8.15729 ± .124018
Ovaries, Paired (g)	0.09299 ± 0.004155	0.09529 ± 0.004337	0.08504 ± 0.003972	* 0.07888 ± 0.003368 ^b
Ovaries, Paired (g) (ANCOVA mean)^a	0.09250 ± .003987	0.09567 ± .003978	0.08417 ± .004037	0.07986 ± .004057
Pituitary (g)	0.00861 ± 0.000529	0.00790 ± 0.000469	0.00771 ± 0.000323	* 0.00692 ± 0.000538 ^b
Pituitary (g) (ANCOVA mean)^a	0.00863 ± .000479	0.00789 ± .000478	0.00774 ± .000485	* 0.00689 ± .000488 ^b

Mean ± SE (n = 15 unless specified in parenthesis by the mean)

^aMean ± SE has been adjusted for body weight at necropsy using ANCOVA.

^bSignificant treatment effect by ANOVA and Statistical Significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

^cSignificant treatment effect by ANCOVA and Statistical Significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

^dNumber of females in each stage of the estrous cycle at necropsy, as characterized by vaginal cytology.

Text Table 12: Body Weight and Organ Weights at Necropsy - Females

Parameter	Methoxychlor (mg/kg/day)			
	0	12.5	25	50
Thyroid/Parathyroid, Post Fixation (g)	0.01649 ± 0.001048	0.01725 ± 0.000628	0.01851 ± 0.000869	0.01693 ± 0.000934
Thyroid/Parathyroid, Post Fixation (g) (ANCOVA mean) ^a	0.01643 ± .000893	0.01730 ± .000891	0.01840 ± .000904	0.01706 ± .000908
Uterus and Cervix without Fluid (g)	0.35533 ± 0.027628	0.34717 ± 0.022768	0.33103 ± 0.018269	0.33157 ± 0.023957
Uterus and Cervix without Fluid (g) (ANCOVA mean) ^a	0.35464 ± .023729	0.34770 ± .023673	0.32980 ± .024026	0.33296 ± .024142
Uterus and Cervix, with Fluid (g)	0.41943 ± 0.049172	0.37781 ± 0.037327	0.35801 ± 0.023003	0.43732 ± 0.055971
Uterus and Cervix, with Fluid (g) (ANCOVA mean) ^a	0.41818 ± .043841	0.37878 ± .043739	0.35579 ± .044389	0.43982 ± .044605

Mean ± SE (n = 15 unless specified in parenthesis by the mean)

^aMean ± SE has been adjusted for body weight at necropsy using ANCOVA.

^bSignificant treatment effect by ANOVA and Statistical Significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

^cSignificant treatment effect by ANCOVA and Statistical Significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

^dNumber of females in each stage of the estrous cycle at necropsy, as characterized by vaginal cytology.

Text Table 12: Body Weight and Organ Weights at Necropsy - Females (continued)

Parameter	Phenobarbital (mg/kg/day)			
	0	25	50	100
Endocrine Status^d				
Diestrus	7	4	7	5
Proestrus	1	2	2	2
Estrus	5	6	3	6
Metestrus	1	3	3	1
Not cycling	0	0	1	3
Terminal Body Weight (g)	171.10 ± 1.953	171.36 ± 3.543	175.37 ± 3.151	171.46 ± 2.753
Adrenal Glands, Paired (g)	0.04136 ± .001746	**0.05203 ± .002524 ^b	**0.05261 ± .001560 ^b	**0.05182 ± .001970 ^b
Adrenal Glands, Paired (g) (ANCOVA mean)^a	0.04128 ± .001994	**0.05197 ± .001993 ^c	**0.05282 ± .002010 ^c	**0.05176 ± .001992 ^c
Kidneys, Paired (g)	1.72900 ± .028952	1.95411 ± .209151	1.74191 ± .073924	1.74029 ± .067031
Kidneys, Paired (g) (ANCOVA mean)^a	1.73311 ± .117595	1.95735 ± .117525	1.73166 ± .118552	1.74319 ± .117502
Liver (g)	8.66695 ± .146905	* 9.78521 ± .312736 ^b	**10.7576 ± .372795 ^b	**11.32150 ± .330428 ^b
Liver (g) (ANCOVA mean)^a	8.76602 ± .192284	**9.86320 ± .192169 ^c	**10.5107 ± .193849 ^c	**11.3913 0 ± .192132 ^c
Ovaries, Paired (g)	0.09299 ± .004155	0.09769 ± .004014	0.10069 ± .003386	0.09420 ± .004302
Ovaries, Paired (g) (ANCOVA mean)^a	0.09313 ± .004007	0.09780 ± .004005	0.10033 ± .004040	0.09430 ± .004004
Pituitary (g)	0.00861 ± .000529	0.00889 ± .000951	0.00790 ± .000520	0.00832 ± .000362 (14)
Pituitary (g) (ANCOVA mean)^a	0.00864 ± .000637	0.00891 ± .000636	0.00785 ± .000641	0.00833 ± .000658 (14)

Mean ± SE (n = 15 unless specified in parenthesis by the mean)

^aMean ± SE has been adjusted for body weight at necropsy using ANCOVA.

^bSignificant treatment effect by ANOVA and Statistical Significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

^cSignificant treatment effect by ANCOVA and Statistical Significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

^dNumber of females in each stage of the estrous cycle at necropsy, as characterized by vaginal cytology.

Text Table 12: Body Weight and Organ Weights at Necropsy - Females (continued)

Parameter	Phenobarbital (mg/kg/day)			
	0	25	50	100
Thyroid/Parathyroid, Post Fixation (g)	0.01649 ± .001048	0.01765 ± .000992	0.01813 ± .001149	* 0.01999 ± .000688 ^b
Thyroid/Parathyroid, Post Fixation (g) (ANCOVA mean) ^a	0.01647 ± .000993	0.01764 ± .000993	0.01818 ± .001001	* 0.01998 ± .000993 ^c
Uterus and Cervix without Fluid (g)	0.35533 ± .027628	0.35147 ± .018169	0.34745 ± .020805	0.35189 ± .023022
Uterus and Cervix without Fluid (g) (ANCOVA mean) ^a	0.35565 ± .022902	0.35172 ± .022888	0.34667 ± .023088	0.35211 ± .022884
Uterus and Cervix, with Fluid (g)	0.41943 ± .049172	0.40701 ± .039135	0.39323 ± .037337	0.40873 ± .036177
Uterus and Cervix, with Fluid (g) (ANCOVA mean) ^a	0.42021 ± .041172	0.40762 ± .041147	0.39128 ± .041507	0.40928 ± .041139

Mean ± SE (n = 15 unless specified in parenthesis by the mean)

^aMean ± SE has been adjusted for body weight at necropsy using ANCOVA.

^bSignificant treatment effect by ANOVA and Statistical Significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

^cSignificant treatment effect by ANCOVA and Statistical Significance for comparison of dosed groups to control (* = P<0.05, **=P<0.01) by Dunnett's Test.

^dNumber of females in each stage of the estrous cycle at necropsy, as characterized by vaginal cytology.

HISTOPATHOLOGY

The Histopathology Report is presented in Appendix 16.

As this was a blinded study, the test article information was disclosed after the processing and histological evaluation of the tissues was completed.

The majority of the gross lesions concerned the accessory sex glands of the males, and dilation of the kidneys of both males and females, which corresponded to hydronephrosis of variable degree. Hydronephrosis was observed in both males and females throughout all groups, except in the 25 mg/kg/day Flutamide-treated males and 50 mg/kg/day Methoxychlor-treated females, and considered an incidental finding.

Microscopic findings observed at each group in both males and females were incidental and expected in animals of similar age and environment, except for 50 mg/kg/day Flutamide treated males. Although 5 animals were microscopically normal in this group, changes in the remaining 10 males included luminal dilation of the testicular tubules, degeneration of the germinal epithelium and edema. Four of these males were also observed with sperm granulomas in the epididymis.

DISCUSSION

This study tested several hypotheses about the effects of endocrine disruptors on pubertal Sprague Dawley rats. Vinclozolin and Flutamide are both androgen receptor antagonists and were expected to delay preputial separation, reduce the weight of androgen sensitive reproductive organs, alter external genitalia, and cause retained nipples when administered to juvenile male Sprague Dawley rats (PND 23-53/54). Treatment did delay preputial separation and reduce the weight of androgen sensitive reproductive organs as expected. Vinclozolin and Flutamide treatment also resulted in higher testis weights and testis lesions (50 mg/kg/day Flutamide only). Treatment did not alter external genitalia or cause retained nipples. The lowest observable effect levels (LOEL) for an endocrine effect were the lowest doses tested, 25 mg/kg/day for Flutamide and 10 mg/kg/day for Vinclozolin.

Phenobarbital is known to be hepatotoxic and affect thyroid function. Treating juvenile males with Phenobarbital was expected to delay preputial separation and reduce the weight of reproductive organs. Treatment did delay preputial separation (100 mg/kg/day), as expected, but did not affect reproductive organ weights. Liver weights were increased in 50 and 100 mg/kg/day Phenobarbital-treated males. In juvenile females, Phenobarbital treatment (PND 22-42/43) was expected to result in delayed vaginal opening and irregular estrous cycling. Treatment did delay vaginal cycling (100 mg/kg/day), but did not disrupt estrous cycling. Treatment in females also increased liver, adrenal and thyroid (100 mg/kg/day) weights. The lowest observable effect level (LOEL) for an endocrine effect was 100 mg/kg/day for Phenobarbital (males and females). No endocrine effect was observed at 25 mg/kg/day.

Methoxychlor and Ethinyl Estradiol are both estrogen receptor agonists and were expected to result in advanced vaginal opening, advanced first estrus and onset of estrous cycling, and/or persistent vaginal estrus. Treatment with Methoxychlor or Ethinyl Estradiol (0.005 mg/kg/day) advanced vaginal opening and first estrus, and disrupted estrous cycling. Ethinyl Estradiol

treatment also resulted in increased adrenal weights. Methoxychlor (50 mg/kg/day) treatment resulted in increased liver, ovary and pituitary weights. The lowest observable effect levels (LOEL) for an endocrine effect were 12.5 mg/kg/day for Methoxychlor and 0.005 mg/kg/day for Ethinyl Estradiol. 12.5 mg/kg/day was the lowest dose tested for Methoxychlor. No endocrine effect was observed at 0.0025 mg/kg/day Ethinyl Estradiol.

CONCLUSIONS

Juvenile male Sprague Dawley rats were treated with Phenobarbital, Vinclozolin, or Flutamide from PND 23 until necropsy on PND 53 or 54. Parameters evaluated included mortality and clinical observations, body weights, day of preputial separation, gross pathology, organ weights and histopathology. The following parameters were affected by treatment.

Test Article	Concentration (mg/kg/day)	Body Weight	Age of Preputial Separation	Organ Weights	Histology
Phenobarbital	25	-	-	↑ liver	-
	50	-	-	↑ liver	-
	100	↓	↑	↑ liver ↓ pituitary	-
Vinclozolin	10	-	↑	-	-
	30	-	↑	↓ repro. organs ↑ testis,	-
	100	-	↑	↓ repro. organs ↑ testis, ↑ adrenal	-
Flutamide	25	-	↑	↓ repro. organs ↑ testis, ↑ liver ↓ kidneys	-
	50	-	↑	↓ repro. organs ↑ testis, ↑ liver ↓ kidneys	testis lesions

- = no effect; ↑ = higher than control means; ↓ = lower than control means
repro. = reproductive

Juvenile female Sprague Dawley rats were treated with Phenobarbital, Methoxychlor or Ethinyl Estradiol from PND 22 until necropsy on PND 42 or 43. Parameters evaluated included mortality and clinical observations, body weights, day of vaginal opening, estrous cyclicity, gross pathology, organ weights and histopathology. The following parameters were affected by treatment.

Test Article	Concentration (mg/kg/day)	Age at Vaginal Opening	Age at First Estrus	Irregular Estrous Cycles	Organ Weights
Phenobarbital	25	-	-	-	↑ liver ↑ adrenal
	50	-	-	-	↑ liver ↑ adrenal
	100	↑	-	-	↑ liver ↑ adrenal ↑ thyroid
Methoxychlor	12.5	↓	↓	-	-
	25	↓	↓	X	-
	50	↓	↓	X	↓ liver ↓ ovary ↓ pituitary
Ethinyl Estradiol	0.0025	-	-	-	↑ adrenal
	0.005	↓	↓	X	↑ adrenal

- = no effect; ↑ = higher than control means; ↓ = lower than control means; X = present

The lowest observable effect level (LOEL) for an endocrine effect was 25 mg/kg/day for Flutamide, 10 mg/kg/day for Vinclozolin, 100 mg/kg/day for Phenobarbital, 12.5 mg/kg/day for Methoxychlor, and 0.005 mg/kg/day for Ethinyl Estradiol.

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ABBREVIATIONS

Not all abbreviations listed are used in this report

↑	greater than control	S.E.	standard deviation
↓	less than control	RSD	relative standard deviation
>	greater than	TK	toxicokinetic
<	less than	PK	pharmacokinetic
≥	greater than or equal to	AUC	area under the curve
≤	less than or equal to	C_{max}	maximum concentration
~	approximately	t_{1/2}	half-life
°	degree	SD	study day
%	percent	GD	gestation day
C	Celsius	PND	post-natal day
F	Fahrenheit	i.p.	intraperitoneal
L	liter	i.v.	intravenous
mL	milliliter	s.c.	subcutaneous
μL	microliter	i.m.	intramuscular
g	gram	EPA	Environmental Protection Agency
kg	kilogram	FDA	Food and Drug Administration
mg	milligram	GLP	Good Laboratory Practices
μg	microgram	GMP	Good Manufacturing Practices
ng	nanogram	IACUC	Institutional Animal Care and Use Committee
pg	picogram	ICH	International Conference on Harmonization
cm	centimeter	MHLW	Ministry of Health, Labor and Welfare
mm	millimeter	NIEHS	National Institute of Environmental Health Sciences
μm	micrometer	NTP	National Toxicology Program
sec	second	OECD	Organization for Economic Co-operation and Development
min	minute	PHS	Public Health Service
h	hour	QA	Quality Assurance
d	day	QAU	Quality Assurance Unit
wk	week	SOP	Standard Operating Procedures
rpm	revolutions per minute	USDA	United States Department of Agriculture
NBF	neutral buffered formalin	LCA	Laboratory Corporation of America
MRI	Midwest Research Institute	RTI	Research Triangle Institute
PAI	Pathology Associates, A Division of Charles River	PPS	Preputial separation
		VO	Vaginal opening