REPORT

ON

ONE-GENERATION EXTENSION STUDY OF VINCLOZOLIN AND DI-N-BUTYL PHTHALATE ADMINISTERED BY GAVAGE ON GESTATIONAL DAY 6 TO POSTNATAL DAY 20 IN CD© (SPRAGUE-DAWLEY) RATS

EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT 2-10

May 7, 2003

Prepared for

JAMES P. KARIYA
WORK ASSIGNMENT MANAGER
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, DC

BATTELLE
505 King Avenue
Columbus, Ohio 43201

FINAL REPORT

TITLE:	One-Generation Extension Study of Vinclozolin and Di-n-Butyl Phthalate Administered by Gavage on Gestational Day 6 to Postnatal Day 20 in CD® (Sprague-Dawley) Rats
AUTHORS:	Julia D. George, Ph.D. Rochelle W. Tyl, Ph.D., DABT Bonnie T. Hamby, B.S. Christina B. Myers, M.S. Melissa C. Marr, B.A., RLATG
PERFORMING LABORATORY:	Center for Life Sciences and Toxicology Chemistry and Life Sciences Group RTI International P. O. Box 12194 Research Triangle Park, NC 27709-2194
SPONSOR:	Battelle Memorial Institute 505 King Avenue Columbus, OH 43201-2693
SPONSOR'S REPRESENTATIVE:	David P. Houchens, Ph.D. EDSP Project Manager Battelle
STUDY INITIATION DATE:	April 25, 2002
IN-LIFE PERFORMANCE DATES:	July 23, 2002 - November 22, 2002
EXPERIMENTAL DATES:	July 29, 2002 - November 22, 2002
FINAL REPORT DATE:	May 5, 2003
RTI IDENTIFICATION NUMBER:	65U-08055.001.012

Author:

_____

Julia D. George, Ph.D. Date
Study Director
Center for Life Sciences and Toxicology
RTI International

Approved:

_____

Satinder K. Sethi, Ph.D. Date
Senior Research Vice President
Chemistry and Life Sciences Group
RTI International

TABLE OF CONTENTS

Page

ABSTRACT 6
OBJECTIVES 9
MATERIALS AND METHODS 9
Test Material and Dose Formulations 9
Animals and Husbandry 9
Text Table 1. Study Schedule 10
Figure 1. Study Design 14
F0 Females 15
Progeny (F1) 15
Necropsy 16
F0 Females and PND 21 F1 Females 16
Necropsy of Pnd 4 Culled Pups 17
Necropsy of F1 Males on PND 21 and 95 17
External and Internal Examination of F1 Males at Necropsy 17
Statistical Analyses 19
Personnel 21
Analytical Report and Protocol/Amendments 21
Storage of Records 21
Compliance 21
RESULTS 22
Dose Formulations 22
Vinclozolin Results 22
F0 Female Observations 22
Fate of F0 Females 22
F0 Female Gestation 23
F0 Female Lactation 23
F0 Female Reproductive Indices 24
Unscheduled F0 Female Necropsy 24
Scheduled F0 Female Necropsy 24
F1 Observations 24
Fate of F1 Animals During Lactation 24
Observations of F1 Pups During Lactation 25
Unscheduled F1 Pup Necropsy During Lacatation 25
Scheduled F1 Pup Necropsy on Pnd 21 25
Fate of Pnd 95 F1 Males 27
F1 Male Post Wean Observations 27
Unscheduled Post Wean F1 Male Necropsy 28
Scheduled Necropsy of F1 Males on Pnd 95 28

TABLE OF CONTENTS

Page

DBP Results 29
F0 Female Observations 29
Fate of F0 Females 29
F0 Female Gestation 29
F0 Female Lactation 30
F0 Female Reproductive Indices 31
Unscheduled F0 Female Necropsy 31
Scheduled F0 Female Necropsy 31
F1 Observations 31
Fate of F1 Aninals During Lactation 31
Observations of F1 Pups During Lactation 32
Unscheduled Necropsy of F1 Pups During Lacatation 32
Necropsy of F1 Pups at Pnd 21 33
Fate of Pnd 95 F1 Males 34
F1 Males Postwean Observations 34
Unscheduled Necropsy of Postwean F1 Males 35
Scheduled Necropsy of F1 Males on Pnd 95 35
DISCUSSION 37
Text Table 2 38
Text Table 3 39
Text Table 4 41
Text Table 5 43
Text Table 6 48
Text Table 7 52
Text Table 8 54
Text Table 9 57
CONCLUSIONS 59
REFERENCES 60
PROTOCOL DEVIATIONS 64

Appendices
Individual Animal Data Tables . . . . . Appendix I
Analytical Chemistry Report . . . . . Appendix II
Protocol and Two Amendments . . . . . Appendix III

LIST OF TABLES

Table	Title	Page
1	Analyses of Dosing Formulations	66
2	Summary of the Fate of the F0 Females	69
3	Summary and Statistical Analysis of F0 Female Body Weights and Weight Change During Gestation	70
4	Summary and Statistical Analysis of F0 Female Feed Consumption During Gestation	73
5	Summary of the F0 Female Clinical Observations During Gestation	77
6	Summary and Statistical Analysis of F0 Female Body Weights and Weight Changes During Lactation and Sacrifice Weight	84
7	Summary and Statistical Analysis of F0 Female Feed Consumption During Lactation	87
8	Summary of the F0 Female Clinical Observations During Lactation and Necropsy Findings	90
9	Summary and Statistical Analysis of F0 Reproductive and Lactational Indexes for the F1 Litters	102
10	Summary and Statistical Analysis of the F1 Litter Size, Pup Body Weights, Anogenital Distance and Nipples During Lactation	106
11	Summary of the F1 Pup Clinical Observations on Postnatal Days 0 Through 21	113
12	Summary of the F1 Pup Necropsy Findings on Postnatal Days 0 Through 21 and Sex Confirmation for Pups Culled on Postnatal Day 4	115
13	Summary and Statistical Analysis of F1 Male Pup Necropsy Data and Necropsy Weights on Postnatal Day 21	118
14	Summary of the F1 Pup Necropsy Findings on Postnatal Day 21and Sex Confirmation for F1 Female pups on Postnatal Day 21	128

15	Summary of the Fate of the F1 Males	130
16	Summary and Statistical Analysis of the F1 Male Preputial Separation Data	131
17	Summary and Statistical Analysis of the F1 Male Body Weights and Weight Changes During the Post Wean Period	133
18	Summary and Statistical Analysis of the F1 Male Feed Consumption During the Post Wean Period	142
19	Summary of the F1 Male Clinical Observations During the Post Wean Period	151
20	Summary and Statistical Analysis of the F1 Male Necropsy Data and Necropsy Weights on Postnatal Day 95	169
21	Summary of the F1 Male Necropsy Findings for Scheduled and Unscheduled Necropsy on Postnatal Day 95	181

RTI Project No.: 65U-08055.001.012
RTI Protocol No.: RTI-807

FINAL REPORT

One-Generation Extension Study of Vinclozolin and Di-n-Butyl Phthalate Administered by Gavage on Gestational Day 6 to Postnatal Day 20 in CD® (Sprague-Dawley) Rats

ABSTRACT

In the standard two-generation test (U.S. EPA, 1998), most F1 animals are sacrificed and examined at postnatal day (pnd) 21; only one animal per sex per litter is usually allowed to continue to maturity. Although the basic two-generation study design was developed to provide information on insult to the reproductive tract, there is concern that certain effects may be missed, simply because the reproductive tract has not had sufficient time to develop before the observations are made on the vast majority of offspring. The study design tested through this work assignment examined whether or not allowing more of the F1 generation males to continue through puberty to adulthood will provide additional information in detecting endocrine-mediated effects than in a standard two-generation design. The objectives of this one-generation extension study are to determine: 1) whether some of the effects from perinatal exposure to Di-n-butyl phthalate (DBP) or to vinclozolin (VIN), that can be easily detected after puberty, are missed in weanling animals of the F1 generation; and 2) whether some of these effects occur at an incidence that would go undetected if only one male per litter is retained past puberty and examined at adulthood. In summary, the hypothesis being tested is that adverse reproductive effects will be detected if three or more F1 males per litter are examined at or after puberty but will be missed if most of the F1 males are examined only at weaning, and only one male/litter is retained to adulthood. The hypothesis will be tested using two known and well-characterized anti-androgens, DBP and VIN, each at two doses, one a known effect level and one a LOAEL (lowest observable adverse effect level) identified by basic research protocols.

Sperm-positive female CD® (Sprague-Dawley) rats (the F0 generation) were administered VIN (CAS No. 5-0471-44-8) or DBP (CAS No. 84-74-2) orally by gavage from gestational day (gd) 6 (sperm detection = gd 0) to pnd 20 (date of birth designated pnd 0), at 50 or 100 mg/kg/day, or 100 and 500 mg/kg/day, respectively at a dose volume of 5 ml/kg/day in Mazola® corn oil. A vehicle control group dosed with corn oil was run concurrently. Twenty-five sperm-positive F0 females were assigned to each treatment group. Body weights and feed consumption for the F0 females were recorded for F0 females during gestation and lactation, and F1 offspring from birth through scheduled sacrifice. Clinical signs were recorded at least once daily for all animals, with twice daily observations during the treatment period. On the day of birth (pnd 0), individual anogenital distance (AGD) and body weights were recorded for all live F1 pups in all litters. F1 litters were standardized on pnd 4 to yield ten pups, maximizing the number of male pups retained. Natural litters with ten or fewer pups were not adjusted. The culled F1 pups were weighed, euthanized, and examined

internally to confirm sex. The remaining F1 pups were counted, (and survival indices were calculated weekly), sexed and weighed throughout lactation to weaning (pnd 21). The presence or absence of retained nipples and areolae on the ventrum was recorded for all F1 males at approximately pnd 11-13. Males with one or more nipples or areolae were uniquely marked until weaning. At weaning, AGD at body weight was documented for all pups. Males were weight ranked within litters and pairs matched by weight. Pairs were assigned either to the pnd 21 necropsy group or the pnd 95 retention group. If the litter had an odd number of male pups or only one male pup, they were weighed and assigned to the retention group. On pnd 21, all males assigned to the pnd 21 necropsy group were euthanized and necropsied. All remaining female pups were euthanized and examined internally to confirm sex. In addition, all F0 maternal females were euthanized, necropsied, and examined for gross lesions. For each retained F1 male offspring, observations for the cleavage of the balanoprepreputial gland (preputial separation) began at 35 days of age and continued until acquisition of preputial separation. Body weight and feed consumption data were collected weekly from pnd 21 to pnd 95. At scheduled sacrifice on pnd 95, the males were euthanized and necropsied. Body and organ weights were recorded, nipples/areolae were counted, and AGD was measured.

Based on the observations, the following conclusions can be made:

- Specific male offspring malformations were detected on pnd 95 but not on pnd 21. Examples include prostate dorsal lobe abnormal/reduced in size (VIN, both doses; DBP, high dose), prostate ventral lobe abnormal/reduced in size (both compounds, both doses), and epispadias (VIN, both doses).
- The incidence of specific male offspring malformations detected on pnd 95 was higher than the incidence of the same malformation observed on pnd 21. Examples include agenesis of all or parts of the epididymis(des) (high dose of both VIN and DBP), hypospadias (low dose VIN), and missing/reduced in size/abnormal seminal vesicles (high dose of both VIN and DBP).
- The effects of VIN on the incidence of hypospadias and ventral prostate agenesis were more obvious at pnd 95 than at pnd 21. This effect was more apparent at the low dose than at the high dose. Specifically, hypospadias was observed in 9.7% vs 15.8% of the animals on pnd 21 and 95, respectively, whereas high dose animals exhibited hypospadias at 80.0% vs 98.6% on pnd 21 and 95, respectively.
- The effects of DBP (high dose) on the incidence of epididymal agenesis on pnd 95 was approximately twice that observed on pnd 21, and thus were more obvious on pnd 95 than on pnd 21.
- Adverse effects on the weight of some male reproductive tissues were more apparent at pnd 95 than on pnd 21. Examples include adjusted right or left testis weight (high dose VIN), absolute right cauda epididymis weight (low dose VIN), adjusted right cauda epididymis weight (low dose VIN and DBP), absolute LABC weight (low dose VIN), adjusted LABC weight (high dose VIN and DBP), and absolute and adjusted Cowper's gland weight (high

dose VIN).

- Adverse reproductive system effects *in toto* (structural malformations and other abnormalities) of the low and high doses of VIN and the high dose of DBP on F1 adult male offspring would most likely be statistically significant with either one or three adult males/litter, and would have been detected with either study design.
- Adverse reproductive system structural effects *in toto* at the low dose of DBP on F1 adult male offspring were clearly biologically significant but not necessarily or likely statistically significant, with either one or three adult males/litter, and provide an example of effects that would not be detected with either study design.
- The more males examined per litter, the better the characterization of the litter as responding or not responding adversely to exposure, and the smaller the variance term for pooled litters within each treatment group. The enhanced sensitivity with more males examined per litter would increase the likelihood of detection of effects as statistically and biologically significant. Also, for effects with low incidence, such as in the low dose DBP group in this study, the risk with fewer males examined per litter is that the effect might be missed, i.e., the litter would be designated as not responding, on the basis of the one male examined, if that male did not exhibit the effect.

OBJECTIVES

The study design tested through this work assignment examined whether or not allowing more of the F1 generation males to continue through puberty to adulthood would provide additional information in detecting endocrine-mediated effects than in a standard two-generation design. Secondary hypotheses to be tested included whether some of the effects easily detected in adults would be missed in the weanling sacrifice, especially those diagnostic of the two potent anti-androgens (see below) and whether examining more males at the weanling and adult necropsy would identify effects and incidences of these effects not identified using the number of weanlings and adults specified in the current EPA OPPTS reproductive toxicity testing guidelines. Assessment of male offspring survival, growth and development through lactation, and weaning through reproductive development until adulthood were evaluated using VIN (50 or 100 mg/kg/day) and DBP (100 or 500 mg/kg/day), two well known male endocrine disruptors, administered to the dams from gd 6 to pnd 20.

MATERIALS AND METHODS

Test Material and Dose Formulations

VIN (CAS No. 50471-44-8) was procured by the sponsor from Chem Service, Inc. (Lot No. 270-71B). GC-FID purity analysis by Battelle-Sequim indicated a purity of 99.75% (Final Chemical Report for WA 2-10, Battelle, February 12, 2003, Appendix II). DBP (CAS No. 84-74-2) was procured by the sponsor from Sigma-Aldrich, Inc. (Lot No. 080K1023). GC-FID purity analysis by Battelle-Sequim indicated a purity of 99.72% (Final Chemical Report for WA 2-10, Battelle, February 12, 2003, Appendix II). Mazola® corn oil (expiration dates 4-03 and 9-03) was purchased by Battelle-Sequim from retail outlets. Peroxide determination of the corn oil was 2.07 meq/kg (expiration date 4-03) and 1.38 meq/kg (expiration date 9-03). The corn oil was stored frozen.

Dose formulations were mixed in corn oil for administration at 5 ml/kg. One vehicle formulation was mixed to be administered to the control group animals concurrently with both DBP and VIN. Stability analysis of dose formulations of VIN in corn oil (10 and 20 mg/ml) indicated that the formulations were stable for at least eight weeks. Formulations assayed (triplicate average) between 90.1 and 99.6% of the target concentration (Appendix II, Table 7). Stability analysis of DBP formulations in corn oil (20 and 100 mg/ml) indicated that these formulations were also stable for at least eight weeks, with assayed concentrations between 91.2 and 98.6% of the target concentration (Appendix II, Table 6).

Animals and Husbandry

One hundred seventy (170) nulliparous female and 110 male outbred albino CD® (Sprague-Dawley) rats (Crl:CD®[SD] IGS BR) were received from Charles River Breeding Laboratories (Raleigh, NC) on July 15, 2002 (Text Table 1). Both the females and males were 70 days old on arrival.

Text Table 1. Study Schedule

Event	Dates[a]
Animals Arrive	07/15/02
Eartag Male (breeders) and Female Rats	07/16-18/02
Mate Females	07/22/02
F_0 Gestation (~3 weeks)	07/23/002 – 08/20/02
F_0 First Day of Dosing (gd 6)	07/29/02
F_0 Lactation (~ 3 weeks)	08/13/02 - 09/10/02
F_1 Anogenital Distance Measurements (pnd 0)	08/13/02 - 08/20/02
F_1 Cull, Retain all Males and Enough Females to = 10 pups (pnd 4)	08/17/02 - 08/24/02
F_1 Culled Pups Visceral to Confirm Sex (pnd 4)	08/17/02 - 08/24/02
F_1 Male Nipple Retention (pnd 11-13)	08/24/02 - 09/02/02
F_1 Anogenital Distance Measurements (pnd 21)	09/03/02 - 09/10/02
F_0 Last Day of Dosing (pnd 20)	09/10/02
F_0 Female Necropsy	09/03/02 - 09/10/02
F_1 Pnd 21 Necropsy (~ 1 weeks)	09/03/02 - 09/10/02
F_1 Pnd 21 Weaning of Males Only (~ 1 weeks)	09/03/02 - 09/10/02
F_1 Post Wean Holding (10 weeks)	09/03/02 – 11/23/02
F_1 Preputial Separation (pnd 35 - ~pnd 50)	09/17/02 - 10/02/02
F_1 Male Sacrifice (pnd 95)	11/16/02 - 11/22/02
F_1 Male Nipple Retention (at sacrifice)	11/16/02 - 11/22/02
F_1 Anogenital Distance Measurements (at sacrifice)	11/16/02 - 11/22/02

The animals were quarantined for one week, during which time they were weighed and examined by a veterinarian. Representative animals were subjected to fecal examination and serum viral antibody analysis. For serum viral antibody analysis, within one day after receipt, five female rats were arbitrarily chosen from the shipment of animals, sacrificed, and blood collected for assessment of viral antibody status. Heat-inactivated serum was sent to BioReliance (Rockville, MD) for their Level 1 Rat Antibody Screen. The viral screen consisted of evaluation for the presence of antibodies against the following: Toolan H-1 virus (H-1), Sendai virus, pneumonia virus of mice (PVM), rat coronavirus/sialodacryoadenitis (RCV/SDA), Kilham rat virus (KRV), CAR *Bacillus* (CARB), and *Mycoplasma pulmonis* (*M. Pul.*) and parvo (PARVO). Results of the physical examination, serology, and parasitology were negative for signs of infectious disease; one animal was equivocal for CARB. The animals were considered to be in good health and suitable for use in this study.

After mating was completed, four additional rats were randomly selected and designated as sentinels. They were singly housed in the study room(s) in polycarbonate solid-bottom cages with bedding and provided feed and water *ad libitum* (as described below for study animals). They were examined once daily by cage-side observation for morbidity or mortality at the same time as clinical observations or morbidity/mortality checks for the study animals. No sentinels exhibited any morbidity or mortality. At the time of necropsy of retained F1 offspring, the sentinels were terminated, blood samples collected, and serum samples prepared. All sentinel serum samples

were submitted to BioReliance (Rockville, MD) for serological evaluation (see above). Analysis of serum (as described above) from sentinels sacrificed during the necropsy of the F0 females was negative, as was the analysis from serum taken during the retained F1 necropsy.

F0 females were individually identified by eartag. One hundred ten (110) male rats of the same strain from the RTI breeding colony, originally from the same supplier, were used to generate timed-mated females. For breeding, individual females were placed in the home cage of singly-housed males (i.e., one male and one female). On the following morning and each morning thereafter, the females were examined for the presence of vaginal sperm or a vaginal copulation plug (Hafez, 1970). The day on which vaginal sperm or plugs were found was designated as gd 0. These females were presumed pregnant. The sperm-positive females (dams), designated the F0 generation, were housed individually or with their litters until scheduled sacrifice. Sperm-negative females were retained in the same male's cage and checked for sperm or vaginal plug on successive mornings until insemination occurred or the treatment groups were filled. When all treatment groups were filled, the remaining sperm-negative females were sacrificed by asphyxiation with CO_2. The fate of all animals was fully documented.

A total of 25 timed-mated females per group were assigned to this study. Confirmed-mated females were assigned to treatment groups by stratified randomization for body weight on gd 0, so that mean body weight on gd 0 did not differ among treatment groups. Selected F1 weanlings were identified by eartag, and F1 pups prior to weaning were not uniquely identified. The method and numbers for identification were documented in the study records.

All adult animals were euthanized by CO_2 asphyxiation. F1 pups culled on pnd 4 were sacrificed by decapitation. Animals received with the initial shipment, but not used in the study, were removed from the study room prior to the start of the treatment period and used for methods development and training of the RTI staff. Records were kept documenting the fate of all animals received for the study.

The experiment was carried out under standard laboratory conditions. The animals were individually housed during the quarantine period and upon the initiation of the treatment period in solid-bottom polycarbonate cages with stainless-steel wire lids (Laboratory Products, Rochelle Park, NJ) with Sani-Chip® cage litter (P.J. Murphy Forest Products Corp., Montville, NJ). F0 females were housed in monogamous breeding pairs during the mating period. Females were caged separately and individually once they were successfully mated (or at the end of the mating period). F0 females were housed with their F1 litters during lactation. Postwean, retained F1 males were housed singly until necropsy. All animals were housed in the RTI Animal Research Facility for the duration of the study. All animal rooms were on a 14:10 hour (light:dark) light cycle per day and were air-conditioned; temperature and relative humidity (RH) were continuously monitored, controlled, and recorded using an automatic system (Siebe/Barber-Colman Network 8000 System, Version 4.4.1, Loves Park, IL). One light cycle deviation occurred (see deviation list). The protocol-mandated temperature range was 66-77°F (22+3°C), and the RH range was 30-70% (NRC, 1996). The F0 animals (and F1 pups during lactation) were housed in Room 303 of the Animal Research Facility, and the F1 animals postweaning were housed in Room 403, 404, 407, and 503. Temperature and

RH readings for the animals rooms, excluding transient deviations (as noted in the Protocol Deviation list) are presented here. Temperature and RH readings for Room 303 from July 23, 2002 to September 25, 2002, were 70.7 to 76.8°F and 45.4 to 63.7% RH. Temperature and RH readings for Room 403 from September 3, 2002 to November 19, 2002 were 70.0 to 74.8°F and 39.3 to 62.2% RH. Temperature and RH readings for Room 404 from September 4, 2002 to November 21, 2002 were 69.6 to 74.5°F and 41.2 to 69.8% RH. Temperature and RH readings for Room 503 from September 6, 2002 to November 22, 2002 were 70.4 to 74.5°F and 44.6 to 60.5% RH. Temperature and RH readings for Room 407 from September 25, 2002 to November 22, 2002 were 69.6 to 75.3°F and 46.3 to 63.5% RH. Two deviations occurred in Rooms 303, 403, 404, and 503, and one deviation occurred in Room 407, in which the RH was above that specified in the protocol for one hour on each occasion (see Protocol Deviations).

Purina Certified Rodent Chow (No. 5002, PMI Feeds, Inc., St. Louis, MO; batch numbers documented in the study records) was available *ad libitum*. All animals in all groups received the same batch/lot (lot #JUN 24 02 1B) of Purina Certified Rodent Chow at all times. The analyses of each feed batch for nutrient levels and possible contaminants were performed by the supplier, examined by the Study Director, and maintained in the study records. The feed was also analyzed at the manufacturer for the phytoestrogens daidzein, genistein, and glycitein. Analysis indicated that the total phytoestrogens in this lot of feed ranged from 341 to 358 ppm (Appendix II; Chemistry report to be amended to include correct feed analysis).

Deionized water (generated in-house from tap water; source: City of Durham, Department of Water Resources, Durham, NC) was available *ad libitum* by plastic water bottles with butyl rubber stoppers and stainless-steel sipper tubes. Contaminant levels of the Durham City water were measured at regular intervals by the supplier per EPA specifications. The deionized water was analyzed by Balazs Analytical Laboratories, Inc. (Sunnyvale, CA). There were no known contaminants that may have affected the outcome of this study.

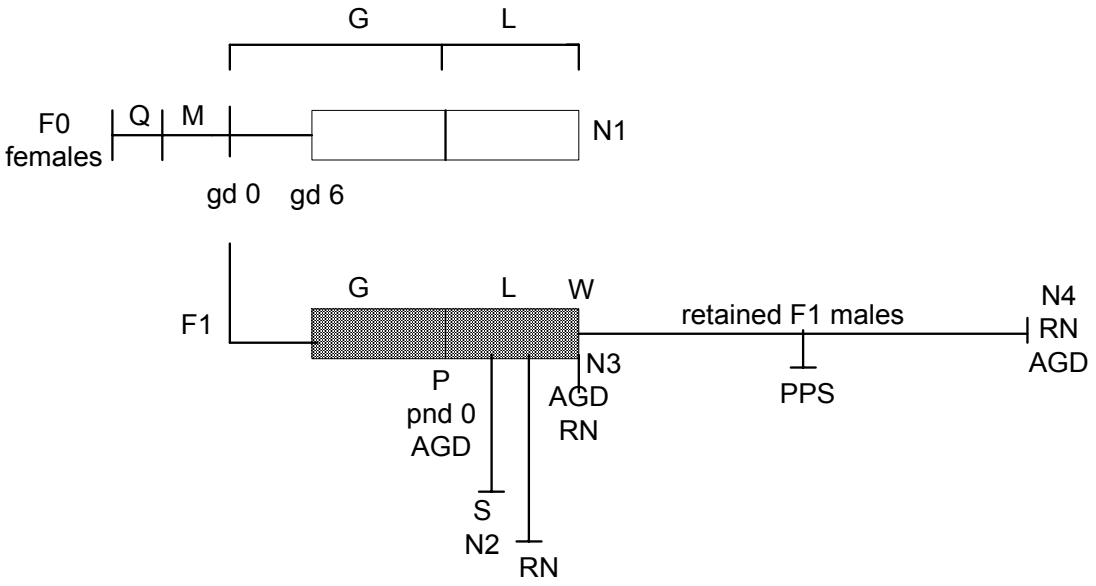
Study Design

A graphic representation of the study design is presented in Figure 1. The study began with 25 timed-mated females/group.

Exposure began for F0 females on gd 6 beginning on July 29, 2002, when they were approximately 12 weeks old. The doses were chosen based on results in the literature. For VIN, Gray et al. (1994) showed that administering VIN in corn oil by gavage once daily on gd 14 through pnd 3 in rats at 0, 100, or 200 mg/kg/day caused dose-related incidences and severities of male reproductive tract malformations and renal system malformations in the offspring. In another dose response publication (Ostby et al., 1997), maternal rats were dosed by gavage on gd 14 through pnd 3 with VIN at 0, 100, and 200 mg/kg/day (first study), with expected male offspring reproductive malformations observed at both doses. In a second study, the doses were 0, 3.125, 6.25, 12.5, 25, 50, and 100 mg/kg/day. In offspring males, reduced AGD was observed at $\geq$3.125 mg/kg/day, retained areolas were observed at $\geq$6.25 mg/kg/day, and reduced ventral prostate weight and hypospadias were observed at 50 mg/kg/day. Ectopic testes were only observed at 100 and 200

mg/kg/day. Effects on serum testosterone levels and spermatogenesis were only observed at ≥100 mg/kg/day. Finally, in a study by Hellwig et al. (2000), Wistar and Long-Evans rats were orally dosed with VIN from gd 14 to pnd 3 at 200, 12, 6, 3, 1, or 0 mg/kg/day. The high dose (200 mg/kg/day) was maternally toxic, and male offspring from both strains exhibited reduced AGD; retained nipples/areolas; hypospadia; penile hypoplasia; development of a vaginal pouch; hypoplasia and chronic inflammation of the epididymides, prostate, seminal vesicles, and coagulating glands; testicular tubule atrophy; and chronic inflammation of the urinary bladder. At 12 mg/kg/day, retained nipples/areolas were present in both strains in preweanling males but persisted only in Long-Evans rats. Long-Evans rats (but not Wistar) also exhibited a low incidence of hypoplasia of accessory sex organs. Based on the results from Gray et al. (1994), Ostby et al. (1997), and Hellwig et al. (2000) in this study, VIN in corn oil was administered by oral gavage once daily on gd 6 through pnd 20 at 0 mg/kg/day (vehicle control; the same control group as for DBP since same vehicle is used for both chemicals), 50 mg/kg/day (considered the LOAEL for male reproductive tract malformations), and 100 mg/kg/day (a clear effect level). An oral dose of 50 mg/kg/day DBP has been defined as the NOAEL (no observable adverse effect level) by Mylchreest et al. (1998a,b, 1999). Male malformations, including shortened AGD, small flaccid testes, agenesis of portions (caput, corpus, cauda) of or the entire epididymis, delayed puberty, retained nipples and areolae, etc., have been observed after doses of 100 to 760 mg/kg DBP (Gray et al., 1998; 2000). Interestingly, *in utero* (gd 15-29) exposure of Dutch-Belted rabbits to DBP at 400 mg/kg in corn syrup by gavage also resulted in reduced testis and accessory gland weights and reduced ejaculated sperm in adult F1 offspring males; F1 males also exhibited reduced serum testosterone, a slight increase in histological alterations of the testis, a doubling of the percentage of abnormal sperm, and one F1 male (out of 17) manifested hypospadias, hypoplastic prostate and cryptorchid testes with carcinoma *in situ*-like cells (Higuchi, et al., 2003). Therefore, for this study, DBP in corn oil was administered by oral gavage once daily on gd 6 through pnd 20 at 0 (vehicle control), 100 mg/kg/day (the LOAEL; lowest observed adverse effect level), and at 500 mg/kg/day (an obvious effect level).

F0 females were assigned to the different groups by means of randomization stratified by body weight, such that the body weights of all groups were homogeneous at study initiation. The range for F0 all females on gd 0 was 211.2 to 294.6 g.



Key:

Direct dosing of F0 parental females, gd 6 - pnd 20

Possible indirect exposure of F1 offspring *in utero* and during lactation from transplacental and/or translactional transfer

No dosing of retained F1 males from weaning on pnd 21 to scheduled necropsy on pnd 95 ± 5

Q	=	quarantine (one week)	L	=	lactation (three weeks)
M	=	mating (one week)	pnd	=	postnatal day
G	=	gestation (~three weeks)	P	=	parturition (date of birth, pnd 0)
gd	=	gestational day	AGD	=	anogenital distance
W	=	wean on pnd 21	PPS	=	acquisition of preputial separation
S	=	standardize litters to 10 (with maximum number of males) on pnd 4			
RN	=	examination of males for retained nipples			
N1	=	necropsy of F0 parental females at weaning of F1 litters			
N2	=	necropsy culled females to confirm sex			
N3	=	necropsy any remaining F1 females (and confirm sex), and necropsy three F1 males per litter at weaning on pnd 21			
N4	=	necropsy of retained F1 males at pnd 95 ± 5			

Figure 1. Study Design

F0 Females

F0 females were dosed with vehicle control, 50 or 100 mg/kg of VIN, or 100 or 500 mg/kg DBP in Mazola® corn oil at 5 ml/kg, adjusted with respect to the most recent body weight. Dosing was done once daily by oral gavage with an appropriate-sized syringe fitted with a 16 g two-inch stainless-steel curved dosing needle (Perfektum®, Popper and Sons, New Hyde Park, NY). F0 females were dosed daily, from gd 6 through pnd 20, and necropsied after weaning of their litters.

Observations for mortality were made twice daily (a.m. and p.m.), and the general condition of all animals was checked daily. Clinical examinations were conducted and recorded daily throughout the course of the study. This record included the degree and duration of symptoms. These cage-side observations included, but were not limited to, changes in: skin and fur, eyes, mucous membranes, respiratory and circulatory system, autonomic and central nervous system, somatomotor activity, and behavioral pattern.

The body weights of the F0 female rats were determined and recorded upon assignment to dose groups. During gestation, F0 females were weighed daily from gd 6 through gd 20. Dams producing litters were weighed daily during lactational (pnd 0 through pnd 21), and body weight gains were computed.

During pregnancy of F0 females, feed weight was recorded for gd 0, 6, 9, 12, 15, 18, and 20. During lactation of the F1 litters, maternal feed weight was recorded for pnd 0, 4, 7, 14, and 21, although maternal feed consumption after pnd 14 was confounded by the contribution from the pups since the pups were self-feeding by this time. (The contribution of self-feeding pups on “maternal” feed consumption during the last week of lactation has been estimated at 30-40%, e.g., Hanley and Watanabe, 1985; Tyl et al., 2002).

Beginning on gd 20, each female was observed twice daily (a.m. and p.m.) for evidence of littering. On the day of birth (pnd 0), AGD was measured and body weight recorded for all live F1 pups in all litters. Body weight was recorded for all live pups on pnd 4 prior to culling and euthanasia. F0 females that had not delivered by gd 26 were euthanized and examined internally for pregnancy status. The F0 dams with litters were allowed to rear their young to pnd 21. On pnd 21, each litter was weaned. All F0 females in all groups were subjected to a complete gross necropsy on pnd 21. No tissues were weighed or retained.

Progeny (F1)

All F1 pups were counted, sexed, weighed, and examined as soon as possible after birth (date of birth designated pnd 0) to determine the number of viable and stillborn pups from each litter. Thereafter, litters were evaluated for survival on pnd 4, 7, 14, and at weaning (pnd 21). Individual AGD and body weight were recorded on pnd 0 for all F1 offspring.

On pnd 4, the size of each litter was adjusted to ten by eliminating extra pups by random selection to maximize the number of males. Natural litters with ten or fewer pups were not culled. F1 pups culled to standardize litters on pnd 4 were euthanized and subjected to a visceral examination to confirm the internal sex. No tissues were retained.

All live pups were counted, sexed, weighed individually, and examined grossly at birth (pnd 0), pnd 4, 7, 14 and at weaning (pnd 21). The body weights and sexes were recorded on an individual basis, with male pups with nipples/areolae uniquely identified on pnd 11-13. AGD was recorded with the individual pup weight on pnd 0 for all F1 pups, and the presence or absence of retained nipples and areolae on the ventrum was recorded for F1 male offspring at approximately pnd 11-13. All pups were examined for physical abnormalities at birth and throughout the preweaning and postwean period. All pups dying during lactation were necropsied, when possible, to investigate the cause of death. At weaning, the F1 males were weight ranked within litters, and pairs were matched and assigned to either the pnd 21 necropsy group or the pnd 95 retention group (e.g., one each of the two heaviest males from each litter went into the pnd 21 necropsy group or the retention group, etc.). For litters with an odd number of males or with more than six males, the extra males were assigned to the pnd 95 retention group. All males (weight matched to the retained males) designated for pnd 21 examination were sacrificed on pnd 21, and any remaining pnd 21 females were sacrificed. The remaining males in each litter (weight matched to the pnd 21 necropsy males) and any extra males were retained until scheduled sacrifice on pnd 95. F1 males assigned to the pnd 95 retention group were held without dosing until scheduled sacrifice.

F1 male postweaning body and feed weights were recorded on pnd 21, 25, 28, 32, 35, 39, 42, 46, 49, 53, 56, 60, 63, 67, 70, 74, 77, 81, 84, 88, 91, and 95. Body weight gain and feed consumption were calculated for pnd 21 to 25, 25 to 28, 28 to 32, 32 to 35, 35 to 39, 39 to 42, 42 to 46, 46 to 49, 49 to 53, 53 to 56, 56 to 60, 60 to 63, 63 to 67, 67 to 70, 70 to 74, 74 to 77, 77 to 81, 81 to 84, 84 to 88, 88 to 91, 91 to 95, and 21 to 95. F1 postweaning observations and procedures for each retained male offspring included observations for the cleavage of the balanoprepreputial gland (preputial separation), which began at 35 days of age and continued until acquisition of preputial separation. Individual body weights were recorded at acquisition. All retained F1 postweanling males were weighed as indicated above (with clinical observations daily) until scheduled necropsy on pnd 95. On pnd 95, F1 males were euthanized and the number of nipples or areolae counted. They were then given a complete external and internal examination. No tissues were saved.

Necropsy

F0 Females and PND 21 F1 Females

F0 females that were moribund were sacrificed by CO_2 asphyxiation, necropsied, and discarded. F0 females that failed to deliver by pnd 27 were sacrificed and necropsied. Uteri from any F0 females who appeared nonpregnant were stained with 10% ammonium sulfide (Salewski, 1964) for confirmation of pregnancy status and a count of implantation sites. F0 females found dead were necropsied. Intact fetuses (*in utero*, delivered prior to the death of the dam) were examined externally and viscerally (with focus on the reproductive system) and discarded. Scheduled sacrifice of the F0 maternal animals occurred after F1 litters were weaned. Maternal females were examined internally for gross lesions. Female F1 offspring were sacrificed at weaning and necropsied to confirm sex by internal examination, then discarded. No tissues or organs were saved.

Necropsy of Pnd 4 Culled Pups

All F1 pups culled on pnd 4, when the litters were standardized, were subjected to a complete external examination and an internal examination of the reproductive organs to determine sex.

Necropsy of F1 Males on Pnd 21 and 95±5

The F1 males selected for necropsy on pnd 21 or retained until pnd 95 were euthanized by CO_2 asphyxiation and then weighed. AGD was measured by Vernier calipers (precision to 0.1 mm) at necropsy.

External and Internal Examination of F1 Males at Necropsy

Each male selected for pnd 21 necropsy or pnd 95 adult necropsy was examined externally at necropsy. Any unusual malformations or anomalies were noted. Then, the ventral surface was shaved from inguinal region to neck, the nipples and areolae were counted, and the number and position of the areolae and nipples was recorded. The animals were checked for hypospadias, epispadias, and cleft phallus, and the AGD was measured. The animals were also examined for obviously undescended testes and preputial separation. The reproductive organs were carefully observed for the following:

- Location of each testis (scrotal, abdominal, gubernaculum attached to abdominal wall)
- Gubernacular cords, present or absent, and length in mm
- Presence of cranial suspensory ligaments
- Testes which were small, absent, fluid filled, enlarged, appeared infected, or other
- Epididymides which were small, absent, or infected (including region of effects)
- Ventral prostate which was small, absent, or infected
- Dorsolateral prostate which was small, absent, or infected
- Seminal vesicles which were small, absent, infected, or one side larger than the other
- Coagulating glands which were small, absent, infected, one side larger than the other, or detached from seminal vesicles

In addition the urinary system was evaluated as follows:

- Kidneys with hydronephrosis or calcium deposits
- Hydroureter(s)
- Urinary bladder stones or blood in urinary bladder

The following organs were weighed:

- Each testis individually
- Each corpus plus caput epididymides
- Each cauda epididymides
- Entire seminal vesicle, plus coagulating glands with fluid as a unit, if possible

- The prostate ventral and dorsolateral lobes separately
- Paired adrenals
- Liver
- Levator ani plus bulbocavernosus muscle complex
- Cowper's (bulbourethral) glands as a pair
- Glans penis (only if preputial separation has occurred)

Because the male necropsies on pnd 21 and 95 were neither routine nor standard, details are provided below. The procedures were the same for both the pnd 21 and 95 necropsies. Each animal was terminated by asphyxiation with CO2 and then weighed. The ventrum was shaved and the numbers of nipples and/or areolae were recorded. The presence or absence of a soiled perineal area was noted. Anogenital distance was then measured by a vernier caliper (precision to 0.2 mm). The animal was then opened with a ventral midline incision and the specified organs removed, trimmed and weighed (to 0.0001g). At the pnd 21 necropsy, the males had not yet achieved puberty (preputial separation, PPS), so the status of the phallus was determined by careful dissection and removal of the foreskin. At the pnd 95 necropsy, PPS had been achieved before the necropsy, so the phallus could be assessed by gentle retraction of the foreskin. Findings of the phallus included cleft phallus (defined as ventral midline cleft from tip; care was taken to distinguish the cleft from a prominent ventral midline furrow along the “seam”), hypospadias (ventral opening was not on the tip but below it, somewhere along the ventral midline seam, usually associated with cleft phallus), and epispadias (ventral opening is not on the tip but below it on the dorsal side of the phallus). Any other gross lesions were also noted.

On pnd 21, the testes had just descended into the scrotal sacs; on pnd 95, the testes had descended long before the necropsy. For both necropsies, the testes were each gently retracted from the scrotal sacs, and the presence of the gubernacular cords was noted. If the cords remained attached to the caudal base of the scrotal sacs, they were not measured. If the cords had detached from the scrotal sac (so they could be measured), they were measured (to 0.1mm). The presence or absence of cranial suspensory ligaments (normally observed only in females, attached to the ovaries and the underside of the diaphragm) was also noted. Any organs to be retained were stored accordingly (e.g., pituitaries were frozen); nonretained organs and the carcass were then discarded.

The whole pituitary was frozen and delivered to Dr. Ralph Cooper at the U.S. EPA's National Health and Environmental Effects Research Laboratory (NHEERL) (RTP, NC). All other organs were examined and weighed, as described above, and discarded.

Two decisions were made for the materials and methods on this study:

1. Andrology (e.g., cauda epididymal sperm number, morphology, and motility, and testicular homogenization-resistant spermatid head counts to calculate daily sperm production and efficiency of daily sperm production) was not included for the F1 male offspring during the pnd 95 necropsy. The rationale is that andrology is not a sensitive endpoint for VIN and DBP in rodents. AGD, retention of nipples/areolae, organ weights and male reproductive system malformations detected by careful dissection and examination are much more sensitive to

these potent anti-androgens. Interestingly, DBP did cause reduced ejaculated sperm counts, as well as reproductive malformations in male offspring rabbits exposed *in utero* to 400 mg/kg/day (Higuchi et al., 2003). These andrology assessments are also very labor intensive (and therefore expensive) and require close timing between the demise of each male and his andrologic assessment.

2. Histopathology of the F1 offspring male reproductive organs from the pnd 95 (or pnd 21) necropsy was not performed. The rationale was that it was anticipated that differences in incidence and severity of effects on male reproductive system organs, at the two different time points, pnd 21 and 95, for the two different chemicals, VIN and DBP, and for the two different doses per chemical, could be adequately detected from the gross dissections and examinations, and by absolute and adjusted (for body weight) organ weights (and because of the expense of and time for the histology procedures and pathological examinations).

Statistical Analyses

The unit of comparison was the pregnant female, the F1 pup, or the retained F1 male offspring, as appropriate. Treatment groups for each chemical were compared to the concurrent control group using either parametric ANOVA under the standard assumptions or robust regression methods (Zeger and Liang, 1986; Royall, 1986; Huber, 1967) that do not assume homogeneity of variance or normality. The homogeneity of variance assumption was examined via Levene's Test (Levene, 1960), which is much more robust to the underlying distribution of the data than the traditional Bartlett's Test. If Levene's Test indicated lack of homogeneity of variance ($p<0.05$), robust regression methods were used to test all treatment effects. The robust regression methods used variance estimators that make no assumptions regarding homogeneity of variance or normality of the data. They were used to test for linear trends across dose within chemicals, as well as overall treatment group differences (via Wald chi-square tests). Significant overall treatment effects were followed by single degree-of-freedom *t*-tests for exposed vs. control group comparisons, if the overall treatment effect was significant. If Levene's Test did not reject the hypothesis of homogeneous variances, standard ANOVA techniques were applied for comparing the treatment groups within chemicals. The GLM procedures in SAS® 8 (SAS Institute Inc., 1999a,b,c,d,e, 2000) were used to test for linear trend, evaluate the overall effect of treatment and, when a significant treatment effect was present, to compare each exposed group within chemicals to the concurrent control group via Dunnett's Test (Dunnett, 1955, 1964). Standard ANOVA methods, as well as Levene's Test, are available in the GLM procedure of SAS® Version 8, and the robust regression methods are available in the REGRESS procedure of SUDAAN® Release 8.0 (RTI, 2001).

The F0 maternal post-implantation loss percentage across dose groups within chemicals was compared using weighted ANOVA techniques. Since percentage data derived from litters tend to have unequal variances, the arcsine of the square root transformation to the litter percentages was applied prior to analysis. The ANOVA was weighted by the number of implants (denominator of the post-implantation loss percentage) in order to further stabilize the variances. In the presence of significant treatment effects, Dunnett's Test was used for pairwise comparisons to control. The

average post-implantation loss percentage (prior to transformation) was presented for each dose group within chemical, with sample size and standard error.

Independent binary endpoints, such as the F0 maternal reproductive indices (e.g., mating, fertility, and live litter indices), were analyzed by Fisher's Exact Test for overall heterogeneity among treatment groups and by an exact version of the Cochran-Armitage Test for linear trend on proportions (Cochran, 1954; Armitage, 1955; Agresti, 1990). When the overall Fisher's Exact Test was significant ($p<0.05$), pairwise comparisons of individual exposed groups within chemicals vs. control were performed using pairwise Fisher's Exact Tests. All of these tests were obtained via the FREQ procedure in SAS 8. The SAS MULTTEST procedure was used to obtain *p*-value adjustments for the multiple treatment comparisons resulting from repeated applications of Fisher's Exact Test. The *p*-value adjustments were based on the bootstrap and permutation resampling techniques of Westfall and Young (1993).

Cluster-correlated data, such as F1 periodic pup body weights during lactation, the periodic pup survival indices, the lactation index, the percentage of stillborns and live births, the sex ratio, the percentage of male pups with areolae and/or nipples on pnd 11-13 and 21, and the AGD were analyzed using GEE regression methods (Zeger and Liang, 1986; Liang and Zeger, 1986) in SAS® 8 or SUDAAN® 8.0 to evaluate overall significance, test for linear trend across dose groups within chemicals, and test pairwise comparisons to the control group values. For AGD, a body weight covariate was included in the regression model. Some of these outcomes were continuous (e.g., body weights, AGD) and some were binary (e.g., periodic pup survival). Ordinal outcomes included those measured on a severity scale, such as none, mild, moderate, and severe. The analyses compared the results for adult offspring with those of weanlings. No matter what type of endpoint was examined, when data from multiple offspring from the same litter were used in the analysis, the resulting correlation was adjusted for responses within litters.

Developmental landmarks, for example, F1 pup age at preputial separation, and the AGD were analyzed using either parametric ANOVA under the standard assumptions or robust linear regression methods, with and without including body weight at acquisition as a covariate in the regression model.

A test for statistical outliers was performed in the UNIVARIATE procedure of SAS® 8 on F0 maternal body weights, feed consumption (in g/day), and retained F1 male body and organ weights. When examination of pertinent study data did not provide a plausible, biologically sound reason for inclusion of the data flagged as "outlier," the data were excluded from summarization and analysis of the data, and the report, and were designated as outliers. When feed consumption data for a given animal for a given observational interval (e.g., pnd 0-4, 4-7 or 7-14 during the lactational exposure period) were designated outliers or unrealistic, then summarized data for this animal encompassing this period (e.g., pnd 0-20 for the lactational exposure period) also did not include this value. For all statistical tests, $p \leq 0.05$ (one- or two-tailed) was used as the criterion for significance.

Personnel

This study was conducted at RTI International, Research Triangle Park, NC, under contract to Battelle, Columbus, OH. Dr. David P. Houchens, EDSP Project Manager, was the Sponsor's Representative. Dr. R. W. Tyl served as Project Toxicologist. Dr. Julia D. George served as Study Director. Reproductive and Developmental Toxicology personnel included Ms. M.C. Marr (Laboratory Supervisor), Ms. C.B. Myers (Reproductive Toxicity Study Supervisor and Data Analyst), Mr. W.P. Ross, Ms. M.C. Rieth, Ms. V.I. Wilson, Ms. L.B Pelletier, Ms. M.P. Gower, Ms. N.M. Kuney, Ms. R.T. Krebs, Ms. S.W. Pearce, Ms. K.D. Vick, Ms. L. McDonald, Ms. A.J. Parham, Mr. M.D. Crews, Mr. C.G. Leach, Ms. A. Goodman, and Mr. T.W. Wiley. Bulk chemical analysis and handling, dose formulation, and dose formulation analysis were provided by the sponsor through Dr. E.A. Crecelius, PNNL, Battelle Marine Sciences Laboratory, Sequim, WA. Mr. M.M. Veselica (Supervisor, RTI Materials Handling Facility), Mr. D.L. Hubbard, and Mr. R.A. Price provided receipt and disbursement of dose formulations at RTI. Animal care was provided by Dr. D.B. Feldman, DVM, ACLAM, Veterinarian, and Mr. F.N. Ali, Manager of RTI Animal Research Facility. RTI Quality Assurance personnel were Ms. D.J. Smith, Ms. M.D. Phillips, and Ms. T.M. Kenney. Ms. Christine Sexsmith, QA Consultant, audited the draft report.

The final report was prepared by Dr. J.D. George and Dr. R.W. Tyl, with assistance from Ms. C.B. Myers on data compilation and statistical analyses, and by Mr. T.W. Wiley on data entry. Ms. C.B. Myers was responsible for all activities concerning organization and custody of the study records. Ms. M.C. Marr was responsible for archiving the study records. Ms. D.B. Bynum provided secretarial assistance.

Analytical Report and Protocol/Amendments

The bulk chemical and dose formulation analytical reports were prepared and signed by the author(s) and included as appendices to the final report. The protocol and two amendments detailing the design and conduct of the study are presented in Appendix III of this final report.

Storage of Records

All original data sheets and records collected during the present study will be stored in the RTI Archives, under the control of the RTI Chemistry and Life Sciences Archivist, and remain the responsibility of RTI. Worksheets and computer printouts, which were generated in the statistical analysis of data, are stored in the RTI Archives. Copies of this report are filed with the RTI Archives and with Battelle. All remaining dose formulations were shipped back to the sponsor. Records and samples from this study in RTI Archives may be released to the Sponsor upon written request.

Compliance

All records, data, biological specimens, and reports will be maintained in storage for the time period specified by the contract or for as long as the quality of the preparation affords evaluation, whichever is less. Quality control (QC) and quality assurance (QA) procedures followed those

outlined in the Quality Assurance Project Plan (QAPP) prepared for this study, and in accordance with the Quality Management Plan (QMP) for this project. The RTI Animal Research Facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), International. At all times, animals were housed, handled, and used according to the NRC Guide (NRC, 1996).

RESULTS

Dose Formulations

Predosing analysis of VIN in corn oil indicated that the formulations were 97.5-97.7% of the target concentrations, whereas DBP formulations assayed at 94.1-94.3% of the target concentrations (Table 1). Aliquots of the dosing solutions and the control formulation were scheduled to be taken on the first day of dosing (first gd 6), and on the first pnd 0, 7, 14, and 20. Aliquots for gd 6, pnd 7, and 14 were taken on the first day of dosing for each designated pnd day. Due to an oversight, the first pnd 0 and 20 samples were not taken until pnd 1 and 21, respectively. This was not considered to have an adverse effect on the study. In addition, the dosing bottles, with the remainder of the dosing solutions, were saved after dosing was completed. These first-day aliquots and postdosing samples in the dosing bottles were shipped back to the sponsor and were analyzed for test chemical concentration. The VIN first-day samples assayed at 93.6-94.6% of the target concentrations, whereas the postdosing samples assayed at 94.8-95.8% of the target concentration (Table 1). The first dosing day samples of DBP assayed at 94.4-94.5% of the target concentrations, whereas the postdosing samples assayed at 94.1-94.3% of the target concentrations (Table 1). The estimated limits of detection are 0.57 µg/ml for DBP and 0.11 µg/ml for VIN. Additional analytical data are presented in Appendix II.

Vinclozolin Results

F0 Female Observations (VIN)

Fate of F0 Females (VIN)

One F0 female in the 100 mg/kg/day VIN group was found dead on pnd 0 due to dystocia, with three delivered pups, and ten pups retained *in utero* (Tables 2 and A-1). In addition, one dam in the control group was euthanized on pnd 3 because her entire litter was missing and presumed dead on pnd 1. Females that were euthanized on gd 26/27, because they had not delivered, included one in the vehicle control group and two in the 100 mg/kg/day VIN group. Therefore, there were 23 females in the vehicle control group, and25 and 22 females in the 50 and 100 mg/kg/day VIN group, respectively, that delivered and reared litters to scheduled sacrifice on pnd 21 (Tables 2 and A-1).

F0 Female Gestation (VIN)

There were no significant differences in the F0 maternal body weights for the VIN- treated animals compared to the control animals on gd 6, 9, 15, 18, or 20 (Tables 3 and A-2). On gd 12, a decreasing linear trend was noted, but there was no treatment-related effect by pairwise comparisons to the concurrent control group value. Maternal body weight change exhibited a decreasing linear trend for gd 6 to 9, 9 to 12, 15 to 18, 18 to 20, and 6 to 20, with the high dose animals gaining significantly less weight for the periods of gd 6 to 9, 18 to 20, and 6 to 20 (gestational treatment period). For gd 15 to 18, the high dose VIN animals gained more weight than the control animals. No significant treatment-related effects were observed across groups for body weight change during the gestational period (gd 0 to 20) (Table 3). There were no treatment-related effects across groups for maternal feed consumption (g/day or g/kg/day) prior to initiation of treatment (Tables 4 and A-3). Feed consumption values expressed as g/day or g/kg/day were significantly decreased in the 100 mg/kg/day VIN group on gd 6 to 9, 9 to 12, 6 to 20 (gestational treatment period), and gd 0 to 20 (gestational period), and in the 50 mg/kg/day group on gd 9 to 12. For gd 12 to 15, 15 to 18, or 18 to 20, there were no treatment-related effects across groups for maternal feed consumption when expressed as g/day or g/kg/day (Table 4). Clinical observations (Tables 5 and A-4) in the control and VIN-treated groups during gestation included weight loss in five females, alopecia in four females, efflux of dosing compound in two females, and piloerection in one female in the control group; rooting postdosing in ten females, alopecia in four females, piloerection in three females, weight loss in two females, and efflux of dosing compound and salivation prior to dosing in one female each in the 50 mg/kg/day VIN group; and rooting postdosing in 15 females, weight loss in 12 females, piloerection in eight females, alopecia in seven females, efflux of dosing compound, salivation prior to dosing, and scabs on one female each at 100 mg/kg/day VIN.

F0 Female Lactation (VIN)

F0 maternal lactational body weights in the VIN-treated animals exhibited a dose-related decreasing trend on pnd 0, 4, 7, and 14 (Tables 6 and A-5). Maternal body weight was significantly decreased in the 100 mg/kg/day VIN group on pnd 0, 4, and 7, and in the 50 mg/kg/day group on pnd 7 and 14. Maternal lactational body weight was not significantly affected by VIN treatment on pnd 21. Maternal lactational body weight change did not exhibit any consistent treatment effect for pnd 0 to 4, 4 to 7, 7 to 14, or 14 to 21 (Table 6). However, when body weight change was calculated for the entire lactational period (pnd 0 to 21), the 100 mg/kg/day VIN group exhibited a significantly larger change in body weight (i.e., increase) compared to the control group. Examination of the data suggests that this increased weight gain in the high dose group was a rebound effect. F0 maternal lactational feed consumption, expressed as g/day and g/kg/day, was largely unaffected by VIN treatment, with only feed consumption from pnd 0 to 4 (g/day) exhibiting a decreasing dose-related trend (Tables 7 and A-6). Maternal clinical observations (Tables 8 and A-7) during lactation included alopecia in six females, rust-colored fur in four females, and piloerection, reddish vaginal discharge, and weight loss due to a faulty water bottle in one female each at 0 mg/kg/day; alopecia in eight

females, rooting postdosing in seven females, and efflux of the dosing compound, piloerection, and salivation prior to dosing in two females each at 50 mg/kg/day; and rooting postdosing in 11 females, alopecia and salivation prior to dosing in nine females each, piloerection in seven females, rust-colored fur in two females, chromodacryorrhea, efflux of the dosing solution, reddish vaginal discharge, and overgrown teeth in one female each at 100 mg/kg/day (Table 8). One female was found dead on pnd 0 (Table 8).

F0 Female Reproductive Indices (VIN)

Pregnancy was confirmed in 24, 25, and 23 F0 females in the 0, 50, and 100 mg/kg/day VIN groups, respectively (Tables 9 and A-8). F0 females in the control and VIN-treated groups were similar with respect to fertility index (Table 9). In addition, there was no effect of exposure to VIN on gestational index or gestational length. There were also no differences across groups for the number of total implantation sites per litter, percent postimplantation loss per litter, or number of total, live, or dead pups per litter at birth (pnd 0) (Table 9).

Unscheduled F0 Female Necropsy (VIN)

The one F0 female in the high dose group that was found dead on pnd 0 apparently died of dystocia. Tan patches on the liver, mottled lungs, multiple red foci on the thymus, and reddened trachea were noted (Tables 8 and A-7). Blood-stained fur around the mouth and vagina, and no food in the stomach were also noted; she had ten retained pups in her uterus.

Scheduled F0 Female Necropsy (VIN)

Necropsy findings for F0 females at scheduled sacrifice were minimal and consisted of alopecia on different parts of the body, occurring in one to three animals in each treatment group (Tables 8 and A-7). Rust-colored fur was noted in two control animals and two high dose animals (Table 8 and A-7).

F1 Observations (VIN)

Fate of F1 Animals During Lactation (VIN)

There were 24, 25, and 22 live litters on pnd 0 at 0, 50, and 100 mg/kg/day, respectively (Tables 9 and A-8). F1 pup mortality for pnd 0-21 was 14, 13, and 23 (including three pups euthanized because their dam was found dead) pups at 0, 50, and 100 mg/kg/day, respectively. By weaning on pnd 21, there were 23, 25, and 22 F1 litters at 0, 50, and 100 mg/kg/day, respectively (Tables 10 and A-8).

Observations of F1 Pups During Lactation (VIN)

Live birth and stillbirth indices were unaffected across all groups, as were the survival indices for pnd 4, 7, 14, and 21, and the lactational index for pnd 4 to 21 (Table 9 and A-8). The mean number of live pups per litter for pnd 0, 4, 7, 14, and 21 was unaffected across all groups (Tables 10 and A-8). Mean F1 female AGDs (absolute or adjusted for body weight) per litter on pnd 0 were equivalent across all groups. However, mean F1 male AGDs (absolute or adjusted for body weight) exhibited a significant dose-related decrease, with AGDs in both the 50 and 100 mg/kg/day VIN groups significantly shorter than the control group (Tables 10 and A-9). Average absolute F1 male AGD was 2.18 mm in the control group, compared to 1.96 and 1.56 mm in the 50 and 100 mg/kg/day VIN groups, respectively. Average adjusted F1 male AGD was 2.15 mm in the control group, compared to 1.94 and 1.58 mm in the 50 and 100 mg/kg/day VIN groups, respectively. Mean F1 pup body weights per litter (sexes combined or separately) were significantly depressed in the 100 mg/kg/day dose group on pnd 0, 4, 7, and 14 (Tables 10 and A-10). On pnd 21, mean F1 pup body weight per litter for the sexes combined and for males was significantly decreased at the high dose. The sex ratio (percent male pups/litter) was significantly decreased at 100 mg/kg/day VIN on pnd 0 but not on pnd 4 (Table 10). Average number of nipples/areolae per male pup and the percent male pups with one or more nipples/areolae were significantly increased in both of the VIN-treated groups compared to the control group (Tables 10 and A-11). Clinical signs observed for pnd 0 through 21, other than dead or missing (presumed dead) pups, were minimal and included a white area on the abdomen (1) in the vehicle control group, hematoma (1), not using hind limbs (1), and left eye not completely open on pnd 14 (1) in the 50 mg/kg/day group, and bruised hindlimbs (1), necrotic tail tip (1), and dehydration (7) in the 100 mg/kg/day group (Tables 11 and A-12).

Unscheduled F1 Pup Necropsy During Lactation (VIN)

Necropsy findings of F1 pups found dead or euthanized moribund on pnd 0-21 included the usual findings: pups that died on pnd 0 exhibited open (fetal state) or closed ductus arteriosus (postnatal state), no air (fetal state) or air (postnatal state) in lungs, no or little milk in stomach, and autolysis of abdominal organs (Tables 12 and A-13). Necropsy findings for F1 pups culled on pnd 4 indicated that the number of pups missexed in the high dose group was 7.8 (3.95 ± 2.19%, out of 75 pups), compared to 0 in the control and low dose groups (Tables 12 and A-13).

Scheduled F1 Pup Necropsy on Pnd 21(VIN)

After selection, there were 74, 82, and 65 F1 male offspring evaluated at scheduled sacrifice on pnd 21 at 0, 50, and 100 mg/kg/day, respectively (Tables 13, A-15, and A-16). F1 male body weight at sacrifice on pnd 21 was significantly reduced at 100 mg/kg/day VIN (Table 13). AGD (absolute or adjusted for body weight) was significantly reduced in both VIN dose groups. The average number of areolae or nipples per pup and the percent male pups with one or more nipples or areolae was increased in both VIN-treated groups. The percentages of male pups necropsied on pnd 21 with hypospadias (83%) or cleft phallus (44%) were increased at the high dose, compared

to 0% for both findings in the control group (Tables 13 and A-15). No effect of VIN treatment was seen on the incidence of epispadias or soiled inguinal region (0% incidence for control and treated groups). In addition, no pnd 21 F1 males were observed to have a partially or entirely detached prepuce, and 100% of the males at 0, 50 and 100 mg/kg were observed to have at least one gubernacular cord, which are appropriate findings at this age (Tables 13 and A-15). There was no treatment effect for the percent males with at least one cranial suspensory ligament or on the length of the right or left gubernacular cord (Table 13). Observation of at least one cranial suspensory ligament occurred in 0, 1.3, and 0% of the male pups at 0, 50 and 100 mg/kg VIN, respectively, on pnd 21.

Mean absolute weights for liver and paired adrenal glands were equivalent across treatment groups for the F1 males on pnd 21. Absolute right or left testis, right or left corpus plus caput epididymis, right or left cauda epididymis, seminal vesicle with coagulating glands, prostate (dorsal, ventral, or whole), levator ani plus bulbocavernosus complex (LABC), and paired Cowper's gland weights were significantly decreased at 100 mg/kg/day VIN. Right corpus plus caput epididymis, seminal vesicle with coagulating glands, ventral prostate, whole prostate, and LABC weights were also decreased at 50 mg/kg/day VIN. Mean adjusted weights (adjusted with respect to body weight) for paired adrenal glands, left testis, and Cowper's gland were equivalent across treatment groups for the F1 males on pnd 21 (Tables 13 and A-16). Adjusted liver weight was increased compared to the controls, whereas adjusted right or left corpus plus caput epididymis, right or left cauda epididymis, seminal vesicle with coagulating glands, prostate (dorsal, ventral, or whole), and LABC were significantly decreased at 100 mg/kg/day VIN. Adjusted right testis weight was increased, whereas adjusted right corpus plus caput epididymis, seminal vesicle with coagulating glands, ventral prostate, whole prostate, and LABC weights were decreased at 50 mg/kg/day VIN (Table 13).

Necropsy findings for F1 pups on pnd 21 at scheduled necropsy included, most notably, missing Cowper's glands (bilateral) in 0, 5, and 43 pups in the 0, 50, and 100 mg/kg/day VIN groups and missing dorsal prostate gland, which occurred in 21 of the high dose animals, compared to 0 in the control and low dose groups (Tables 14 and A-17). Missing right or left Cowper's glands was observed in one or two animals (left; 50 or 100 mg/kg/day, respectively) or in three or two animals (right; 50 or 100 mg/kg/day, respectively). Findings observed only in the high dose animals included missing right caput (1), missing left corpus and caput (1), missing or spongy LABC (1 each), penis reduced in size or reduced in size and soft (3 and 1), missing ventral prostate (4), missing left lobe of ventral prostate (1), misshapen seminal vesicles (6), misshapen and reduced seminal vesicles (1), and testis in the abdominal cavity (1-2) (Table 14). Necropsy of F1 females on pnd 21 indicated that 4% of the female pups in the high dose group were missexed (i.e., identified as females, but internally were males). F1 females in the control and 50 mg/kg/day VIN group were all correctly sexed (Tables 14 and A-18).

Fate of Pnd 95 F1 Males(VIN)

During the postwean period, one high dose F1 male was euthanized moribund on pnd 64, and another high dose F1 male was found dead on pnd 80 (Tables 15 and A-19). There were 82, 95, and 74 F1 males at 0, 50, and 100 mg/kg/day, respectively, evaluated at scheduled necropsy on pnd 95 (Table 15).

F1 Male Post Wean Observations (VIN)

Absolute F1 male age at acquisition of preputial separation exhibited a dose-related increasing trend, with both the 50 and 100 mg/kg/day VIN-treated males achieving preputial separation later than the control males (Tables 16 and A-20). The absolute mean postnatal day of preputial separation was 40.6 days for the 50 mg/kg/day group and 42.2 days for the 100 mg/kg/day dose group, compared to 40.0 days for the vehicle control group. When postnatal day of preputial separation was adjusted for body weight, only the high dose group was significantly delayed compared to the control group (42.1 vs. 40.1 days, respectively; Table 16). F1 male body weight on the day of acquisition of preputial separation increased in a dose-related manner and was significantly greater at the high dose.

F1 male body weight was significantly reduced at 100 mg/kg/day VIN throughout the postwean period, up until pnd 95 (Tables 17, A-21, and A-22). Differences in body weight change were minimal, with the high dose group gaining significantly less weight than the control group for the periods of pnd 25 to 28, 32 to 35, and 42 to 46. A dose-related increasing trend was observed for body weight change on pnd 84 to 88, but there was no overall treatment effect. Body weight change for pnd 46 to 49 exhibited an overall treatment effect but no dose-related pattern or pairwise differences between the treated and the control groups (Table 17).

Feed consumption (expressed as g/day) exhibited a decreasing trend and overall treatment effect on pnd 21-25, with the high dose group slightly but significantly lower than the controls (10.5 g/day vs. 11.2 g/day; Tables 18, A-23, and A-24). A decreasing trend was also noted for feed consumption (g/day) for pnd 42 to 46, but there was no overall treatment effect or pairwise differences between the treated groups and the contols. Feed consumption for pnd 25 to 28 exhibited a significant overall treatment effect but no dose-related trend (Table 18). There were no significant treatment effects on feed consumption (g/day) from pnd 28 through pnd 42, pnd 46 through pnd 74, pnd 77 to 81, or for the postwean period as a whole (pnd 21 to 95). However, feed consumption (g/day) exhibited a dose-related increasing trend and overall treatment effect for pnd 74 to 77, 81 to 84, 84 to 88, 88 to 91, and 91 to 95, with the high dose group significantly higher compared to the control group (Table 18). When feed consumption was expressed as g/kg/day, feed consumption was significantly increased over the control group at every time period except pnd 21-25 (Table 18). Feed consumption was increased at the lower dose for pnd 25 to 28, but not at any other time point. Clinical observations included sore(s) in four males, dehydration or rust-colored fur in two males each, alopecia, chromodacryorrhea, cleft phallus, torn ear, and weight loss due to a sipper tube malfunction in one male each at 0 mg/kg/day; cleft phallus in 48 males, hypospadias in 14 males, sore(s) in six males, dehydration in two males, and torn ear in one male

at 50 mg/kg/day; and cleft phallus in 75 males, hypospadias in 74 males, vaginal pouch in 40 males, undescended testes in 17 males, torn ear or sore(s) in three males, chromodacryorrhea in two males, alopecia, dehydration, red ear, morbundity, pinpoint pupils and tearing, lethargy, bleeding penis, prostration, rectal discharge, labored breathing, and death in one male each at 100 mg/kg/day (Tables 19 and A-25).

Unscheduled PostWean F1 Male Necropsy (VIN)

Necropsy of the one high dose male sacrificed moribund and the one male found dead indicated that they both had cleft phallus, hypospadias, and reduced seminal vesicle size (Table 21). Missing Cowper's glands, missing LABC, missing or reduced prostate, undescended testes, and vaginal pouch were also observed in one or the other of these pups (Tables 21 and A-28).

Scheduled Necropsy of F1 Males on PND 95 (VIN)

At necropsy on pnd 95, the average body weight of the F1 males was significantly lower at 100 mg/kg/day (Tables 20 and A-27). Average AGD (absolute and adjusted for body weight) exhibited a dose-related decreasing trend and overall treatment effect, and was significantly decreased at both 50 and 100 mg/kg/day. The average adjusted AGD was 35.8 mm and 30.0 mm for the 50 and 100 mg/kg/day groups, compared to 37.4 mm for the control group (Tables 20 and A-26). The incidence of control males with one or more nipples or areolae, hypospadias, epispadias, or soiled inguinal region was 0%. However, the incidence of these parameters and cleft phallus (control = 1.2%) was clearly increased after treatment with VIN. The number of males with one or more nipples or one or more areolae exhibited a dose-related increasing trend and overall treatment effect, with significant increases at both 50 and 100 mg/kg/day. Approximately 50 - 59% of the low dose animals and 95 - 97% of the high dose animals had one or more nipples or areolae on pnd 95, compared to 0% for the control group (Tables 20 and A-26). The percent males with hypospadias, epispadias, cleft phallus, or soiled inguinal region also increased in a dose-related manner. A significant increase in the percent affected males in both VIN-treated groups compared to the control group was observed for hypospadias and cleft phallus, reaching 99 -100% affected at the high dose (Table 20). Increased incidence of epispadias and soiled inguinal region of 15 and 51%, respectively, was observed at 100 mg/kg/day, but not at 50 mg/kg/day. The percent males with a partially or entirely attached prepuce was not affected by VIN treatment, nor was there a treatment effect for the percent males with at least one cranial suspensory ligament (Table 20). The incidence of at least one cranial ligament was 0, 0, and 1.35% in the 0, 50, and 100 mg/kg/day groups, respectively. The percent males with at least one gubernacular cord exhibited an increasing dose-related trend but no pairwise differences between the treated groups and the control group (Table 20). The percent males with at least one gubernacular cord was 6.2, 11.6, and 17.6 in the 0, 50, and 100 mg/kg/day groups, respectively. The average length of the gubernacular cord was not affected by treatment. Mean absolute weights for liver and paired adrenal glands were equivalent across treatment groups for the F1 males on pnd 95 (Tables 20 and A-27).

Absolute right or left testis, right or left corpus plus caput epididymis, right or left cauda epididymis, seminal vesicle with coagulating glands, prostate (dorsal, ventral, or whole), levator ani plus bulbocavernosus complex (LABC), and paired Cowper's gland weights exhibited a decreasing linear trend and overall treatment effect, and were significantly decreased at 100 mg/kg/day VIN. Decreased right cauda epididymis and LABC weight were also observed at 50 mg/kg/day VIN. Mean adjusted weights (adjusted with respect to terminal body weight) for liver, paired adrenal glands, right or left testis, right or left corpus plus caput epididymis, right or left cauda epididymis, seminal vesicle with coagulating glands, prostate (dorsal, ventral, or whole), LABC and Cowper's glands also exhibited a decreasing trend and overall treatment effect and were significantly decreased at the higher dose. Adjusted LABC weight was also decreased at 50 mg/kg/day VIN. No other significant differences from controls were observed for adjusted organ weights at 50 mg/kg/day, although all the values at this dose were decreased slightly (Table 20).

Necropsy findings for F1 pups at scheduled necropsy on pnd 95 were more varied than those observed at pnd 21, and included missing Cowper's glands (bilateral) in 0, 3, and 49 pups, reduced seminal vesicles in 0, 3, and 50 pups, vaginal pouch in 0, 2, and 43 pups, reduced ventral prostate in 0, 4, and 40 pups, LABC with reduced size in 0, 2, and 36 pups, glans penis not entirely detached in 0, 3, and 20 pups, reduced dorsal prostate in 0, 2, and 19 pups, missing dorsal prostate in 0, 0, and 17 pups, missing ventral prostate in 0, 0, and 12 pups, and testis reduced and/or undescended in 1, 2, and 19 pups at 0, 50, and 100 mg/kg/day, respectively (Tables 21 and A-28). Additional observations are presented in Table 21 and A-28).

DBP Results

F0 Female Observations (DBP)

Fate of F0 Females (DBP)

Females that were euthanized on gd 26/27, because they had not delivered, included one in the vehicle control group (as noted above), two in the 100 mg/kg/day DBP group, and one in the 500 mg/kg/day DBP group. One female at 0 mg/kg was euthanized on pnd 3 since her entire litter was missing on pnd 1 and presumed dead and cannibalized (Tables 2 and A-1). Therefore, there were 23, 23, and 24 F0 females in the vehicle control group, 100, and 500 mg/kg/day DBP group, respectively, that delivered and reared litters to scheduled sacrifice on pnd 21.

F0 Female Gestation (DBP)

There were no significant differences in the F0 maternal body weights for the DBP- treated animals compared to the control animals on gd 0, 6 , 9, or 12 (Tables 3 and A-2). On gd 15, a decreasing linear trend was noted, but there were no significant treatment-related effects by pairwise comparisons compared to the control group rats. Maternal body weight significantly decreased in a dose-related manner on gd 18 and 20 also, with the 500 mg/kg/day DBP group significantly below the control group (Table 3). Maternal body weight change exhibited a

decreasing linear trend for gd 6 to 9, 15 to 18, 18 to 20, 6 to 20 (treatment period), and 0 to 20 (gestation period), with the high dose animals gaining significantly less weight for the periods of gd 18 to 20, 6 to 20, and 0 to 20. There were no treatment-related effects across groups for maternal feed consumption (g/day or g/kg/day) prior to initiation of treatment (Tables 4 and A-3). Feed consumption values expressed as g/day exhibited a decreasing trend for gd 6 to 9, 9 to 12, 12 to 15, 15 to 18, 6 to 20, and 0 to 20 (Table 4). Feed consumption (g/day) was significantly decreased in the 500 mg/kg/day DBP group on gd 6 to 9, 15 to 18, and 6 to 20 (treatment period). Feed consumption on gd 18 to 20 exhibited no treatment-related effects. When maternal feed consumption was expressed as g/kg/day, a decreasing trend was noted for gd 6 to 9, 12 to 15, 15 to 18, and 6 to 20 (Table 4). Feed consumption (g/kg/day) was significantly decreased at 500 mg/kg/day DBP for gd 6 to 9 (Table 4). Maternal feed consumption (g/kg/day) was increased compared to the control group at 100 mg/kg/day DBP for gd 15 to 18. No effect of DBP treatment was observed for maternal feed consumption (g/kg/day) on gd 9 to 12, 18 to 20, or 0 to 20 (Table 4). Clinical observations in the control and DBP-treated groups during gestation included weight loss in five females, alopecia in four females, efflux of dosing compound in two females, and piloerection in one female in the control group; alopecia in nine females, weight loss in five females, efflux of dosing compound or rooting postdosing in four females each, piloerection in three females, and sore(s) or struggling during dosing in one female each in the 100 mg/kg/day DBP group; and rooting postdosing in nine females, alopecia in seven females, weight loss in six females, piloerection in five females, efflux of dosing compound, salivation prior to dosing, scabs, sore(s), rough coat or umbilical hernia in one female each at 500 mg/kg/day DBP (Tables 5 and A4).

F0 Lactation (DBP)

F0 maternal lactational body weights in the DBP-treated animals exhibited a dose-related decreasing trend on pnd 4 and 7, with a significant depression in maternal body weight at 500 mg/kg/day DBP on pnd 7 (Tables 6 and A5). Maternal lactational body weight was not significantly affected by DBP treatment on pnd 0, 14, or 21. Maternal lactational body weight change did not exhibit any consistent treatment effect for any of the intervals measured (Table 6). However, significant pairwise differences from the control group were noted at 100 mg/kg/day DBP for pnd 0 to 4 (decrease) and 7 to 14 (increase). F0 maternal lactational feed consumption, expressed as g/day and g/kg/day, was largely unaffected by DBP treatment, with feed consumption from pnd 0 to 4 and 7 to 14 (g/day) exhibiting a decreasing dose-related trend (Tables 7 and A6). Overall treatment effects were observed for maternal feed consumption on pnd 7 to 14 (g/day and g/kg/day), but there were no pairwise differences between the values in the DBP-treated groups and in the control group for these periods (Table 7). Maternal clinical observations during lactation included alopecia in six females, rust-colored fur in four females, and piloerection, weight loss, and reddish vaginal discharge in one female each at 0 mg/kg/day, alopecia in 11 females, rust-colored fur in nine females, efflux of dosing solution or piloerection in four females, rooting postdosing in two females, and salivation prior to dosing or sore(s) in one female each at 100 mg/kg/day; and alopecia in eight females, piloerection in four females, rust-colored fur in three females, rooting

postdosing in two females, and chromodacryorrhea, red ear(s), efflux of dosing solution, swollen genital area, salivation prior to dosing, and sore(s) in one female each at 500 mg/kg/day (Tables 8 and A7).

F0 Female Reproductive Indices (DBP)

Pregnancy was confirmed in 24, 24, and 25 F0 females in the 0, 100, and 500 mg/kg/day DBP groups, respectively (Tables 9 and A8). F0 control and DBP-treated groups were similar with respect to fertility index, gestational index, and gestational length (Table 9). There were also no differences across groups for the number of total implantation sites per litter, percent postimplantation loss per litter, or number of total, live, or dead pups per litter at birth (pnd 0) (Table 9).

Unscheduled F0 Female Necropsy (DBP)

There were no unscheduled necropsies for the F0 females treated with DBP (Tables 8 and A-7).

Scheduled F0 Female Necropsy (DBP)

Necropsy findings for F0 females at scheduled sacrifice were minimal and consisted of alopecia on different parts of the body, occurring in four to eight animals in the control and treated groups (Tables 8 and A-7). Rust-colored fur was noted in two control animals, eight low dose animals, and three high dose animals. One retained pup in uterine horn and one pup lodged in the vagina were noted in one low dose F0 female (No. 4), and a mammary mass was noted in one low dose female (No. 123) (Table 8).

F1 Observations (DBP)

Fate of F1 Animals During Lactation (DBP)

There were 24, 23, and 24 live litters on pnd 0 at 0, 100, and 500 mg/kg/day, respectively (Tables 9 and A-8). F1 pup mortality for pnd 0-21 was 14, 16, and 36 pups at 0, 100, and 500 mg/kg/day, respectively. By weaning on pnd 21, there were 23 F1 litters each at 0, 100, and 500 mg/kg/day (Tables 10 and A-8).

Observations of F1 Pups During Lactation (DBP)

Live birth and stillbirth indices were unaffected across all groups, as were the survival indices for pnd 7, 14, and 21, and the lactational index for pnd 4 to 21 (Tables 9 and A-8). The survival index for pnd 4 exhibited a decreasing trend and overall treatment effect but no significant difference between the control and the 100 or 500 mg/kg/day DBP groups (Table 9). The mean number of live pups per litter for pnd 0, 7, 14, and 21 was unaffected across all groups (Tables 10 and A-8). A decreasing linear trend was observed for the number of live pups per litter on pnd 4, but there was no overall treatment effect. Absolute average F1 female AGDs per litter on pnd 0 exhibited a slight increasing trend, with the mean AGD in the control group measuring 1.07 mm, compared to 1.13 mm and 1.15 mm for the 100 and 500 mg/kg/day groups, respectively (Tables 10 and A-9). When F1 female AGDs were adjusted for body weight, an increasing linear trend was observed, with the high dose exhibiting significantly longer AGDs than the control group (1.19 mm vs. 1.06 for controls; Table 10). However, mean F1 male AGDs (absolute or adjusted for body weight) exhibited a significant dose-related decrease, with AGDs in the 500 mg/kg/day DBP group significantly shorter than the control group (Table 10). Average absolute F1 male AGD was 2.18 mm in the control group, compared to 2.16 and 1.91 mm in the 100 and 500 mg/kg/day DBP groups, respectively. Average adjusted F1 male AGD was 2.15 mm in the control group, compared to 2.14 and 1.95 mm in the 100 and 500 mg/kg/day DBP groups, respectively. Mean F1 pup body weights per litter (sexes combined or separately) were significantly depressed in the 500 mg/kg/day dose group on pnd 0, 4, 7, 14, and 21 (Tables 10 and A-10). The sex ratio (percent male pups/litter) was unaffected by DBP treatment on both pnd 0 and 4 (Table 10). The average number of nipples per male pup and the percent male pups with one or more nipples were significantly increased in the 500 mg/kg/day DBP group compared to the control group (Tables 10 and A-11). With respect to areolae, the number per pup and percent pups with one or more were increased at both 100 and 500 mg/kg/day DBP. Clinical signs observed for pnd 0 through 21, other than dead or missing (presumed dead) pups, were minimal and included a white area on the abdomen (1) in the vehicle control group; hematoma (2), sore (s), scabs, and/or scars at the umbilicus (11), and small, pale, and dehydrated (1) at 100 mg/kg/day; and missing tail (1), clubbed left hindlimb (1), and pale in color (1) at 500 mg/kg/day DBP (Tables 11 and A-12).

Unscheduled Necropsy of F1 Pups During Lactation (DBP)

Necropsy findings of F1 pups found dead or euthanized moribund on pnd 0-21 included the usual findings: pups that died on pnd 0 exhibited open (fetal state) or closed ductus arteriosus (postnatal state), no air (fetal state) or air (postnatal state) in lungs, no or little milk in stomach, and autolysis of abdominal organs (Tables 12 and A-13). Pups that died later during lactation primarily exhibited no or little milk in stomach and autolysis of abdominal organs. Necropsy findings for F1 pups culled on pnd 4 indicated that all pups in the control and 100 and 500 mg/kg/day DBP groups were sexed correctly (Table 12).

Necropsy of F1 Pups at Pnd 21 (DBP)

After selection on pnd 21, there were 74, 71, and 65 F1 male offspring evaluated at scheduled sacrifice on pnd 21 at 0, 100, and 500 mg/kg/day DBP, respectively (Tables 13, A-15, and A-16). F1 male body weight at sacrifice on pnd 21 exhibited a dose-related decreasing trend and was significantly reduced at 500 mg/kg/day DBP (Tables 13 and A-16). AGD (absolute or adjusted for body weight) was significantly reduced at the high dose of DBP. Similarly, the average number of areolae or nipples per pup and the percent male pups with one or more nipples or areolae were also increased at the high dose of DBP. The percent male pups necropsied on pnd 21 with cleft phallus was increased at the high dose at 3%, compared to 0% for the control group (Tables 13 and A-15). DBP treatment resulted in an increasing trend for the incidence of hypospadias, but no effect on the incidence of epispadias, or soiled inguinal region. No pnd 21 F1 males were observed to have a partially or entirely detached prepuce, which is an appropriate observation at this age (Table 13), nor was there a treatment effect for the percent males with at least cranial suspensory ligament or the length of the right or left gubernacular cord (Table 13). The percent males with at least one gubernacular cord exhibited a dose-related decreasing trend, with the 500 mg/kg/day DBP group significantly less than the control group (91% vs 100%).

Mean organ weights (absolute or adjusted with respect to body weight) for paired adrenal, absolute right or left testis, right or left corpus plus caput epididymis, right or left cauda epididymis, seminal vesicle with coagulating glands, prostate (dorsal, ventral, or whole), levator ani plus bulbocavernosus complex (LABC), and paired Cowper's gland weights were significantly decreased at 500 mg/kg/day DBP, whereas adjusted liver weight was increased. At 100 mg/kg/day DBP, adjusted liver weight was increased, whereas absolute right or left corpus plus caput epididymis, and paired Cowper's gland weights were decreased, as were adjusted right or left testis, right or left corpus plus caput epdidymis, and paired Cowper's gland weight (Tables 13 and A-16).

Necropsy findings for F1 pups on pnd 21 at scheduled necropsy included, most notably, missing Cowper's glands (bilateral) in 0, 1, and 6 pups in the 0, 100, and 500 mg/kg/day DBP groups (Tables 14 and A-17). Hydronephrosis (right kidney) was observed in 2, 3, or 5 animals in the 0, 100, or 500 mg/kg/day DBP. High dose males exhibited an array of epididymal anomalies, including missing caput (right or bilateral, 1 each), missing corpus and caput (right, left, or bilateral, 1 each), missing corpus and caput on one side and whole epididymis on the other side (4), missing whole epididymis (right or bilateral, 2 each), epididymis partially developed or reduced in size (5). Other findings observed only in the high dose animals included missing left Cowper's gland (1), missing prostate (dorsal, ventral, ventral left lobe; 1 each), missing or misshapen seminal vesicles (7 animals), testis undescended or in abdominal cavity (4), and testis reduced and brown in color (1). Necropsy of F1 females on pnd 21 indicated that all females were correctly sexed (Tables 14 and A-18).

Fate of Pnd 95 F1 Males (DBP)

During the postwean period, one high dose F1 male was euthanized moribund on pnd 72, and another high dose F1 male was euthanized moribund on pnd 79 (Tables 15 and A-19). There were 82, 81, and 74 F1 males at 0, 100, and 500 mg/kg/day, respectively, evaluated at scheduled necropsy on pnd 95 (Table 15).

F1 Male Postwean Observations (DBP)

Absolute F1 male age at acquisition of preputial separation exhibited a dose-related increasing trend, with both the 100 and 500 mg/kg/day DBP-treated males achieving preputial separation later than the control males (Tables 16 and A-20). The absolute mean postnatal day of preputial separation was 40.7 days for the 100 mg/kg/day group and 42.2 days for the 500 mg/kg/day dose group, compared to 40.0 days for the vehicle control group. When postnatal day of preputial separation was adjusted for body weight, both DBP-treated groups were still significantly delayed compared to the control group in a dose-related manner (40.6 and 42.3 at 100 and 500 mg/kg/day, respectively, vs. 40.1 days for the control group; Table 16). F1 male body weight on the day of acquisition was not affected by DBP treatment (Table 16).

F1 male body weight exhibited a dose-related decreasing trend and was significantly depressed at 500 mg/kg/day DBP during the postwean period from pnd 21 to 81, but not from pnd 84 to pnd 95 (Tables 17, A-21, and A-22). F1 male body weight at 100 mg/kg/day DBP was significantly decreased only on pnd 32. Differences in body weight change (pnd 21 to 95) were less pronounced, with the high dose group gaining significantly less weight than the control group for the periods of pnd 21 to 25, 25 to 28, 28 to 32, 32 to 35, and 42 to 46. F1 male body weight change was also significantly less than the control group at 100 mg/kg/day for pnd 25 to 28. A dose-related decreasing trend and overall treatment effect, with no significant pairwise differences between the treated groups and the control group, was observed for body weight change on pnd 35 to 39. Body weight change for pnd 74 to 77 exhibited an overall treatment effect but no dose-related pattern or pairwise differences between the treated and the control groups (Table 17). Finally, F1 male body weight change exhibited an overall treatment effect, with the 100 mg/kg/day group significantly greater than the control group on pnd 81 to 84 and 21 to 95 (Table 17).

Feed consumption (expressed as g/day) exhibited a decreasing trend and overall treatment effect on pnd 21-25, 25 to 28, 28 to 32, 32 to 35, 35 to 39, 39 to 42, and 42 to 46, with the high dose group significantly lower than the controls (Tables 18, A-23, and A-24). Feed consumption (g/day) was increased at 100 mg/kg/day on pnd 39 to 42. Feed consumption (g/day) exhibited a treatment effect that was determined by the 100 mg/kg/day DBP-treated group for all intervals from pnd 49 to pnd 95 and for pnd 21 to 95 (postweaning period) (Table 18). The 100 mg/kg/day DBP group was significantly increased for feed consumption (g/day) over the control group for all intervals from pnd 49 to pnd 95 and pnd 21 to 95. Significant linear trends were noted for feed consumption on pnd 46 to 49 and 49 to 53. Feed consumption at 500 mg/kg/day was increased for pnd 74 to 77 (Table 18). When feed consumption was expressed as g/kg/day, an increasing

trend and overall dose effect was noted for all intervals except pnd 21 to 25, 35 to 39, 63 to 67, and 91 to 95. For those intervals exhibiting both a significant trend and overall effect of treatment, feed consumption (g/kg/day) was increased compared to the control group in both the 100 and 500 mg/kg/day DBP groups, with the exception of pnd 25 to 28 and 28 to 32, for which only the higher dose was significantly increased. For pnd 21 to 25, a treatment effect was noted but no pairwise differences between the treated groups and the control group. Feed consumption (g/kg/day) on pnd 35 to 39, 63 to 67, and 91 to 95 exhibited a treatment effect, with both DBP groups significantly greater than the controls, although the pattern was not strictly dose related (Table 18).

Clinical observations included, most notably, cleft phallus in 1, 1, and 31 males in the 0, 100, and 500 mg/kg/day dose groups, and hypospadias in 0, 0, and 12 males at 0, 100, and 500 mg/kg/day DBP (Tables 19 and A-25). Other clinical observations included sore(s) in four males, dehydration or rust-colored fur in two males each, and alopecia, chromodacryorrhea, torn ear, and weight loss due to a sipper tube malfunction in one male each at 0 mg/kg/day; chromodacryorrhea or sore(s), in three males each, scar at the umbilicus in two males, and alopecia or penis pointed anteriorly in one male each at 100 mg/kg/day; and undescended testes in four males, vaginal pouch or dehydration in three males, ataxia, chromodacryorrhea or piloerection in two males, and scruffy appearance, red ear, dragging hind limb(s), skinny apearance, labored breathing, rust- colored fur, sore(s), or dark testes (external appearance) in one each of the males at 500 mg/kg/day DBP (Table 19).

Unscheduled Necropsy of Postwean F1 Males (DBP)

Necropsy of the two males sacrificed moribund indicated that they both had reduced epididymides (left or bilateral) and testes that were undescended and/or reduced in size (left or bilateral; Table 21). One male had additional urogenital anomalies, including cleft phallus, bilaterally missing Cowper's glands, hypospadias, missing LABC, missing dorsal and ventral prostate, and reduced seminal vesicles (Tables 21 and A-28). Other observations are noted in Table 21.

Scheduled Necropsy of F1 Males on Pnd 95 (DBP)

At necropsy on pnd 95, the average body weight of the F1 males did not exhibit any dose-related changes (Tables 20 and A-27). Average AGD (absolute) exhibited a dose-related decreasing trend and overall treatment effect, and was significantly decreased at 500 mg/kg/day DBP. The average absolute AGD was 37.0 and 33.0 mm for the 100 and 500 mg/kg/day groups, compared to 37.4 mm for the control group (Tables 20 and A-26). When AGD was adjusted for body weight, only the high dose was significantly less than the control group (33.1 mm vs. 37.4), although AGD for the low dose group was also slightly decreased at 36.8 mm (98.4% of the control value). The incidence of control males with one or more nipples or areolae, or hypospadias, was 0%. However, the incidence of these parameters and cleft phallus (control = 1.2%) was clearly increased after treatment with 500 mg/kg/day DBP. The number of males with one or more nipples

or one or more areolae exhibited a dose-related increasing trend and overall treatment effect, with significant increases at 500 mg/kg/day. Approximately 32 and 36% of the high dose animals had one or more nipples or areolae on pnd 95, compared to 0% for the control group (Table 20). The percent males with hypospadias or cleft phallus also increased in a dose-related manner. A significant increase in the percent affected males at 500 mg/kg/day DBP, compared to the control group, was observed for hypospadias and cleft phallus, reaching 16 and 35%, respectively, compared to 0 and 1.2 % in the control animals (Table 20). Epispadias or soiled inguinal region were not observed in any animals in the control or DBP treated groups. The percent males with a partially or entirely attached prepuce was not affected by DBP treatment, nor was there a treatment effect for the percent males with at least one gubernacular cord. The average length of the right or left gubernacular cord exhibited a significant trend and overall treatment effect, respectively, but these effects were not clearly dose-related (Table 20). The percent males with at least one cranial suspensory ligament was 0, 0, and 8.4 for the 0, 100, and 500 mg/kg/day DBP groups, which was a significant increase at the high dose (Table 20). Mean absolute weights for paired adrenal glands or Cowper's glands were equivalent across treatment groups for the F1 males on pnd 95 (Tables 20 and A-27). Absolute liver weight was significantly increased above the control group at 100 (but not 500) mg/kg/day. Absolute right or left testis, right or left corpus plus caput epididymis, right or left cauda epididymis, seminal vesicle with coagulating glands, prostate (dorsal, ventral, or whole), and LABC weights exhibited a decreasing linear trend and overall treatment effect, and were significantly decreased at 500 mg/kg/day DBP. Mean adjusted weights (adjusted with respect to terminal body weight) for liver was significantly increased at the high dose compared to the control animals, and adjusted paired adrenal gland and Cowper's gland weights were unaffected by DBP treatment. However, weights of all other tissues, including right or left testis, right or left corpus plus caput epididymis, right or left cauda epididymis, seminal vesicle with coagulating glands, prostate (dorsal, ventral, or whole), and LABC exhibited decreasing trends and overall treatment effects and were significantly decreased at the higher dose. With the exception of adjusted right cauda epididymis weight, no significant differences from controls were observed for adjusted organ weights at 100 mg/kg/day, and all of the values (except liver weight) at this dose were similar to those seen in control animals (Table 20). Adjusted right cauda epididymis weight was significantly lower than the control value.

Necropsy findings for F1 pups at scheduled necropsy on pnd 95 were more varied than those observed at pnd 21 and were primarily observed at the high dose (Tables 21 and A-28). They included anomalies of the Cowper's glands (12 males), epididymides (61 males), dorsal prostate (11 males), ventral prostate (11 males), seminal vesicles (32 males), and testis (56 males). Additional observations are presented in Tables 21 and A-28.

DISCUSSION

This study was designed to test the hypothesis that the current U.S. EPA OPPTS testing guideline for Reproduction and Fertility Effects (OPPTS 870.3800; U.S. EPA, 1998) is relatively insensitive for detection of male reproductive system malformations since it mandates necropsy of three animals/sex/litter on pnd 21 (at weaning), and only one animal/sex/litter retained through acquisition of puberty to adulthood, with functional and structural reproductive system evaluations and andrological endpoints assessed on these F1 animals (one per litter) as adults. A secondary hypothesis was whether specific effects and incidences of effects identified in the adults would be missed in the weanling sacrifice. These hypotheses was tested by employing two known potent antiandrogens – VIN and DBP – each at a high effect level and at a low effect level, based on U.S. EPA in-house data, with the F0 dams (25/group) dosed from gd 6 through pnd 20. The F1 litters were standardized on pnd 4 to ten pups with retention of all male F1 pups. AGD was recorded at birth, retained nipples/areolae were recorded on pnd 11-13, and AGD and retained nipples/areolae recorded at weaning and adult sacrifices. On pnd 21, approximately one-half of the F1 males per litter were carefully necropsied. On pnd 95, the remaining F1 males per litter were carefully necropsied.

The hypotheses can be broken down into a series of questions (and the answers from this study) as follows:

1. Can this study design and the performing laboratory detect both doses of both test compounds (VIN and DBP) as effect levels? Both doses of both test chemicals were detected as effect levels by pnd 13 from data on AGD at birth (both doses of VIN and the high dose of DBP)and on retention of areolae in preweanling males on pnd 11-13 (both doses of both test compounds). Interestingly, the incidence of nipples on pnd 11-13 males was significantly increased for both doses of VIN and for the high dose (but not the low dose) of DBP. Since retained nipples are not observed in control males, and retained areolae are (at 0-3.5% incidence in the performing laboratory), the anticipation was that retained nipples would be a more sensitive indicator of anti-androgenic activity than retained areolae. See Text Table 2 for AGDs and retained nipples and/or areolae from birth to pnd 95.
2. Were male reproductive system malformations detected at the pnd 21 necropsy for both test chemicals at both doses? *In utero*/lactational exposure to both doses of both test chemicals resulted in male reproductive system malformations in a dose-related incidence and severity. The male reproductive malformations at the low dose of DBP were biologically significant (never observed in controls) but clearly not statistically significant. They included missing Cowper's glands and presence of cranial suspensory ligaments (normally observed only in females). Text Table 3 presents the data in support of this answer.

Text Table 2. Anogenital Distance and Retention of Nipples and Areolae in F1 Males[a]

	Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
Parameter	0	50	100	100	500
Male absolute AGD (mm): pnd 0	2.18	1.96***	1.56***	2.16	1.91***
pnd 21	15.31	14.52*	11.01***	15.47	12.55***
pnd 95	37.44	35.81**	29.95***	37.00	32.96***
Female AGD (mm)					
pnd 0:					
absolute	1.07	1.10	1.09	1.13	1.15
adjusted	1.06	1.11	1.11	1.12	1.19**
Pnd 11-13					
No. nipples/male	0.00	0.43*	5.97***	0.02	0.99***
Males with ≥ 1 nipples[b]	0 (0.00)	11 (13.14)**	56 (86.76)***	0.9 (1.31)[c]	15 (22.86)***
No. areolae/male	0.23	6.43***	7.66***	0.94**	4.01***
Males with ≥ 1 areolae[b]	6 (7.69)[d]	77 (93.71)***	64 (97.79)***	21 (29.41)**	49 (75.71)***
Pnd 21					
No. nipples/male	0.0	0.46	4.36***	0.01	0.63***
Males with ≥ 1 nipple[b]	0 (0.0)	15 (18.29)**	59 (90.63)***	1 (1.43)	23 (35.38)***
No. areolae/male	0.0	0.73*	4.02***	0.0	0.71***
Males with ≥ 1 areolae[b]	0 (0.0)	25 (30.49)***	58 (89.06)***	0 (0.0)	16 (24.62)***
Pnd 95					
No. nipples/male	0.0	1.60***	5.86***	0.01	1.04***
Males with ≥ 1 nipple[b]	0 (0.0)	48 (50.53)***	70 (94.59)***	1 (1.23)	24 (32.43)***
No. areolae/male	0.0	2.14***	6.57***	0.04	1.14***
Males with ≥ 1 areolae[b]	0 (0.0)	56 (58.95)***	72 (97.30)***	2 (2.47)	27 (36.49)***

a Data taken from summary tables 10, 13, and 20.

[b] Data presented as number (%)

[c] Not statistically significantly increased

[d] The performing laboratory's historical control incidence range of % males with ≥ areola(e) is 0.0-3.7%

*, **, *** = $p < 0.05, 0.01, 0.001$ relative to concurrent control group value

Text Table 3. F1 Male Offspring Reproductive System Malformations at the Pnd 21 Necropsy

	Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
Parameter	0	50	100	100	500
No. pups	74	82	65	71	65
Cowper's gland missing:					
Left	0 (0.0)[a]	1 (1.2)	2 (3.1)	0 (0.0)	1 (1.5)
Right	0 (0.0)	3 (3.7)	2 (3.1)	0 (0.0)	0 (0.0)
Bilateral	0 (0.0)	5 (6.1)	43 (66.2)	1 (1.4)	6 (9.2)
Total	**0 (0.0)**	**9 (11.0)**	**47 (72.3)**	**1 (1.4)**	**7 (10.8)**
Epididymis(mides) missing, reduced in size:					
Left	0 (0.0)	0 (0.0)	1 (1.5)	0 (0.0)	8 (12.3)
Right	0 (0.0)	0 (0.0)	1 (1.5)	0 (0.0)	8 (12.3)
Bilateral	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (7.7)
Total	**0 (0.0)**	**0 (0.0)**	**2 (3.1)**	**0 (0.0)**	**21 (32.3)**
Epispadias	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Hypospadias	0 (0.0)	8.0 (9.7)	52 (80.0)	0 (0.0)	2 (3.1)
Males with $\geq$ 1 gubernacular cord	74 (100.0)	82 (100.0)	65 (100.0)	69 (97.2)	59 (90.8)
Males with $\geq$ 1 cranial suspensory ligament	0 (0.0)	1 (1.3)	0 (0.0)	2.2 (3.1)	2.2 (3.4)
Levator ani bulbo-cavernosus complex:					
Missing	0 (0.0)	0 (0.0)	1 (1.5)	0 (0.0)	0 (0.0)
Spongy	0 (0.0)	0 (0.0)	1 (1.5)	0 (0.0)	0 (0.0)
Penis reduced in size	0 (0.0)	0 (0.0)	3 (4.6)	0 (0.0)	0 (0.0)
Phallus, cleft	0 (0.0)	4 (4.9)	25 (38.5)	0 (0.0)	2 (3.1)
Prostate missing:					
Dorsal	0	0	21 (32.3)	0	1 (1.5)
Ventral	0	0	4 (6.2)	0	1 (1.5)
V left lobe	0	0	1 (1.5)	0	1 (1.5)
Total	**0 (0.0)**	**0 (0.0)**	**26 (40.0)**	**0**	**3 (4.6)**
Seminal vesicles:					
Missing	0 (0.0)	0 (0.0)	0 (0.0)	0	4 (6.2)

Parameter	Vehicle Control (mg/kg/day) 0	VIN (mg/kg/day) 50	VIN (mg/kg/day) 100	DBP (mg/kg/day) 100	DBP (mg/kg/day) 500
Misshapen	0 (0.0)	0 (0.0)	7 (10.8)	0	3 (4.6)
Total	**0 (0.0)**	**0 (0.0)**	**7 (10.8)**	**0**	**7 (10.8)**
Testes undescended	0 (0.0)	0 (0.0)	3 (4.6)	0	4 (6.1)
Hydronephrosis: [b]					
Left	0 (0.0)	1 (1.2)	0 (0.0)	0	0 (0.0)
Right	3 (4.0)	4 (4.9)	0 (0.0)	2 (2.8)	6 (9.2)
Bilateral	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.4)	0 (0.0)
Total	**3 (4.0)**	**5 (6.1)**	**0 (0.0)**	**3 (4.2)**	**6 (9.2)**

[a] Number (and %) with the indicated finding. A male may be counted more than once if he exhibited more than one malformation.

[b] The incidence of hydronephrosis, a common finding in male CD® (Sprague-Dawley) rats is provided for internal quality control. There was no chemical- or dose-related incidence.

3. Were male reproductive system malformations detected at pnd 95 for both test chemicals at both doses? *In utero*/lactational exposure to both test chemicals at both doses resulted in male reproductive system malformations in a dose- and chemical-related incidence and severity on pnd 95. For the low dose DBP, findings included cleft phallus, dorsal and ventral prostate lobes reduced in size, and enlarged testes. Admittedly, these were observed at a low incidence at this dose, but they were biologically significant although not likely statistically significant. Text Table 4 presents the data in support of this answer.

4. Did the F1 males that died or were sacrificed moribund also exhibit male reproductive malformations? There were two F1 males each at the VIN high dose and the DBP high dose that died or were sacrificed moribund. They did exhibit the same male reproductive system malformations as those observed in the adult males at scheduled necropsy (see Text Table 5 below).

5. Were the incidences of findings present at both pnd 21 and 95 necropsies different? Were they greater on pnd 95? Would they have been detected on pnd 95 with only one male evaluated per litter? As is obvious from Text Table 6, in almost every case for findings present at both pnd 21 and 95, the incidence is higher on pnd 95. In addition, some observations were present only on pnd 95 (such as vaginal pouch on Text Table 6 and findings in Text Table 3 marked with footnote “b”).

Text Table 4. F1 Male Offspring Reproductive System Malformations at the Pnd 95 Necropsy[a]

	Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
Parameter	0	50	100	100	500
No. males	82	95	74	81	74
Cowper's glands:					
Missing	0 (0.0)	6 (6.32)	56 (75.7)	0 (0.0)	5 (6.8)
Reduced in size	0 (0.0)	1 (1.0)	8 (10.8)	0 (0.0)	3 (4.0)
Epididymis missing	0 (0.0)	0 (0.0)	4 (5.4)	0 (0.0)	33 (44.6)
Reduced in size	1 (1.2)	0 (0.0)	12 (16.2)	0 (0.0)	52 (70.3)
Epispadias	0 (0.0)	4 (4.3)	11 (14.9)	0 (0.0)	0 (0.0)
Glans penis not completely detached [b]	0 (0.0)	3 (3.2)	20 (27.0)	0 (0.0)	7 (9.5)
Hypospadias	0 (0.0)	15 (15.8)	73 (98.6)	0 (0.0)	12 (16.2)
LABC: [b]					
Missing	0 (0.0)	0 (0.0)	2 (2.7)	0 (0.0)	0 (0.0)
Reduced in size	0 (0.0)	2 (2.1)	38 (51.4)	0 (0.0)	4 (5.4)
Malformed	0 (0.0)	1 (1.0)	5 (6.7)	0 (0.0)	0 (0.0)
Males with $\geq$ 1 gubernacular cord	5.5 (6.2)	11 (11.6)	13 (17.6)	1.0 (1.2)	6 (8.1)
Males with $\geq$ 1 cranial suspensory ligament	0 (0.0)	0 (0.0)	1.0 (1.4)	0 (0.0)	6.2 (8.4)
Phallus, cleft	1 (1.20)	41 (43.2)	74 (100.0)	2.0 (2.5)	26 (35.1)
Prepuce partially or fully detached	81 (98.8)	94 (99.0)	74 (100.0)	81 (100.0)	74 (100.0)
Preputial glands, pus filled [b]	0 (0.0)	1 (1.2)	0 (0.0)	0 (0.0)	0 (0.0)
Prostate, dorsal:					
Missing	0 (0.0)	0 (0.0)	17 (23.0)	0 (0.0)	3 (4.0)
Reduced in size	0 (0.0)	2 (2.4)	20 (27.0)	2 (2.5)	7 (9.5)
Abnormal/infected	0 (0.0)	2 (2.4)	3 (4.0)	0 (0.0)	1 (1.4)

(Continued)

Text Table 4 (continued)

Parameter	Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
	0	50	100	100	500
Prostate, ventral:					
Missing	0 (0.0)	0 (0.0)	12 (16.2)	0 (0.0)	3 (4.0)
Reduced in size	0 (0.0)	4 (4.9)	43 (58.1)	1 (1.2)	4 (5.4)
Abnormal/infected	1 (1.2)	3 (3.1)	5 (6.7)	1 (1.2)	6 (8.1)
Seminal vesicles:					
Missing	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	8 (10.8)
Misshapen/infected	0 (0.0)	0 (0.0)	6 (8.1)	0 (0.0)	5 (6.8)
Reduced in size	0 (0.0)	5 (6.1)	58 (78.4)	0 (0.0)	27 (36.5)
Testes					
Undescended	0 (0.0)	1 (1.0)	15 (20.3)	0 (0.0)	10 (13.5)
sc in abdom. wall[c]	0 (0.0)	0 (0.0)	8 (10.8)	0 (0.0)	2 (2.7)
Reduced in size	1 (1.2)	1 (1.0)	17 (23.0)	0 (0.0)	45 (60.8)
Flaccid/soft	0 (0.0)	0 (0.0)	1 (1.4)	0 (0.0)	32 (43.2)
Enlarged	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.2)	2 (2.7)
Abnormal/infected	0 (0.0)	0 (0.0)	3 (4.0)	0 (0.0)	1 (1.4)
Urinary bladder: [b]					
Adhered to prostate	0 (0.0)	0 (0.0)	1 (1.4)	0 (0.0)	0 (0.0)
Calculi present	1 (1.2)	0 (0.0)	3 (4.0)	0 (0.0)	1 (1.4)
Vaginal pouch [b]	0 (0.0)	2 (2.4)	43 (58.1)	0 (0.0)	1 (1.4)

[a] Data are presented as number (%) with the indicated finding; data from summary tables 20 and 21. On this table, a male may be counted more than once if he exhibited more than one malformation.

[b] Findings not reported at the pnd 21 necropsy.

[c] Undescended testes imbedded subcutaneously (sc) in the abdominal wall.

Text Table 5. Gross Findings at Unscheduled Necropsy [a]

	Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
Parameter	0	50	100	100	500
No. males	0	0	2 [b]	0	2 [c]
Cleft phallus	–	–	2	–	1
Cowper's gland, missing	–	–	1	–	1
Epididymis, reduced in size	–	–	0	–	2
Hypospadias	–	–	2	–	1
LABC, missing	–	–	1	–	1
Penis, pus	–	–	1	–	0
Prostate:					
Dorsal missing	–	–	0	–	1
Ventral missing	–	–	0	–	1
Whole gland missing	–	–	1	–	0
Whole gland reduced in size	–	–	1	–	0
Seminal vesicles, reduced in size	–	–	2	–	1
Testis:					
Undescended	–	–	1	–	2
sc in abdomen	–	–	1	–	0
Reduced in size	–	–	1	–	2
Urinary bladder calculi	–	–	1	–	0
Vaginal pouch	–	–	1	–	0

[a] Data from summary table 21. On this table, a male may be counted more than once if he exhibited more than one malformation.

[b] One high dose VIN male was euthanized moribund on pnd 64, and one high dose VIN male was found dead on pnd 80.

[c] Both high dose DBP males were euthanized moribund, one each on pnd 72 and 79.

The incidences of the findings for the epididymides are presented separately for missing (agenesis of caput, corpus and/or cauda or of the entire organ) and for reduced in size/abnormal. The description “abnormal” i.e., changes in color, appearance, texture or presence of infection is almost exclusively documented on pnd 95. Missing parts or whole epididymis(des) were noted at the high dose of both VIN and DBP on both pnd 21 and 95, with incidences for both time points higher in DBP than in VIN and with higher incidences on pnd 95 than on pnd 21 for both chemicals. Epididymis(des), reduced in size/abnormal, were not noted in any VIN group nor in the low dose of DBP on pnd 21. At the high dose of DBP on pnd 21, the finding was present but the incidence was low (6.2%). On pnd 95, over 25% of the males at the high dose VIN exhibited the finding, while the incidence for the high dose DBP males was over 70% (Text Table 6). Therefore, for both time points, the incidence was higher at 500 mg/kg DBP than at 100 mg/kg VIN.

The incidence of the findings for the prostate were presented separately for dorsal and ventral lobes and for missing (agenesis of part[s] or the entire lobe), versus reduced in size/abnormal. “Abnormal”, i.e., change in color, appearance, texture, or presence of infection is almost exclusively documented on pnd 95.

For the prostate, missing dorsal lobes were documented at a relatively high incidence at 100 mg/kg/day on pnd 21 (32.3%) and on pnd 95 (23.0%) for VIN, and at a much lower incidence for DBP at 500 mg/kg/day on pnd 21 (1.5%) and on pnd 95 (4.0%). Prostate dorsal lobe, reduced in size/abnormal, was not present on pnd 21 for any VIN or DBP doses. On pnd 95, this finding was present at 50 mg/kg (33.2%) and at 100 mg/kg /day (27.0%) VIN, and at 100 mg/kg/day (2.5%) and at 500 mg/kg (10.8%) DBP. The agenesis of the dorsal lobe of the prostate was much more common in VIN than in DBP at their high doses on both pnd 21 and 95. Small/abnormal dorsal prostate lobes were present only on pnd 95 with higher incidences in the VIN low and high doses than in the DBP low and high doses.

Prostate ,ventral lobe missing, was not present in controls or at either VIN or DBP low doses, with a 7.7% incidence at 100 mg/kg/day VIN and a 3.1% incidence at 500 mg/kg/day DBP on pnd 21. Agenesis of the ventral lobe was present at both high doses of VIN and DBP on pnd 95. Ventral lobe, reduced in size/abnormal, was not present in any group on pnd 21 and was present on pnd 95 at 0 mg/kg (1.2%; one male with ventral lobe “brown and hard”), at 50 mg/kg/day (7.4%) and 100 mg/kg/day (60.8%) VIN, and at 100 mg/kg/day (2.5%) and 500 mg/kg/day (10.8%) DBP. The ventral lobe of the prostate was detected as missing in the high doses of both chemicals at a low incidence on pnd 21. On pnd 95, the incidence at 100 mg/kg/day VIN was increased over two-fold (to 16.2%) relative to the incidence on pnd 21. The incidence was similar for both time points (3-4.0%) for 500 mg/kg/day DBP.

Hypospadias was not detected in the vehicle control group or in the low dose DBP group on pnd 21 or pnd 95. At the low dose VIN, the incidence on pnd 95 (15.8%) was almost twice that of the incidence on pnd 21 (9.7%). At the high dose VIN, the incidences were very high at both pnd 21 (80.0%) and at pnd 95 (98.6%). At the high dose DBP, the incidence on pnd 95 (16.2%) was over fivefold greater than at pnd 21 (3.1%). The incidence of hypospadias was higher for VIN than

for DBP and higher on pnd 95 than on pnd 21 for both chemicals. Epispadias was not detected in any group on pnd 21, and was not detected in the vehicle control group or in either DBP group on pnd 95. Epispadias was detected in the low dose VIN (4.3%) and in the high dose VIN (14.9%) on pnd 95. Thus, the incidence of epispadias was also greater for VIN than for DBP (it was undetected in DBP groups) and was higher on pnd 95 than on pnd 21 for VIN (it was undetected in VIN groups on pnd 21).

Effects on seminal vesicles (missing/reduced in size/abnormal) were not present in the control group or in the low dose VIN or DBP groups on pnd 21, and not in the controls or the low dose DBP on pnd 95. These effects were observed in approximately equal incidences in the high dose VIN (12.3%) and the high dose DBP (10.8%) on pnd 21. On pnd 95, the incidence in the low dose VIN was 6.1%, with a very high incidence in the high dose VIN (85.1%, sevenfold higher than on pnd 21); at the high dose DBP, on pnd 95, the incidence was 52.7% (a fivefold increase over the incidence in this group on pnd 21).

The presence of gubernaculum and cranial suspensory ligaments in the F1 males on pnd 21 and 95 and their relationship with undescended testes requires further discussion.

Imajima et al. (1997) and Shono et al. (2000) reported postnatal cryptorchidism, preceded by delays in the transabdominal descent of testes in rat fetuses, after gestational exposure to a very high oral dose (1000 mg/kg/day) of monobutyl phthalate (the major intestinal metabolite of DBP) on gd 15-18 (Imajima et al., 1997) or on gd 7-10, 11-14, or 15-18 (Shono et al., 2000). Testes normally descend to the inguinal ring by term and into the scrotal sacs during late lactation (typically pnd 16-20 in the performing laboratory). Cryptorchidism (undescended testes) was observed in the present study in both the weanling (pnd 21) and adult (pnd 95) necropsies for both VIN and DBP high dose groups.

Very recent data have indicated that male perinatal reproductive development is regulated not just by testosterone (and DHT; made by the Leydig cells) and Müllerian-inhibiting substance (MIS; made by the Sertoli cells) which cause the Müllerian ducts, which form female reproductive structures to regress in males, but also by a peptide hormone, now designated as insulin-like factor 3 (ISNL3) produced by the Leydig cells (Kubota et al., 2002). ISNL13 was discovered in the 1980s, but its function in regulating the development of the gubernaculum (which attaches to the caudal portion of the testis and epididymis and is responsible, in whole or part, for testis descent into the lower abdomen to the inguinal ring *in utero* and into the scrotal sacs during late lactation in rodents) was not identified until ISNL13-knockout mice were constructed (with normal T and MIS), which exhibited bilateral cryptorchidism (Nef and Parada, 1999; Zimmermann et al., 1999). ISNL13 has also been shown to play a role in DES-induced cryptorchidism in mice (Emmen et al., 2000a), which implies estrogen involvement. The role of ISNL13 in human cryptorchidism is not clear, with evidence both for its role (Tomboc et al., 2000) and against any role (Baker et al., 2002). Emmen et al. (2000b) have shown that both androgen and Ins13 are required for rodent gubernuclear outgrowth *in vitro*.

Dr. L. Early Gray's laboratory at U.S. EPA (NHEERL) (Gray et al., 2000; Gray and Foster, 2002; Wilson et al., 2003) has reported that gavage administration to rats of DBP (or DEHP or BBP) at 750 mg/kg/day on gd 14 to pnd 3 results in male reproductive system malformations, including cryptorchidism associated with abnormalities of the gubernaculum. In addition, examination of fetal testes from dams exposed to DEHP at 750 mg/kg/day on gd 14-18 indicated that not only was testosterone production reduced, but also that Ins13 mRNA was inhibited by approximately 80% (Wilson et al., 2003). When both DBP and BBP, each by gavage at 500 mg/kg/day, on gd 14-18 (Gray et al., in preparation) were administered to rat dams, no offspring males in the combination group exhibited normal gubernacula, and a few males exhibited retained cranial suspensory ligaments, which normally maintain the ovaries in the upper abdomen in females. In some cases, only the gubernaculum was affected, with no other male reproductive system malformations or vice versa, indicating an effect of the phthalates at high oral doses on both testosterone and ISNL13 synthesis. Consistent with this interpretation, DBP administered by gavage at a high oral dose (500 mg/kg/day) to dams in the present study during gestation and lactation, produced a small but biologically significant incidence of undescended testes, as well as other male reproductive system malformations in male offspring on pnd 21 and 95.

Therefore, the cryptorchidism observed by Imagima et al. (1997), Shono et al. (2000), Gray and Foster (2002), Wilson et al. (2003), and in this study from administration of various phthalates (monobutyl phthalate, DBP, DEHP, BBP, etc.) resulted only from very high oral "bolus" doses during the sensitive period.

In the present study, the presence of cranial suspensory ligaments in males and the reduced number of males with at least one gubernaculum on pnd 21, were observed at the low and high doses of DBP (and in one male at the low dose VIN on pnd 21). Cranial suspensory ligaments were also observed on pnd 95 at the high dose of VIN (one male) and at the high dose of DBP (six males); cranial suspensory ligaments are normally observed only in females, attached to the ovaries and the under side of the diaphragm. Undescended testes were observed on pnd 21 at the high dose VIN (three) and at the high dose DBP (four); and on pnd 95 at the low dose VIN (one), the high dose VIN (15), and at the high dose DBP (10) but not in the low dose DBP. Of the males with undescended testes at the high VIN and the high DBP doses, eight of 15 at 100 mg/kg/day VIN and two at 500 mg/kg/day DBP exhibited testes imbedded subcutaneously in the abdominal wall, strong evidence for abnormal/absent gubernacula.

The diagnostic offspring male reproductive malformations observed after *in utero* exposure to VIN are hypospadias and agenesis (absence) of the ventral prostate. In Text Table 6, which compares the percentage and incidence of malformations at pnd 21 and 95, the incidence of hypospadias was increased on pnd 95 over the incidence on pnd 21 for both doses of VIN (and for the high dose of DBP). Agenesis of the ventral prostate exhibited a higher incidence on pnd 95 than on pnd 21 for the high dose of VIN; it was not detected at either time point for the low dose of VIN. It was also detected at the high (but not the low) dose of DBP at both time points at approximately equal incidences. When one separates the data on missing ventral prostate lobe

from data on ventral prostate lobe "reduced in size/abnormal," the sensitivity of the pnd 95 necropsy (over the pnd 21 necropsy) becomes very clear for the latter finding. In Text Table 6 on pnd 21, there were no incidences of ventral prostate lobe reduced in size/abnormal. However, on pnd 95, the incidences were increased at the low (7.4%) and at the high dose (60.8%) on VIN and at the low (2.5% and high (10.8%) doses of DBP. Therefore, this effect was missed entirely on pnd 21.

Also for Text Table 6, those male malformations observed on pnd 95 but not on pnd 21 in either or both test chemicals or in both dose groups per chemical include the following:

- epididimydes reduced in size/abnormal (not observed in either VIN groups on pnd 21)
- epispadias (not detected in any group on pnd 21)
- levator ani bulbocavernosus (LABC) complex, missing/reduced in size (not observed in the low dose of VIN or either dose of DBP on pnd 21)
- cleft phalllus (not observed in the low dose of DBP on pnd 21)
- dorsal and/or ventral lobes of prostate reduced in size/abnormal (not detected in any group on pnd 21)
- seminal vesicles missing/reduced in size/abnormal (not observed in the low dose of VIN on pnd 21)
- undescended testes (not observed in the low dose of VIN on pnd 21)
- testes reduced in size or flaccid/soft (not detected in any group on pnd 21)
- and vaginal pouch (not detected in any group on pnd 21)

An approach to determining whether effects observed on pnd 95 would have been observed if we only examined one adult male per litter in each group is presented in Text Table 7. In this table, the number (and %) of male reproductive system malformations and the number(and %) of males with one or more reproductive system malformations are presented for pnd 21 and 95. Then, the incidence, based on the number of males with one or more reproductive system malformations (taken from the individual animal data tables; Appendix I, Tables A-26 and A-28) and on the number of litters in each group, with $\geq$1 male with $\geq$1 reproductive malformations, is presented. These numbers are used to detemine whether one male randomly selected to represent his litter on pnd 95 would have been malformed.

Text Table 6. Comparison of the Incidence of Male Reproductive System Malformations on pnd 21 Versus pnd 95

	Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
Parameter	0	50	100	100	500
No. males, pnd 21	74	82	65	71	65
No. males, pnd 95	82	95	74	81	74
Cowper's gland missing/ reduced in size:					
Pnd 21	0 (0.0) [a]	9 (11.0)	47 (72.3)	1 (1.4)	7 (10.8)
Pnd 95	1 (1.2)	7 (7.4)	63 (85.1)	0 (0.0)	8 (10.8)
Epididymides missing					
Pnd 21	0 (0.0)	0 (0.0)	2 (3.1)	0 (0.0)	14 (21.5)
Pnd 95	0 (0.0)	0 (0.0)	4 (5.4)	0 (0.0)	33 (44.6)
Epididymides reduced in size/abnormal					
Pnd 21	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (6.2)
Pnd 95	1 (1.2)	0 (0.0)	19 (25.7)	0 (0.0)	52 (71.6)
Epispadias:					
Pnd 21	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Pnd 95	0 (0.0)	4 (4.3)	11 (14.9)	0 (0.0)	0 (0.0)
Hypospadias:					
Pnd 21	0 (0.0)	8.0 (9.7)	52 (80.0)	0 (0.0)	2 (3.1)
Pnd 95	0 (0.0)	15 (15.8)	73 (98.6)	0 (0.0)	12 (16.2)
LABC missing/reduced in size:					
Pnd 21	0 (0.0)	0 (0.0)	1 (1.5)	0 (0.0)	0 (0.0)
Pnd 95	0 (0.0)	2 (2.1)	40 (54.0)	0 (0.0)	4 (5.4)

(Continued)

	Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
Parameter	0	50	100	100	500
Phallus, cleft:					
Pnd 21	0 (0.0)	4 (4.9)	25 (38.5)	0 (0.0)	2 (3.1)
Pnd 95	1 (1.2)	41 (43.15)	74 (100.0)	2.0 (2.47)	26 (35.14)
Prostate dorsal lobe missing					
Pnd 21	0 (0.0)	0 (0.0)	21 (32.3)	0 (0.0)	1 (1.5)
Pnd 95	0 (0.0)	0 (0.0)	17 (23.0)	0 (0.0)	3 (4.0)
Prostate dorsal lobe reduced in size/abnormal					
Pnd 21	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Pnd 95	0 (0.0)	3 (3.2)	20 (27.0)	2 (2.5)	8 (10.8)
Prostate ventral lobe missing					
Pnd 21	0 (0.0)	0 (0.0)	5 (7.7)	0 (0.0)	2 (3.1)
Pnd 95	0 (0.0)	0 (0.0)	12 (16.2)	0 (0.0)	3 (4.0)
Prostate ventral lobe reduced in size/abnormal					
Pnd 21	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Pnd 95	1 (1.2)	7 (7.4)	45 (60.8)	2 (2.5)	8 (10.8)
Seminal vesicles missing/reduced in size/ abnormal					
Pnd 21	0 (0.0)	0 (0.0)	8 (12.3)	0 (0.0)	7 (10.8)
Pnd 95	0 (0.0)	5 (6.1)	63 (85.1)	0 (0.0)	39 (52.7)
Testes undescended					
Pnd 21	0 (0.0)	0 (0.0)	3 (4.6)	0 (0.0)	4 (6.1)
Pnd 95	0 (0.0)	1 (1.0)	15 (20.3)	0 (0.0)	10 (13.3)
Testes reduced in size					
Pnd 21	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Pnd 95	1 (1.2)	1 (1.0)	17 (23.0)	0 (0.0)	45 (60.8)

(Continued)

Parameter	Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
	0	50	100	100	500
Testes flaccid/soft					
Pnd 21	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Pnd 95	0 (0.0)	0 (0.0)	1 (1.4)	0 (0.0)	32 (43.2)
Males with >1 gubernuclear wt.[b]					
Pnd 21	74 (100.0)	82 (100.0)	65 (100.0)	69 (97.2)	59 (90.8)
Pnd 95	5.5 (6.2)	11 (11.6)	13 (17.6)	1.0 (1.2)	6 (8.1)
Males with >1 cranial suspensory ligament[b]					
Pnd 21	0 (0.0)	1 (1.32)	0 (0.0)	2.2 (3.1)	2.2 (3.4)
Pnd 95	0 (0.0)	0 (0.0)	1 (1.4)	0 (0.0)	6.2 (8.1)
Vaginal pouch					
Pnd 21	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Pnd 95	0 (0.0)	2 (2.4)	43 (58.1)	0 (0.0)	1 (1.4)

[a] Data presented as number (%) of males exhibiting the finding. Males may be counted more than once if they exhibited more than one finding.

[b] The incidences of these findings are taken from Summary Task 13 (and Text Table 3) for pnd 21, and from Summary Task 20 (and Text Table 4) for pnd 95.

For the pnd 95 necropsy, approximately 50% of the males and 84% of the litters at the low dose of VIN and all of the males in all of the litters at the high dose of VIN exhibited at least one reproductive malformation. Approximately 90% of the males and 30% of the litters at the low dose of DBP and 92% of the males and 96% of the litters at the high dose of DBP also exhibited at least one reproductive malformation. These values indicate that the number of affected males per affected litter would be 1.0, 2.2, 3.4, 1.0, and 3.1 at 0, 50, and 100 VIN and at 100 and 500 DBP, respectively (Text Table 7). This information is interpreted to mean that affected males in the low and high dose VIN and the high dose DBP would have been detected at either the pnd 21 or 95 necropsies (under the current testing guidelines). The incidence at the low dose DBP (1.0 affected males per affected litter) implies that detection of males with reproductive system malformations on pnd 21 (three males examined out of five males present at weaning in the current guidelines) and at pnd 95 (one male examined out of two males remaining after the pnd 21 necropsy in the

current guidelines) would likely be detected but at an erroneously lower incidence. Since approximately three males/litter were examined on pnd 21 (as specified by the 1998 OPPTS testing guideline), the likelihood of detecting effects on pnd 21 is not changed, and the calculation for results on pnd 21 for this protocol, versus the current testing guidelines, was not performed.

The best approach to determine whether examination of more male pups at weaning, and, more importantly, retention of more male offspring to adulthood (versus the one/sex/litter in the standard two-generation study design) would allow detection of more male reproductive malformations and higher incidences of these malformations, would be to start with the full offspring data set from this study. Then, repeated random samples of three pups/litter on pnd 21 and one male/litter on pnd 95 would be selected (using mathematical random number generators from SAS®). Approximately 1000 such random samples would be obtained and then analyzed to determine whether retention of more pups/litter would result in smaller variances (increased precision) for estimated differences and therefore more significant p-values. This repeated analysis is basically a Monte-Carlo simulations assessment (and use of a one-tailed t-test). The results should provide the probability of detecting significant effects (for the parameters selected) with the randomly selected three male pups/litter on pnd 21 and with the randomly selected one male/litter on pnd 95 versus the probability of detection with the entire data set from this study.

6. Were there differences in the effects on weights of various male reproductive system organs between the two chemicals at the same time point, and within each chemical at the two different time points? Text Table 8 presents the absolute and adjusted (adjusted for body weight as covariate) weights of all the reproductive organ weights on pnd 21 and 95 for all groups.

For testis weights, effects were more profound from DBP with significant reductions at the low dose DBP (but not at the low dose VIN) on pnd 21 and greater reductions at the high dose DBP than at the high dose VIN for both pnd 21 and pnd 95.

For epididymal corpus plus caput together and for cauda epididymis, reductions were greater at the high dose DBP than at the high dose VIN for both pnd 21 and pnd 95. Interestingly, the weight of the corpus plus caput was reduced for both the low dose VIN and DBP for pnd 21 but not for pnd 95. The cauda epididymis weight was reduced for the right (but not left) cauda for the low dose VIN only on pnd 95. The adjusted (but not the absolute) weight of the right (but not the left) cauda was also reduced for the low dose DBP only for pnd 95.

Seminal vesicle plus coagulating gland weights were more profoundly reduced at the high dose VIN than at the high dose DBP for both pnd 21 and 95. In addition, the absolute weights were significantly reduced at the low dose VIN (but not DBP) for pnd 21, but not for pnd 95.

Text Table 7. Evaluation of Detection of Male Reproductive System Malformations in this Protocol Versus in the OPPTS Testing Guidelines

	Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
Parameter	0	50	100	100	500
Pnd 21[a]					
No. males examined	74	82	65	71	65
No. litters examined	23	25	22	23	23
Mean number of F1 male offspring examined per litter	3.22	3.28	3.38	3.24	3.54
Pnd 95[b]					
No. males examined	82	95	74	81	74
No. litters examined	23	25	22	23	23
Mean number of F1 male offspring examined per litter	3.56	3.80	3.36	3.52	3.22
Incidence of total no. of malformations based on no. F1 males examined					
Pnd 21 [c,d]	0 (0.0)	21 (256)	169 (260.0)	1 (1.4)	43 (66.2)
Pnd 95 [c,e]	8 (9.8)[f]	95 (100.0)	532 (718.9)	11 (13.6)	237 (320.3)
Total number of F1 males with $\geq$1 malformation on pnd 95 [c]	7 (8.5)[f]	47 (49.5)	74 (100.0)	7 (8.6)	68 (91.9)
Total no. of F1 litters with $\geq$ 1 male with $\geq$ 1 malformations on pnd 95 [c]	7 (30.4)[f]	21 (84.0)	22 (100.0)	7 (30.4)	22 (95.7)
No. malformed males per affected litters on pnd 95	1.0[f]	2.24	3.36	1.0	3.09

[a] The number of F1 male weanlings/litter examined in this protocol versus the numbers from the 1998 OPPTS testing guideline are approximately equivalent.

[b] The OPPTS testing guideline specifies one male/litter retained to adulthood. Therefore, this protocol provides at least three times the power to detect adult male malformations.

[c] Data presented as number (%).

[d] Pnd 21 data taken from Summary Table 14 and Individual Animal Tables A-15 and A-17.

[e] Pnd 95 data taken from Summary Table 21 and Individual Animal Tables A-26 and A-28.

[f] Predominantly minor effects (e.g., epididymis and testis reduced in size, prostate abnormal [hard and brown], etc.)

The whole prostate weight was more profoundly reduced for the high dose VIN than for the high dose DBP for both pnd 21 and 95; it was also significantly reduced as absolute and adjusted weights at the low dose VIN on pnd 21 but not on pnd 95. The ventral lobe weights paralleled the intact gland weights exactly. The dorsal lobe weight was significantly reduced only at the high dose of both VIN and DBP, with the weights at the high dose VIN more reduced than the weights at the high dose DBP on both pnd 21 and 95.

The levator ani bulbocavernosus (LABC) weights (absolute and adjusted) were significantly reduced at the low and high dose VIN, and at the high dose (but not the low dose) DBP, with the reductions greater for VIN than for DBP at both pnd 21 and 95.

Cowper's gland weights were unaffected at the low dose VIN and significantly reduced for absolute weights on both pnd 21 and pnd 95, and for adjusted weights only for pnd 95 at the high dose VIN. For DBP groups, absolute and adjusted Cowper's gland weights were reduced at the high and low doses only for pnd 21 (and not for pnd 95).

In summary, testes and epididymides weights were more affected (reduced) by DBP. Seminal vesicle and coagulating gland, prostate, LABC, and Cowper's gland weights were more affected (reduced) by VIN (Text Table 8).

In addition to addressing the specific objectives stated above, the data in this study invite comparison of the effects of these two dissimilar antiandrogens.

As an example of this, the kinds and incidences of F1 male reproductive malformations observed at pnd 95 in the low and high dose groups of VIN versus DBP are presented in Text Table 9. The enhanced pnd 95 data resulting from the present study design provide an opportunity to compare male reproductive anomalies that are most likely persistent. There were dose-related incidences in both chemicals, with the low dose of VIN more effective (more malformations observed) than the low dose of DBP (incidence of specific malformations was less or none). This assessment is obviously related to the doses selected for each chemical and the different mechanisms of action of the two chemicals. VIN is a dicarboximide fungicide, considered an androgen-receptor antagonist (Gray et al., 1994). DBP is an industrial chemical used as a coalescing agent in latex adhesives, as a plasticizer in cellulose plastics, and as a solvent for dyes (Kavlock et al., 2002). DBP or its monoester, mBuP, does not bind to the androgen receptor (Kavlock et al., 2002), and it does not act on reproductive development via its peroxisomal proliferating activity (Ward et al., 1998). Its developmental anti-androgenic mechanism is via inhibition of fetal testicular testosterone biosynthesis in the fetal interstital Leydig cells, perhaps by delaying Leydig cell differentiation and resulting in Leydig cell hyperplasia *in utero* (Parks et al., 2000). DBP may also act by its inhibition of the transcription of the Insl3 gene also in the Leydig cells (Kubota et al., 2002). The peptide hormone Insl3 is responsible for the normal development and function of the gubernaculum in fetal male rats. Knockout mice with normal T and MIS

Text Table 8. Comparison of Male Reproductive System Organ Weights on pnd 21 versus pnd 95[a]

	Vehicle Control (mg/kg/day)	VIN (mg/kg/day)		DBP (mg/kg/day)	
Parameter	0	50	100	100	500
Testis					
Right Ab pnd 21	— (0.1323)	—	↓↓ (0.1198)	—	↓↓↓ (0.1017)
Right Ab pnd 95	— (1.7702)	—	↓↓↓ (1.5284)	—	↓↓↓ (1.2383)
Right Aj pnd 21	— (0.1295)	↑ (0.1334)	—	↓ (0.1221)	↓↓↓ (0.1105)
Right Aj pnd 95	— (1.7680)	—	↓↓↓ (1.5335)	—	↓↓↓ (1.2416)
Left Ab pnd 21	— (0.1305)	—	↓↓ (0.1186)	—	↓↓↓ (0.0983)
Left Ab pnd 95	— (1.7688)	—	↓↓↓ (1.4970)	—	↓↓↓ (1.3982)
Left Aj pnd 21	— (0.1281)	—	—	↓↓ (0.1189)	↓↓↓ (0.1078)
Left Aj pnd 95	— (1.7649)	—	↓↓↓ (1.5056)	—	↓↓↓ (1.4089)
Corpus and Caput Epididymis					
Right Ab pnd 21	— (0.0139)	↓ (0.0125)	↓↓↓ (0.0100)	↓ (0.0123)	↓↓↓ (0.0088)
Right Ab pnd 95	— (0.3623)	—	↓↓↓ (0.3267)	—	↓↓↓ (0.2405)
Right Aj pnd 21	— (0.0137)	↓ (0.0123)	↓↓↓ (0.0105)	↓ (0.0121)	↓↓↓ (0.0094)
Right Aj pnd 95	— (0.3617)	—	↓↓↓ (0.3282)	—	↓↓↓ (0.2402)
Left Ab pnd 21	— (0.0137)	—	↓↓↓ (0.0099)	↓↓↓ (0.0111)	↓↓↓ (0.0084)
Left Ab pnd 95	— (0.3563)	—	↓↓ (0.3329)	—	↓↓↓ (0.2548)
Left Aj pnd 21	— (0.0130)	—	↓↓↓ (0.0103)	↓↓↓ (0.0108)	↓↓↓ (0.0092)
Left Aj pnd 95	— (0.3617)	—	↓ (0.3282)	—	↓↓↓ (0.2543)

(Continued)

Cauda Epididymis					
Right Ab pnd 21	— (0.0099)	—	↓↓↓ (0.0069)	—	↓↓↓ (0.0062)
Right Ab pnd 95	— (0.2857)	↓ (0.2719)	↓↓↓ (0.2309)	—	↓↓↓ (0.1128)
Right Aj pnd 21	— (0.0097)	—	↓↓↓ (0.0072)	—	↓↓↓ (0.0069)
Right Aj pnd 95	— (0.2854)	↓ (0.2716)	↓↓↓ (0.2316)	↓ (0.2735)	↓↓↓ (0.1143)
Left Ab pnd 21	— (0.0096)	—	↓↓↓ (0.0076)	—	↓↓↓ (0.0064)
Left Ab pnd 95	— (0.2720)	—	↓↓↓ (0.2263)	—	↓↓↓ (0.1362)
Left Aj pnd 21	— (0.0095)	—	↓↓↓ (0.0079)	—	↓↓↓(0.0070)
Left Aj pnd 95	— (0.2714)	—	↓↓↓ (0.2277)	—	↓↓↓ (0.1363)
Seminal vesicles plus coagulating glands					
Ab pnd 21	— (0.0180)	↓↓↓ (0.0145)	↓↓↓ (0.0082)	—	↓↓↓ (0.0097)
Ab pnd 95	— (1.4707)	—	↓↓↓ (0.7097)	—	↓↓↓ (1.1111)
Aj pnd 21	— (0.0178)	↓↓↓ (0.0143)	↓↓↓ (0.0085)	—	↓↓↓ (0.0102)
Aj pnd 95	— (1.4585)	—	↓↓↓ (0.7378)	—	↓↓↓ (1.1186)
Prostate					
Whole Gland					
Ab pnd 21	— (0.0483)	↓ (0.0441)	↓↓↓ (0.0235)	—	↓↓↓ (0.0292)
Ab pnd 95	— (1.0810)	—	↓↓↓ (0.4226)	—	↓↓↓ (0.8601)
Aj pnd 21	— (0.0478)	↓ (0.0436)	↓↓↓ (0.0249)	—	↓↓↓ (0.0322)
Aj pnd 95	— (1.0799)	—	↓↓↓ (0.4261)	—	↓↓↓ (0.8689)
Ventral lobe					
Ab pnd 21	— (0.0251)	↓ (0.0225)	↓↓↓ (0.0117)	—	↓↓↓ (0.0163)
Ab pnd 95	— (0.6418)	—	↓↓↓ (0.1897)	—	↓↓↓ (0.4762)

(Continued)

Aj pnd 21	— (0.0246)	↓ (0.0220)	↓↓↓ (0.0127)	—	↓↓↓ (0.0178)
Aj pnd 95	— (0.6417)	—	↓↓↓ (0.1899)	—	↓↓↓ (0.4812)

Dorsal lobe					
Ab pnd 21	— (0.0232)	—	↓↓↓ (0.0118)	—	↓↓↓ (0.0126)
Ab pnd 95	— (0.4393)	—	↓↓↓ (0.2303)	—	↓↓ (0.3791)
Aj pnd 21	— (0.0229)	—	↓↓↓ (0.0126)	—	↓↓↓ (0.0139)
Aj pnd 95	— (0.4381)	—	↓↓↓ (0.2337)	—	↓ (0.3829)
LABC					
Ab pnd 21	— (0.0562)	↓↓ (0.0474)	↓↓↓ (0.0313)	—	↓↓↓ (0.0419)
Ab pnd 95	— (1.3577)	↓↓↓ (1.1463)	↓↓↓ (0.5539)	—	↓↓↓ (0.9411)
Aj pnd 21	— (0.0551)	↓↓ (0.0459)	↓↓↓ (0.0337)	—	↓↓ (0.0456)
Aj pnd 95	— (1.3508)	↓↓↓ (1.1400)	↓↓↓ (0.5703)	—	↓↓↓ (0.9547)
Cowper's glands					
Ab pnd 21	— (0.0036)	—	↓↓ (0.0021)	↓ (0.0030)	↓↓ (0.0027)
Ab pnd 95	— (0.1480)	—	↓↓↓ (0.0688)	—	—
Aj pnd 21	— (0.0035)	—	—	↓ (0.0030)	↓ (0.0029)
Aj pnd 95	— (0.1479)	—	↓↓↓ (0.0697)	—	—

[a] The control values are presented (in parentheses) for comparison with the values from the treated groups; if there is a statistically significant change, the degree of significance is presented by up/down arrows and the value is in parentheses.

pnd 21 organ weights in grams from Summary Table 13

pnd 95 organ weights in grams from Summary Table 20

Ab = Absolute organ weight in grams

Aj = Adjusted organ weight in grams (adjusted for body weight as covariate).

LABC = Levator ani bulbocavernosus (LABC) muscle

↓, ↓↓, ↓↓↓, statistically significantly reduced at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, by appropriate statistical tests (see summary tables and text for details).

↑, statistically significantly increased at $p < 0.05$ by appropriate statistical tests (see summary tables and text for details).

—, no statistically significant difference from the control group value.

exhibited bilateral cryptorchidism (Nef and Parada, 1999; Zimmerman et al., 1999). The gubernaculum attaches to the caudal portion of the testis and the caudal epididymis and to the inguinal ring (and scrotal sac). It is responsible for the descent of the testis to the inguinal ring *in utero* and, with androgen, for testicular descent into the scrotal sac during later lactation. When it is absent or abnormal (e.g., threadlike), the testis(es) do not descend to the inguinal ring during gestation and do not normally descend into the scrotal sacs postnatally. DBP at high oral doses (750 mg/kg/day, gd 14-pnd 3; Gray et al., 2000) has been shown to cause cryptorchidism with abnormal gubernacula (Gray and Foster, 2002) in rats. Both VIN (at both doses) and DBP (at the high dose) in the present study caused undescended testes, with some of these males exhibiting testes embedded in the abdominal wall at the high doses of VIN (eight males) and DBP (two males).

Additionally, differences between the range and severity of effects caused by the two test chemicals are evident (Text Table 9). VIN causes a low incidence of flaccid/soft testes or retention of cranial suspensory ligaments in males, while DBP causes a midrange incidence. In contrast, VIN causes a higher incidence of epispadias, hypospadias, missing or small ventral prostate, or vaginal pouch than DBP does. It appears as if VIN more strongly affects those structures under DHT control (lower reproductive tract, prostate, seminal vesicles, external genitalia). The other findings on Text Table 9 occurred with approximately equal incidences in the high dose groups of both chemicals (and with a slightly less concordance in the low dose groups of both chemicals).

Text Table 9. Comparison of the Kinds and Incidences of F1 Male Reproductive Malformations by Chemical and by Dose Observed on pnd 95[a]

	VIN Dose		DBP Dose	
Finding	Low	High	Low	High
Cowpers gland missing/reduced in size	##	##	–	##
Epididymides, missing/reduced in size	–	##	–	##
LABC[b] missing/reduced in size/malformed	–	#	–	#
Hypospadias	##	###	–	##
Cleft phallus	##	###	#	##
Prostate: Dorsal	#	##	#	##
Ventral	#	###	–	##
Seminal vesicles, missing/misshapen	#	###	–	##
Epispadias	#	##	–	–
Vaginal pouch	#	##	--	–
Testes undescended	#	##	–	##
Testes embedded in abdominal wall	–	##	–	#
Testes reduced in size	#	##	–	##

	VIN Dose		DBP Dose	
Finding	Low	High	Low	High
Testes flaccid/soft	–	#	–	##
Glans penis not completely detached	#	##	–	##
Males with ≥ 1 cranial suspensory ligament	–	#	–	##

– = no incidence

= small incidence (1-5%), ## = mid range incidence (6-74%), ### = high incidence (>75%)

[a] PND 95 data taken from Individual Animal Tables 20 and 21.

[b] LABC = Levator Ani plus Bulbocavernosus Complex

CONCLUSIONS

1. Specific male offspring malformations were detected on pnd 95 but not on pnd 21. Examples include prostate dorsal lobe abnormal/reduced in size (VIN, both doses; DBP, high dose), prostate ventral lobe abnormal/reduced in size (both compounds, both doses), and epispadias (VIN, both doses).
2. The incidence of specific male offspring malformations detected on pnd 95 was higher than the incidence of the same malformation observed on pnd 21. Examples include agenesis of all or parts of the epididymis(des) (high dose of both VIN and DBP), hypospadias (low dose VIN), and missing/reduced in size/abnormal seminal vesicles (high dose of both VIN and DBP).
3. The effects of VIN on the incidence of hypospadias and ventral prostate agenesis were more obvious at pnd 95 than at pnd 21. This effect was more apparent at the low dose than at the high dose. Specifically, hypospadias was observed in 9.7% vs 15.8% of the animals on pnd 21 and 95, respectively, whereas high dose animals exhibited hypospadias at 80.0% vs 98.6% on pnd 21 and 95, respectively.
4. The effects of DBP (high dose) on the incidence of epididymal agenesis on pnd 95 was approximately twice that observed on pnd 21, and thus were more obvious on pnd 95 than on pnd 21.
5. Adverse effects on the weight of some male reproductive tissues were more apparent at pnd 95 than on pnd 21. Examples include adjusted right or left testis weight (high dose VIN), absolute right cauda epididymis weight (low dose VIN), adjusted right cauda epididymis weight (low dose VIN and DBP), absolute LABC weight (low dose VIN), adjusted LABC weight (high dose VIN and DBP), and absolute and adjusted Cowper's gland weight (high dose VIN).
6. Adverse reproductive system effects *in toto* (structural malformations and other abnormalities) of the low and high doses of VIN and the high dose of DBP on F1 adult male offspring would most likely be statistically significant with either one or three adult males/litter, and would have been detected with either study design.
7. Adverse reproductive system structural effects *in toto* at the low dose of DBP on F1 adult male offspring were clearly biologically significant but not necessarily or likely statistically significant, with either one or three adult males/litter, and provide an example of effects that would not be detected with either study design.
8. The more males examined per litter, the better the characterization of the litter as responding or not responding adversely to exposure, and the smaller the variance term for pooled litters within each treatment group. The enhanced sensitivity with more males examined per litter would increase the likelihood of detection of effects as statistically and biologically significant. Also, for effects with low incidence, such as in the low dose DBP group in this study, the risk with fewer males examined per litter is that the effect might be missed, i.e., the litter would be designated as not responding, on the basis of the one male examined, if that male did not exhibit the effect.

REFERENCES

Agresti, A. (1990). *Categorical Data Analysis*. John Wiley and Sons, NY.

Armitage, P. (1955). Test for linear trends in proportions and frequencies. *Biometrics* **11**, 375-386.

Baker, L.A., S. Nef, M.T. Nguyen, R. Stapleton, H. Pohl, and L.F. Parada (2002). The insulin-3 gene: lack of a genetic basis for human cryptorchidism. *J. Urol.* **167(6),** 2534-2537.

Cochran, W. (1954). Some methods for strengthening the common $\chi 2$ tests. *Biometrics* **10**, 417-451.

Dunnett, C.W. (1955). A multiple comparison procedure for comparing several treatments with a control. *J. Am. Stat. Assoc*. **50**, 1096-1121.

Dunnett, C.W. (1964). New tables for multiple comparisons with a control. *Biometrics* **20**, 482-491.

Emmen, J.M., A. McLuskey, I.M. Adham, W. Engel, M. Verhoef-Post, A.P. Themmen, J.A. Grootegoed, and A.O. Brinkman (2000a). Involvement of insultin-like factor 3 (Insl3) in diethylstilbestrol-induced cryptorchidism. *Endocrinol.* **141(2),** 846-849.

Emmen, J.M., A. McLuskey, I.M. Adham, W. Engel, J.A. Grootegoed, and A.O. Brinkman (2000b). Hormonal control of gubernaculum development during testis descent: gubernaculum outgrowth *in vitro* requires both insulin-factor and androgen. *Endocrine* **14(12),** 4720-4727.

Gray, L.E. Jr., and P. Foster (2002). Significance of experimental studies for assessing adverse effects of endocrine disrupting chemicals. SCOPE/IUPAC International Symposium on Endocrine Auto Substances and Supplementary Workshop, Yokohama, Japan, November 17-21, 2002. Program and Collection Abstract, Subtopic 14, pp. 40-41.

Gray, L.E., Jr., J. Ostby, J. Furr, M. Price, D.N.R. Veeramachaneni, and L. Parks (2000). Perinatal exposure to the phthalates DEHP, BBP and DINP, but not DEP, DMP or DOTP alters sexual differentiation of the male rat. *Toxicol. Sci*. **58(2)**, 350-365.

Gray, L.E., Jr. J.S. Ostby, E. Mylchreest, and P.M. Foster (1998). Dibutyl phthalate (DBP) induces antiandrogenic but not estrogenic in vivo effects in LE hooded rats. *Toxicologist* **42(1-S)**, 176.

Gray, L.E., Jr., J.S. Ostby, and W.R. Kelce (1994). Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rats. *Toxicol. Appl. Pharmacol.* **129(1)**, 46-52.

Hafez, E.S.E. (ed.) (1970). *Reproduction and Breeding Techniques for Laboratory Animals*. Lea and Febiger, Philadelphia, PA.

Hanley, T.R., Jr., and P.G. Watanabe (1985). Measurement of solid food consumption patterns in neonatal rats by ^{141}Ce-radiolabeled microspheres. *Toxicol. Appl. Pharmacol.* **77(3)**, 496-500.

Hellwig, J., B. van Ravenzwaay, M. Mayer, and C. Gembardt (2000). Pre- and postnatal oral toxicity of vinclozolin in Wistar and Long-Evans rats. *Regul. Tox. Pharm*. **32(1)**, 42-50.

Higuchi, T.T., J.S. Palmer, L.E. Gray, Jr., and D.N. Veeramachaneni (2003). Effects of dibutyl phthalate in male rabbits following *in utero*, adolscent, or postpubertal exposure. *Toxicol Sci.* **72(2**), 301-313.

Huber, P.J. (1967). The behavior of maximum likelihood estimates under nonstandard conditions. In: *Proceedings of the Fifth Berkeley Symposium on Mathematical Statistics and Probability* **1**, 221-233.

Imajima, T., T. Shono, O. Zakaria, and P. Suita (1997). Prenatal phthalate causes cryptorchidism postnatally by inducing transabdominal ascent of the testis in fetal rats. *J. Pediatr.* Surg. **32,** 18-21.

Kavlock R, Boekelheide K, Chapin R, Cunningham M, Faustman E, Foster P, Golub M, Henderson R, Hinberg I, Little R, Seed J, Shea K, Tabacova S, Tyl R, Williams P, Zacharewski T. (2002). NTP Center for the Evaluation of Risks to Human Reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di-n-octyl phthalate. *Reprod. Toxicol*. **16(5)**:721-34.

Kubota, Y., C. Temelcos, R.A. Bathgate, K.J. Smith, D. Scott, C. Zhao, and J.M. Hutson (2002). The role of insulin 3, testosterone, Müllerian inhibiting substance and relaxin in rat gubernacular growth. *Mol. Hum. Reprod.* **8(10),** 900-905.

Levene, H. (1960). Robust tests for the equality of variance. In: *Contributions to Probability and Statistics* (I. Olkin, S.G. Ghurye, W. Hoeffding, W.G. Madow, and H.B. Mann, Eds.), Palo Alto, CA, Liang, K., and S. Zeger (1986). Longitudinal data analysis using generalized linear models. *Biometrika* **73**, 13-22.

McIntyre B.S., N.J. Barlow, and P.M. Foster (2001). Androgen-mediated development in male rat offspring exposed to flutamide *in utero*: permanence and correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues. *Toxicol. Sci.* **62(2)**, 236-249.

McIntyre B.S., N.J. Barlow, P.M. Foster (2002). Male rats exposed to linuron *in utero* exhibit permanent changes in anogenital distance, nipple retention, and epididymal malformations that result in subsequent testicular atrophy. *Toxicol. Sci.* **65(1)**, 62-70.

Mylchreest, E., R.C. Cattley, M. Sar, and P.M. Foster (1998a). The effects of di(n-butyl) phthalate on prenatal androgen-regulated male sexual differentiation are not mediated by direct interaction with the androgen receptor. *Teratology* **57(4/5)**, 199 (Abstract No. 25).

Mylchreest, E., R.C. Cattley, and P.M. Foster (1998b). Male reproductive tract malformations in rats following gestational and lactational exposure to di(n-butyl) phthalate: an antiandrogen mechanism? *Toxicol. Sci*. **43(1)**, 47-60.

Mylchreest, E., M. Sar, R.C. Cattley, and P.M. Foster (1999). Disruption of androgen-regulated male reproductive development by di(n-butyl) phthalate during late gestation in rats is different from flutamide. *Toxicol. Appl. Pharmacol*. **156**, 81-95.

Nef, S., and L.F. Parada (1999). Cryptorchidism in mice mutant for Insl3. *Nat. Genet.* **22(3),** 295-299.

NRC (1996). *Guide for the Care and Use of Laboratory Animals*. Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council. Revised 1996.

Ostby, J.S., L.E. Gray, W.R. Kelce, C.J. Wolf, and O.P. Huey (1997). Sexual differentiation in male

Parks, L.G., J.S. Ostby, C.R. Lambright, B.D. Abbott, G.R. Klinefelter, N.J. Barlow, and L.E. Gray, Jr. (2000). The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicol. Sci.* **58,** 339-349.

RTI (2001). ***SUDAAN User's Manual, Release 8.0.*** Research Triangle Park, NC: Research Triangle Institute.

Royall, R.M. (1986). Model robust confidence intervals using maximum likelihood estimators. *International Statistical Review* **54,** 221-226.

Salewski, E. (1964). Farbemethode zum makroskopischen Nachweis von Implantationsstellen am Uterus der Ratte. *Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmakol*. **247**, 367.

SAS Institute Inc. (1999a). *SAS® Language Reference: Concepts*, Version 8, Cary, NC: SAS Institute Inc. 554 pp.

SAS Institute Inc. (1999b). *SAS/STAT® Users' Guide*, Version 8, Cary, NC: SAS Institute Inc. 3884 pp.

SAS Institute Inc. (1999c). *SAS® Language Reference: Dictionary*, Version 8, Cary, NC: SAS Institute Inc. 1244 pp.

SAS Institute Inc. (1999d). *SAS® Procedures Guide*, Version 8, Cary, NC: SAS Institute Inc. 1643 pp.

SAS Institute Inc. (1999e). *SAS® Companion for the Microsoft Windows Environment*, Version 8, Cary, NC: SAS Institute Inc. 562 pp.

SAS Institute Inc. (2000). *SAS/STAT® Software: Changes and Enhancements, Release 8.1*, Cary, NC: SAS Institute Inc. 554 pp.

Shono, T., H. Kai, and H. Sulta Send Nawata (2000). Time-specific effects of mono-n-butyl phthalate on the transabdominal descent of the testis in rat fetuses. *BJU International* **86,** 121-125.

Tomboc, M., P.A. Lee, M.F. Mitwally, F.X. Schneck, M. Bellinger, and S.F. Witchel (2000). Insulin-like 3/relaxin-like factor gene mutations are associated with cryptorchidism. *J. Clin. Endocrinol. Metab.* **85(11),** 4013-4018.

Tyl, R.W., C.B. Myers, M.C. Marr, B.F. Thomas, A.R. Keimowitz, D.R. Brine, M.M. Veselica, P.A. Fail, T.Y. Chang, J.C. Seely, R.L. Joiner, J.H. Butala, S.S. Dimond, S.Z. Cagen, R.N. Shiotsuka, G.D. Stropp, and J.M. Waechter (2002). Three-generation reproductive toxicity study of dietary Bisphenol A (BPA) in CD® (Sprague-Dawley) rats. *Toxicol. Sci.* **68(1),** 121-146.

U.S. EPA (1998). U.S. Environmental Protection Agency, Prevention, Pesticides and Toxic Substances. Health Effects Test Guidelines, OPPTS 870.3700, Prenatal Developmental Toxicity Study, EPA 712-C-98-207. August 1998.

Ward, J.M., J.M. Peters, C.M. Perella, and F.J. Gonzalez (1998). Receptor and nonreceptor-mediated organ-specific toxicity of Di(2-ethylhexyl) phthalate (DEHP) in peroxisome proliferator-activated receptor α-null mice. *Toxicol. Pathol.* **26,** 240-246.

Westfall, P., and S. Young (1993). *Resampling-Based Multiple Testing*. NY: John Wiley and Sons.

Wilson, V.S., C. Lambright, J. Furr, J. Ostby, C. Wood, G. Held, and L.E. Gray, Jr. (2003). Phthalate ester-induced gubernacular ligament lesions are associated with reduced Insl3 gene expression in the fetal rat testis during sexual differentiation, in press.

Zeger, S. and K. Liang (1986). Longitudinal data analysis for discrete and continuous outcomes. *Biometrics* **42**, 121-130.

Zeger, S., K.Y. Liang, and P.S. Albert (1988). Models for longitudinal data: a generalized estimating equation approach. *Biometrics* **44**, 1049-1060.

Zimmermann, S., G. Steding, J.M. Emmen, A.O. Brinkman, K. Nayernia, A.F. Holstein, W. Engel, and I.M. Adham (1999). Targeted disruption of the Insl3 gene causes bilateral cryptorchidism. *Mol. Endocrinol.* **13(5),** 681-691.

PROTOCOL DEVIATIONS

1. Nine instances of deviations from the protocol were noted, all involving relative humidity levels in the animal rooms, as follows:

Room	Date	%RH	Duration
303	8/15/02	71.0	1 hour
303	9/17/02	85.5	1 hour
403	9/17/02	80.4	1 hour
403	10/9/02	85.0	1 hour
404	9/17/02	85.3	1 hour
404	10/9/02	78.9	1 hour
503	9/17/02	78.4	1 hour
503	10/9/02	76.1	1 hour
407	10/9/02	79.9	1 hour

2. On 8/2/02, the lights in the ARF did not go off at 8 pm as scheduled. The lights were turned off manually at 9:30 pm. Thus, the lights were on 15.5 hours instead of 14. This deviation occurred during the gestational period. Based on the pregnancy rates, successful deliveries, and subsequent rearing of the young, this deviation in the light cycle did not appear to have an adverse effect on the study.

3. Samples of the dose formulations were scheduled to be taken on the first day of dosing on gd 6, pnd 0, 7, 14, and 21. Due to an oversight by the laboratory staff, the samples for pnd 0 and 20 were not taken until the second day of dosing for those time points. Results of the analysis (see Appendix II) suggest that the dosing formulation had not been compromised by this oversight, and that it did not adversely affect the results of the analysis or the study.

4. The post-dosing time for Animal #98, Rx Code 31036 was inadvertently not recorded on 8/16/02.

5. The post-dosing observation time for 4 females in Rx 01822 was from 1-5 minutes over the 2 hours post-dosing time on 8/5/03.

6. On 8/15/02, two female pups were found dead and one male pup. External and visceral findings were recorded for one female and one male. Documentation for external and visceral exam for one female was inadvertently not recorded.

7. Presence or absence of cranial suspensory ligament was inadvertently not recorded at necropsy for Male 1348, Rx Code 77491, and Male 1038, Rx Code 31038.

8. Weight of Glans Penis inadvertently not recorded at necropsy for Male #1044, Rx Code 31038.

9. The presence or absence of gubernacular cords was not recorded at necropsy for Male #1158, Rx Code 31038.

10. The weight of the Glans Penis was taken at necropsy even though the Glans Penis was noted as partially detached for Male 1156, Rx Code 31038. The weight was not entered.

11. The presence or absence of cranial suspensory ligaments was not recorded at pnd 21 necropsy for 6 pups in Rx Code 77491, for 6 pups in Rx Code 31036, for 9 pups in Rx Code 01822, for 6 pups in Rx Code 24038, and for 7 pups in Rx Code 17225.

12. Animals were ordered at 10-11 weeks of age with a weight range of 200-300 g and should have been ordered at age of 9-10 weeks with a weight range of 200-225 g.

13. Presence or absence of hypospadias not recorded for: dam 10 pup 2, dam 21 pup 2 in Rx 77491; dam 12 pup 5, dam 41 pup 2 in Rx 31036; dam 42 pup 1, dam 23 pup 5 in Rx 01822; dam 7 pup 2, dam 84, pup 4 in Rx 24038; dam 6 pup 1, dam 16 pup 2 in Rx 17725.

14. Presence or absence of epispadias not recorded for: dam 10 pup 2, dam 21 pup 2 in Rx 77491; dam 12 pup 5, dam 41 pup 2 in Rx 31036; dam 3 pup 1, dam 8 pup 3, dam 8 pup 4, dam 13 pup 3, dam 23 pup 3, dam 23 pup 5, dam 42 pup 1, dam 51 pup 3 in Rx 01822; dam 7 pup 2 dam 84 pup 4 in Rx 24038; dam 6 pup 1, dam 16 pup 2, dam 114 pup 2 in Rx 17725.

15. Presence or absence of cleft phallus not recorded for: dam 10 pup 2, dam 21 pup 2 in Rx 77491; dam 12 pup 5, dam 41 pup 2 in Rx 31036; dam 3 pup 1, dam 8 pup 3, dam 8 pup 4, dam 13 pup 3, dam 23 pup 3, dam 23 pup 5, dam 42 pup 1, dam 42 pup 2 in Rx 01822; dam 7 pup 2, dam 84 pup 4 in Rx 24038; dam 6 pup 1, dam 16 pup 2 in Rx 17725.

16. Inguinal regions soiled with urine not recorded for: dam 10 pup 2 in Rx 77491; dam 41 pup 2 in Rx 31036; dam 13 pup 2, dam 13 pup 3, dam 23 pup 5, dam 42 pup 1, dam 51 pup 3 in Rx 01822; dam 7 pup 2, dam 84 pup 4 in Rx 24038; dam 16 pup 2, dam 77 pup 1 in Rx 17725.

17. The length of the right gubernacular cord was not recorded because the cord was inadvertently cut for dam 23 pup 2 in Rx 01822. The length of neither gubernacular cord was not recorded because both were inadvertently cut for dam 115 pup 2 in Rx 17725.

18. F1 males 1266 through 1323 were weighed on 9/15/02 instead of 9/14/02 as required. Note: neither body or feed weights were done on 9/15/02.

19. Presence or absence of gross necropsy findings not recorded for: dam 3 pup 1, dam 42 pup 1 in Rx 01822; dam 24 pup 1 in Rx 24038; dam 6 pup 1, dam 15 pup 2 in Rx 17725.

20. Seminal vesicles were lost prior to weighing for dam 52 pup 1 in Rx 31036.

21. Number of nipples and number of areola not recorded for: dam 23 pup 5 in Rx 01822; dam 34 pup 3 in Rx 24038.

22. Levator ani plus bulbocavernosus lost prior to weighing for dam 109 pup 1 in Rx 24038.

23. One cowpers gland lost prior to weighing for dam 16 pup 1 in Rx 17725.

In the Study Director's professional opinion, these deviations did not affect the study design, performance, or interpretation and are presented for completeness.

_____ _____

Julia D. George, Ph.D. Date

Study Director

Table 1. Analyses of Dose Formulations[a]

Test chemical	RTI Rx Code	RTI Color Code	Battelle Sample Code	Sample Type[b]	Nominal Concentration (mg/ml)	Analytical Concentration (mg/ml)[c]	Mean % of Nominal ± RSD[d]
vehicle	NA	NA	2-10-A	preship	0	ND[e]	--
DBP	NA	NA	2-10-b	preship	20	18.8	94.1±2.52
			2-10-c	preship	100	94.2	94.3±2.09
Vinclozolin	NA	NA	2-10-d	preship	10	9.77	97.7±2.28
	NA	NA	2-10-e	preship	20	19.5	97.5±1.89
DBP	24038-GD6	purple	1778-12-1	first dosing day	20	19.0	94.4±2.01
	20438-PND0	purple	1778-12-2	first dosing day	20	18.3	
	20438-PND7	purple	1778-12-3	first dosing day	20	18.7	
	20438-PND14	purple	1778-12-4	first dosing day	20	19.0	
	20438-PND21	purple	1778-12-5	first dosing day	20	19.3	
	17725-GD6	red	1778-13-1	first dosing day	100	93.8	94.5±2.16
	17725-PND0	red	1778-13-2	first dosing day	100	93.6	
	17725-PND7	red	1778-13-3	first dosing day	100	97.7	
	17725-PND14	red	1778-13-4	first dosing day	100	92.3	
	17725-PND21	red	1778-13-5	first dosing day	100	95.0	
Vinclozolin	31036-GD6	green	1779-86-1	first dosing day	10	9.48	94.6±1.26
	31036-PND0	green	1779-86-2	first dosing day	10	9.27	
	31036-PND7	green	1779-86-3	first dosing day	10	9.58	
	31036-PND14	green	1779-86-4	first dosing day	10	9.47	
	31036-PND21	green	1779-86-5	first dosing day	10	9.51	
	01822-GD6	orange	1779-87-1	first dosing day	20	18.7	93.6±1.76
	01822-PND0	orange	1779-87-2	first dosing day	20	18.9	
	01822-PND7	orange	1779-87-3	first dosing day	20	18.8	

(Continued)

Test Chemical	RTI Rx Code	RTI Color Code	Battelle Sample Code	Sample Type[b]	Nominal Concentration (mg/ml)	Analytical Concentration (mg/ml)[c]	Mean % of Nominal ± RSD[d]
	01822-PND14	orange	1779-87-4	first dosing day	20	18.1	
	01822-PND21	orange	1779-87-5	first dosing day	20	19.0	
DBP	10303-05-01	NA	1778-10-1	postdose	20	18.8	94.3±0.49
	10303-05-01	NA	1778-10-2	postdose	20	18.9	
	10303-06-01	NA	1778-11-1	postdose	100	83.5	94.1±1.36
	10303-06-01	NA	1778-11-1 R-1[f]	postdose	100	93.8	
	10303-06-01	NA	1778-11-1 R-2	postdose	100	95.2	
	10303-06-01	NA	1778-11-1 R-3	postdose	100	95.9	
	10303-06-01	NA	1778-11-2	postdose	100	93.2	
Vinclozolin	10303-07-01	NA	1779-84-1	postdose	10	8.79	95.8±3.34
	10303-07-01	NA	1779-84-1 R-1	postdose	10	9.75	
	10303-07-01	NA	1779-84-1 R-2	postdose	10	9.95	
	10303-07-01	NA	1779-84-1 R-3	postdose	10	9.71	
	10303-07-01	NA	1779-84-2	postdose	10	9.35	
	10303-08-01	NA	1779-85-1	postdose	20	18.2	94.8±5.79
	10303-08-01	NA	1779-85-2	postdose	20	17.7	
	10303-08-01	NA	1779-85-2 R-1	postdose	20	19.5	
	10303-08-01	NA	1779-85-2 R-2	postdose	20	19.5	
	10303-08-01	NA	1779-85-2 R-3	postdose	20	20.1	

(Continued)

[a] Dosing solutions were formulated on 7/9/02 in corn oil vehicle for administration at 5.0 ml/kg. The doses were therefore 0, 50, and 100 mg/kg/day vinclozolin, and 100, and 500 mg/kg/day DBP.

[b] Samples were taken prior to shipping from Battelle to RTI (preship), on the first day dosing for gd 6 and pnd 7 and 14, on the second day of dosing on pnd 0 and 20, and after dosing was completed (postdose).

[c] n=3 for individual determinations.

[d] Data are presented as mean % (% relative standard deviation).

[e] ND = not detected; estimated limit of detection is 0.57 microg/ml for DBP and 0.11 microg/ml for vinclozolin.

[f] Analyses labeled “R” were reanalyses of samples for which less than 90% of the nominal concentration was recovered.