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Toxicology Portfolio

Pubertal Development and Thyroid Function in Intact Juvenile Rats Exposed to 3-Nitro-1,2,4-Trazol-5-One (NTO), February–June 2012

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14. ABSTRACT

NTO was administered to male (0, 250, and 500 mg/kg-day) and female (0, 500, and 1000 mg/kg-day) Sprague-Dawley rats (15/sex/group) via oral gavage from weaning through puberty. Animals were examined daily for onset of puberty. Estrous cyclicity was evaluated daily upon completion of vaginal opening (VO). Age and body weight at VO and PPS, and all measures of estrous cyclicity were not affected by treatment with NTO. Testis mass was reduced by 30% and 65% compared to control in the 250 and 500 mg/kg-day groups, respectively. The reduction in testis mass was associated with tubular degeneration/atrophy. Less pronounced reductions in the mass of androgen-dependent accessory reproductive tissues were also observed in the 500 mg/kg-day group; however, the reduction was only significant in the epididymides. There were no differences in hormone levels or thyroid follicle and colloid scores between NTO treatment groups and the control groups. These findings suggest that NTO is not acting as an estrogen or thyroid active compound, but may indicate effects on steroidogenesis and/or direct testicular toxicity

15. SUBJECT TERMS

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Pubertal Development and Thyroid Function in Intact Juvenile Rats Exposed to 3-Nitro-1,2,4-Trazol-5-One (NTO), February-June 2012

Data Requirement

Endocrine Disruptor Screening Program Test Guidelines
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STATEMENT OF DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this report on the basis of its falling within the scope of TSCA § 790.7 (a) – (d).

Company:_____

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Good Laboratory Practice Compliance Statement

The study described in this report was conducted in compliance with Title 40, Code of Federal Regulations (CFR), Part 792, Good Laboratory Practice Standards, except for the following:

1. The test article characterization (purity) was conducted by the manufacturer and it is not known whether the testing was done in compliance with the above regulation.

Submitted By:

Study Director:

Emily May Lent

EMILY MAY LENT
Toxicologist
Toxicity Evaluation Program (TEP)

2/14/2014
Date

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FEBRUARY–JUNE 2012

1 Summary

1.1 Purpose

The primary objective of this study was to assess the endocrine disrupting potential of 3-nitro-1,2,4-triazol-5-one (NTO) through the use of two screening assays. The purpose of these assays was to determine whether NTO has the potential to interact with the endocrine system *in vivo* by identifying effects on pubertal development and thyroid function in the intact juvenile rat. These assays can detect anti-thyroid, estrogenic, anti-estrogenic, androgenic, and anti-androgenic activity as well as agents acting via gonadotropins, prolactin, or hypothalamic function.

1.2 Conclusions

NTO was administered to male (0, 250, and 500 milligrams per kilogram per day (mg/kg-day)) and female (0, 500, and 1000 mg/kg-day) Sprague-Dawley rats (15/sex/group) via oral gavage from weaning through puberty. Animals were examined daily for onset of puberty. Estrous cyclicity was evaluated daily upon completion of vaginal opening (VO). Detailed gross necropsy was conducted; weights were recorded for reproductive tissues, accessory reproductive tissues, thyroid, pituitary, adrenals, liver, and kidneys. Trunk blood was collected and clinical chemistry and hormone assays (testosterone, thyroxine (T4), thyroid-stimulating hormone (TSH)) conducted. Final body weight and weight gain of females in the 1000 mg/kg-day group were reduced (7% and 10%, respectively) compared to the control. Body weight of male rats was unaffected by NTO treatment. Food consumption did not differ among treated and control groups at any time for males or females. Age at VO and body weight at VO, as well as all measures of estrous cyclicity (age at first estrus, cycle length, percent cycling, percent cycling regularly) were not affected by treatment with NTO at 500 and 1000 mg/kg-day. Age at preputial separation (PPS) and body weight at PPS did not differ between NTO treated rats and the control group. Testis mass was reduced by 30% and 65% compared to control in the 250 and 500 mg/kg-day groups, respectively. The reduction in testis mass was associated with tubular degeneration/atrophy. Less pronounced reductions in the mass of androgen-dependent accessory reproductive tissues, including epididymides, seminal vesicles, and dorsolateral prostate were also observed in the 500 mg/kg-day group; however, these reductions were only significant for the epididymides. NTO did not significantly affect clinical chemistry parameters in female rats. In male rats total bilirubin (TBIL) was reduced in the 500 mg/kg-day dose group. There were no differences in hormone levels between NTO treatment groups and the control groups for males or females. Thyroid follicle and colloid scores did not differ between NTO treated and control groups. These findings suggest that NTO is not acting as an estrogen or thyroid active compound under the test conditions. The observed testicular toxicity and the effects on the androgen-dependent reproductive tissues may indicate effects on steroidogenesis and/or direct testicular toxicity.

2 References

See Appendix A for a listing of references.

3 Authority

Military Interdepartmental Purchase Request (MIPR) number MIPRIJDATHR142. This toxicology study addresses, in part, the environmental safety and occupational health requirements outlined in Army Regulations (AR) 200-1, AR 40-5, and AR 70-1; Department of Defense Instruction 4715.4; and Army Environmental Requirements and Technology Assessments (AERTA) (DA 2007a; b; 2003, 2008; DOD 1996; USAEC 2009). It was performed as part of an on-going effort by the U.S. Army Environmental Quality Technology (EQT), Ordnance Environmental Program Pollution Prevention Team, to produce safer ordnance. This program is under the direction of the U.S. Army Research, Development, and Engineering Command Environmental Acquisition Logistics & Sustainment Program and EQT Pollution Prevention.

4 Background

NTO (3-nitro-1,2,4-triazol-5-one) is a member of a new generation of insensitive munitions (IM) being developed to replace conventional explosives (e.g., 2,4,6-trinitrotoluene (TNT), research department explosive (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)) which are susceptible to unintentional initiation (Mathieu and Stucki 2004; Smith and Cliff 1999; Spear, Louey, and Wolfson 1989). NTO, a white crystalline compound produced by nitration of 1,2,4-triazol-5-one, was first synthesized in 1905. Testing of the IM properties of NTO dates to the mid-1980s (Lee and Coburn 1985). Characterized as much less sensitive than RDX and TNT with limited reduction in performance in initial tests, NTO has undergone evaluation for use in varied IM-formulations (Lee and Coburn 1985; Smith and Cliff 1999; Spear, Louey, and Wolfson 1989). Although the chemical, physical and explosive properties of NTO have been extensively tested, only limited toxicological testing has been conducted.

Toxicity testing of conventional explosives has often focused on determining effects in personnel working with explosives and development of safe worker levels. Occupationally relevant toxicity of nitroaromatic explosives (e.g., TNT, dinitrotoluenes (DNTs)) typically includes methemoglobinemia, liver and spleen damage, cataracts, and/or skin irritation (Cenas et al. 2001; Kalderis et al. 2011). Similar testing of NTO demonstrated that NTO causes mild skin irritation, but not eye irritation or dermal sensitization (London and Smith 1985). Subacute and subchronic oral studies in rats demonstrated limited hematological effects (slight anemia) and liver hyperplasia only at the highest doses tested, 1000 mg/kg-day of NTO. The most pronounced effects of NTO following subchronic oral exposure were testicular and epididymal toxicity and hypospermia, occurring at doses as low as 315 mg/kg-day (Crouse et al. 2010).

The toxicity of nitro compounds (e.g., nitro-aromatic and nitro-heterocyclic) is typically attributed to metabolic intermediates produced through nitroreduction and, to a lesser extent, denitrification (LeCampion et al. 1997). *In vitro* metabolism studies using rat liver microsomes indicate that NTO is metabolized to 5-amino-1,2,4-triazol-3-one (ATO) via nitroreduction and 5-hydroxy-triazolone (urazole) via denitrification (LeCampion et al. 1997). *In vitro* enzymatic reactivity was much lower for NTO than for TNT, resulting in lower bioreductive activation of NTO (Sarlauskas et al. 2004). The low toxicity of NTO (LD_{50}>5 grams per kilogram (g/kg)) (London and Smith 1985) has been ascribed to the limited production of reactive metabolic intermediates and weak electron accepting potency of NTO (Sarlauskas et al. 2004). ATO was found at very low concentrations and urazole was not detected in urine samples collected from primates orally exposed to NTO. The authors suggest that the high concentration of NTO in these samples may have contributed to *ex vivo* ATO formation. NTO is quickly filtered into the urine and is unlikely to undergo significant metabolism in

the liver (Hoyt et al. 2013). Low production of NTO reactive intermediates may similarly indicate a low potential for reactivity with proteins and DNA and low genotoxicity and carcinogenicity risk. This theory was supported in a battery of genotoxicity tests in which NTO was not mutagenic in the Ames *Salmonella* test (TA98, TA100, TA1535, and TA1537), with and without metabolic activation (S9), or in *Escherichia coli* (WP2*uvrA*), did not induce chromosomal aberrations in Chinese Hamster Ovary (CHO) cells (CHO-W-B1), and was negative in the L5178YTK$^{+/-}$ mouse lymphoma test (Reddy et al. 2011). NTO was also non-genotoxic in the *in vivo* rat peripheral blood micronucleus assay at doses up to 2000 mg/kg-day for 14 days (Reddy et al. 2011).

Detection of explosive compounds and their degradation products in soil and groundwater near ammunition plants raises issues of potential effects on surrounding populations (Kalderis et al. 2011). For example, TNT in groundwater in Germany has been linked to increased incidences of leukemia in local populations (Cenas et al. 2001). Similar links between NTO contamination and effects in local populations have not been established. Although NTO has demonstrated limited toxicity in previously conducted testing, testicular toxicity reported at the lowest doses tested raises concern about potential reproductive and endocrine disrupting effects. In the present study, we therefore conducted several Tier 1 U.S. Environmental Protection Agency (EPA) Endocrine Disruptor Screening Program tests to assess the *in vivo* endocrine disrupting potential of NTO.

The following table identifies the date of critical study events.

Table 1. Critical Study Events

Critical Event	**Date of Event**
Animal Use Protocol Approved	02/07/2012
Timed-Pregnant Females Received	02/29/2012
Delivery Period (Post Natal Day (PND) 0)	03/13/2012 – 03/14/2012
PND3 Litter Size Standardization	03/16/2012 - 03/17/2012
PND21 Pups Selected for Study and Assigned to Experimental Groups	04/03/2012
Experimental Start	04/04/2012
Female Sexual Maturation Evaluation (PND 22 – Vaginal Opening)	04/04/2012 – 04/23/2012
Estrous Cyclicity Evaluation (Vaginal Opening – PND 42/43)	04/14/2012 – 04/25/2012
Male Sexual Maturation Evaluation (PND 30 – Preputial Separation)	04/12/2012 – 04/30/2012
Female Necropsies (PND 42/43)	04/24/2012 – 04/25/2012
Male Necropsies (PND 53/54)	05/05/2012 – 05/06/2012
Thyroid Hormone Assays	06/06/2012 – 06/11/2012
Experimental Completion	06/11/2012
Study Completion	02/14/2014

5 Materials and Methods

5.1 Test Substance

Neat NTO (CAS # 932-64-9; lot 11L375-061; purity: 99.4%) was produced by BAE Systems, Ordnance Systems, 4509 West Stone Drive, Kingsport, TN 37660. Dosing solutions/suspensions were prepared by weighing the required amount of NTO and a measured volume of corn oil (lot Feb072013D). NTO was then wetted with a small amount of the measured volume of corn oil, ground using a mortar and pestle to a fine consistency, and mixed with the remaining measured volume of corn oil. Three dosing solutions/suspensions, 50, 100, and 200 milligrams per milliliter (mg/ml), were prepared at the start of the study in sufficient volume for use throughout the study. A one milliliter sample was taken from each dosing solution/suspension and analyzed by Army Institute of Public Health (AIPH) Laboratory Sciences Portfolio via high performance liquid chromatography to verify the concentration. In addition, the homogeneity of the solutions/suspensions was verified by determining the concentration of samples taken from the top, middle, and bottom of the highest concentration (200 mg/ml) suspension. Samples were collected from a representative suspension (6 mg/ml) at weekly intervals prior to the study to determine the stability of the dosing suspensions. Results from the stability test indicated that the test compound was stable for at least six weeks when stored at room temperature. The dosing solutions/suspensions were mixed on a stir plate for approximately one hour prior to taking analytical samples, prior to dosing, and continued to be mixed throughout the dosing procedure.

5.2 Animals*

This study was conducted using F1 generation male and female Sprague Dawley (Crl:CD(SD) CD®) rats delivered at the testing facility. Twenty-two P generation timed-pregnant dams were obtained from Charles River Laboratories, Wilmington, Massachusetts on gestation day (GD) nine. Dams were not considered part of the test system. All animals were housed in temperature-, relative humidity-, and light-controlled rooms. The target conditions of the rooms were 68-79 °F and 30-70 percent humidity. An automatically controlled 14:10-hour light/dark cycle was maintained, with the dark period beginning at 1900 hours. Temperature was out of range on one day for approximately two hours, resulting in a temperature range of 61-79 °F. Relative humidity was out of range on several occasions, with a range of 24-77 percent humidity. A certified pesticide-free rodent chow with genistein plus daidzein content of less than 300 micrograms per gram (µg/g) (Harlan Teklad®, 2020XC Certified Rodent Diet) was available *ad libitum*. Deionized water was provided *ad libitum* in glass water bottles with silicone stoppers. P generation dams were individually housed. After weaning, the F1 generation rats were housed in same sex groups of two or three by dosage group. All rats were housed in suspended polycarbonate cages with ALPHA-dri® bedding. Each P generation rat and weaned F1 generation rat was uniquely identified by number via cage card and tail marking. Pups were not individually identified during the postpartum nursing period. (CD® is a registered trademark of Charles River Laboratories International, Inc.; Teklad® is a registered trademark of Harlan, Teklad; ALPHA-dri® is a registered trademark with Shepard Specialty Papers.)

* Animal use procedures were approved by the United States Army Public Health Command (USAPHC) Institutional Animal Care and Use Committee. Animal care and use was conducted in accordance with *The Guide for the Care and Use of Laboratory Animals* and all applicable Federal and DOD regulations. The USAPHC Animal Care and Use Program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Litters were weighed and pups were counted and sexed on post natal day (PND) zero or one. Litters with more than 10 pups were standardized by culling on PND 3 to 10 pups per litter. One litter was abandoned by the dam and was euthanized. Two dams had fewer than 10 pups and were excluded from use on the study. Litters were weighed weekly until PND 21.

5.3 Contract Studies

Tissues were preserved, packaged, and transported to the United States Army Medical Research Institute of Chemical Defense (USAMRICD), Aberdeen Proving Ground, MD, for processing, slide preparation, and staining. Slides were returned to the USAPHC for evaluation by an American College of Veterinary Pathology board-certified military veterinary pathologist.

5.4 Quality Assurance

The AIPH Quality Systems Office audited critical study phases. Appendix B provides the dates of these audits, the phases audited and the dates that the results of the inspections were reported to the Study Director (SD) and Management.

5.5 Study Personnel

Appendix C lists the names of individuals contributing to the study performance.

5.6 Test Group Assignment

Forty-five female and forty-five male Sprague Dawley (Crl:CD(SD) CD®) rats were used for this study. At the start of test substance administration, females were at PND 22 and weighed 53.8 ± 4.05 grams (g), while males were at PND 23 and weighed 60.5 ± 4.09 g. On PND 21, fifteen rats were assigned to each of two NTO treatment groups and a vehicle control group (corn oil control). Assignment to test groups was accomplished using a stratified random procedure, with animals stratified according to body mass and litter of origin. Littermates were not assigned to the same treatment group. Body mass did not differ among test groups prior to initiation of dosing. Animals were divided into two time-separated necropsy groups, with animals from each test group approximately evenly distributed across necropsy groups.

5.7 Dose Selection and Test Substance Administration

Dosage levels were selected based on the pattern of effects observed in the 14- and 90-day oral toxicity studies (Crouse et al. 2010). Limited abnormal clinical chemistry was observed in the 14-day study at doses at or above 1000 mg/kg-day. Testes weights and weight ratios were significantly reduced compared to controls in male rats administered 500 mg/kg-day NTO and above in the 14-day study. Histopathology was not performed on any tissues from the 14-day study. No effects on body weight were observed in the 90-day study and effects on blood parameters were limited to slight anemia and reduced albumin and protein in males at or above 1000 mg/kg-day. The 90-day study on NTO revealed significant reductions in testes and epididymides weights and sperm counts at dosages of 315 mg/kg-day and above. Histopathology performed on the 90-day tissues revealed significant incidences of testicular hypoplasia at dosages of 315 mg/kg-day and above as well as insignificant, less severe, testicular hypoplasia at dosages of 100 mg/kg-day and below. The only effect noted for females was hepatocellular hypertrophy at doses at or above 1000 mg/kg-day. Based on these results, it was determined that the maximum

tolerated dose (MTD) differed between males and females and, thus, the doses selected for this study differed between the sexes. A MTD of 1000 mg/kg-day was selected for females based on the very limited effects at this dose and the lack of effects observed below this dose in either the 14- or 90-day study. The low dose for females was calculated as ½ the MTD, or 500 mg/kg-day. A MTD of 500 mg/kg-day was selected for the males based on the presence of abnormal clinical chemistry/hematology and liver histopathology at 1000 mg/kg-day. The pattern of changes in reproductive tissue weights and histopathology was used in selecting the dose; however, the MTD was not selected to be below doses causing reproductive effects as doing so would likely ensure a negative outcome for the assay. The low dose for males was calculated as ½ the MTD, or 250 mg/kg-day.

All NTO doses and the control were administered based on body mass and volume of solution/suspension at a rate of 5 milliliter per kilogram (ml/kg). Dosages were adjusted daily for changes in body mass. The NTO solution/suspensions and corn oil control were administered between 0700 and 0900 daily from PND 22 through PND 42/43 for females and from PND 23 through PND 53/54 for males. Oral dosing was performed using a stainless steel 18 gauge x 1-2 inch gavage needle with a 2.25 millimeter (mm) ball tip; needle length was selected based on rat size.

5.8 Observations, Body Mass, Food Consumption

Litters were evaluated for viability daily. Pups were sexed and counted and litter body mass was recorded on PNDs 0/1, 3, 7, 14 and 21.

During the dosing period, observations for mortality and signs of toxic effects were made at least twice daily, once in the morning and once in the afternoon, except on weekends when observations occurred only in the morning. Additionally, each animal was removed from its cage daily and given a clinical examination. Examinations included evaluation of skin and fur, eyes and mucous membranes. Respiratory and circulatory effects, autonomic effects such as salivation, central nervous system effects, including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypes or bizarre behavior (e.g., self-mutilation and walking backwards) were recorded. Clinical examinations were made concurrently with dosing.

Animals were weighed daily during the dosing period. Feed was provided *ad libitum* seven days per week in weighed feeder bins. Feeders were reweighed at least weekly and the mass of the empty feeder was subtracted from the mass of the full feeder to determine the grams of food consumed for each group of animals. Per rat feed consumption was calculated as total feed consumed per cage divided by the number of rats per cage. Although this does not reflect individual feed consumption, differences in the number of rats per cage necessitated standardizing feed consumption data per rat rather than per cage.

5.9 Sexual Maturation Evaluation – Vaginal Opening (VO) and Estrous Cyclicity

Beginning on PND 22, females were examined at approximately the same time each day after daily dosing for VO. Females were examined for the appearance of a small “pin hole,” a vaginal thread, or complete VO. The PND and body mass at initiation of VO and completion of VO were recorded. Beginning on the day of complete VO through the day of necropsy, vaginal smears were obtained daily to evaluate estrous cyclicity. Vaginal smears were collected via vaginal lavage (Marcondes,

Bianchi, and Tanno 2002; OECD 2009). Using a pipette with a disposable tip, a small amount (approximately 0.1 ml) of sterile saline was repeatedly (approximately 2-3 times) flushed in and out of the vagina until the fluid appeared cloudy. The cell suspension was then placed in a labeled microcentrifuge tube and stored at 4±2 ºC, if slides were not made immediately. Several drops of the cell suspension were then expelled onto a labeled glass slide and covered with a cover slip. Slides were evaluated within 6 hours of collection to obviate fixing and staining and were discarded after evaluation.

Slides were examined under low-power (10-20X) using a light microscope for the presence of leukocytes, nucleated epithelial cells, or cornified epithelial cells (Marcondes, Bianchi, and Tanno 2002; OECD 2009; USEPA 2009). The vaginal smears were classified as diestrus (predominance of leukocytes mixed with some cornified epithelial cells), proestrus (predominance of clumps of round, nucleated epithelial cells), or estrus (predominance of cornified epithelial cells). The estrous stage was determined daily after VO and the age at first vaginal estrus noted.

5.10 Sexual Maturation Evaluation – Preputial Separation (PPS)

Beginning on PND 30, males were examined at approximately the same time each day after daily dosing for PPS. The PPS was determined by attempting to manually retract the prepuce using gentle pressure (Korenbrot, Huhtaniemi, and Weiner 1977). The appearance of partial and complete PPS, or a persistent thread of tissue between the glans and prepuce, was recorded on the days they were observed. The PND and body mass at initiation and completion of PPS were recorded.

5.11 Necropsy

Post-weaning, P generation dams were euthanized via carbon dioxide (CO_2) and were not further evaluated. Pups culled from litters on PND 3 were sacrificed using CO_2 followed by decapitation and were not further evaluated. Pups not selected for study on PND 21 were transferred to another approved protocol.

On the day of necropsy, rats were removed one at a time from the dosing room to a separate room for euthanasia to minimize stress-related responses in other animals which may affect hormone measurements. On PND 42/43, female rats were given an intramuscular injection of ketamine (70-80 mg/kg) in combination with xylazine (7-10 mg/kg) immediately prior to decapitation. On PND 53/54, male rats were anesthetized by exposure to CO_2 for no more than sixty seconds and immediately euthanized by decapitation. Upon decapitation, blood from the trunk of the animals was collected immediately into serum separation tubes. Blood was allowed to clot at room temperature for 30 to 40 minutes, was centrifuged for approximately 10 minutes at 2,325 x g. Serum was removed and immediately analyzed for clinical chemistry parameters, testosterone, and total thyroxine (T4). Aliquots (100 microliters (µl)) were also placed in siliconized tubes and stored at approximately -35 °C for subsequent (TSH) assays. Rats were euthanized beginning two hours after dosing and blood collection was completed by 1300 hours to minimize the effects of diurnal fluctuations in thyroid hormone levels. Necropsy order was evenly distributed across treatment groups.

A full, detailed gross necropsy including a careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents was performed on all experimental animals following euthanasia. At necropsy, the ovaries (without oviducts; with and without fluid), uterus, testes, epididymides, ventral prostate, dorsolateral prostate, seminal vesicle

(with coagulating glands, with and without fluid), levator ani plus bulbocavernosus muscles (LABC), thyroid (with attached portion of trachea), liver, kidneys, pituitary, and adrenals were removed and weighed (except the thyroid/trachea, which was preserved prior to weighing). Kidneys, adrenals and ovaries were weighed as pairs. The left and right testes and the left and right epididymides were weighed individually. Small tissues such as the adrenals and pituitary, as well as tissues that contain fluid, were placed in a weigh-boat and covered with a moist paper towel to prevent tissues from drying out prior to weighing. Any observed lesions were removed for processing. Sections of the intestines were collected based on observations of pale intestines at necropsy. The vagina was collected for confirmation of estrus stage at time of necropsy.

The kidneys, ovaries, uterus, thyroid (with attached trachea), and vagina were placed in 10% buffered formalin for at least 24 hours for fixation. The thyroid (with parathyroids) was then dissected from the trachea, blotted and weighed to the nearest 0.01 mg. The left testis and epididymis from each animal was placed in Davidson's fixative overnight (no longer than 24 hours). After fixation, all tissues were rinsed and stored in 70% ethanol until embedded in paraffin.

5.12 Histopathology

Tissues were selectively trimmed and placed in cassettes labeled with protocol number, animal identification number, and laboratory assigned accession number. Cassettes were placed in labeled bottles and transported to the USAMRICD for processing. Tissues were routinely processed and paraffin embedded. All processed and embedded tissues were microtomed at 5 micrometers (µm) thick and stained routinely with hematoxylin and eosin.

The pathologist examined slides for compound-induced histopathologic changes via light microscopy. The prevalence and severity of findings were graded as compared to controls. Control animals were examined for background findings and all findings were recorded. Findings, in all animals and groups, were assigned as none, minimal, mild, moderate, or severe. Testis, epididymis, uterus, thyroid, one ovary, and one kidney were evaluated for pathologic abnormalities and potential treatment-related effects for all treated and control animals. The estrus stage at time of necropsy was determined in the uterus, ovary, and vagina.

The thyroid gland was paraffin embedded and a minimum of two sections per lobe, 5 µm apart, were placed per slide. Thyroid sections were subjectively evaluated for follicular cell height and colloid area using a five point grading scale (1 = shortest/smallest; 5 = tallest/largest) and any abnormalities/lesions were noted. A minimum of two sections of each of the two lobes of the thyroid were evaluated, and a representative thyroid score was determined for each animal based on the evaluation of all serial sections.

Ovaries were evaluated for follicular development (including presence/absence of tertiary/antral follicles, presence/absence of corpora lutea, changes in corpus luteum development, and changes in number of both primary and atretic follicles) in addition to any abnormalities/lesions such as ovarian atrophy. Five random sections were evaluated using the method of Smith et al. (Smith et al. 1991). The ovary was paraffin embedded and 5 sections, 5 µm apart, were placed on each slide. Five slides per ovary were generated and one section per slide was selected at random for evaluation. Follicles were counted and recorded per examined ovary section as primordial, growing, antral or atretic using the method of Smith et al. (Smith et al. 1991) and as referenced by Pedersen et al. (Pedersen and Peters 1968).

Uterine hyper- or hypotrophy as characterized by changes in uterine horn diameter and myometrial, stromal, or endometrial gland development was documented. Estrus stage at time of death was determined through histologic evaluation of the vagina, uterus, and ovary (Westwood 2008).

For each testis and epididymis evaluated, two step sections, 5 µm apart, were mounted per slide. The testis was examined for lesions including atrophy and tumors as well as treatment related effects including retained spermatids, missing germ cell layers or types, multinucleated giant cells, or sloughing of spermatogenic cells into the lumen. A longitudinal section of the intact epididymis, to include the caput, corpus, and cauda, was examined in order to identify such lesions as sperm granulomas, leukocytic infiltration (inflammation), aberrant cell types within the lumen, or the absence of clear cells in the cauda epididymal epithelium (Foley 2001; Creasy 2001).

5.13 Clinical Chemistry

Clinical chemistry parameters evaluated included the following: alkaline phosphatase (ALKP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), cholesterol (CHOL), creatinine (CREA), glucose (non-fasting) (GLU), total bilirubin (TBIL), total protein (TP), sodium (Na), potassium (K), and chloride (Cl). Results were determined using the Idexx VetTest® 8008 Chemistry Analyzer and the VetLyte® Electrolyte Analyzer on all serum samples. (VetTest® and VetLyte® are registered trademarks of IDEXX Laboratories, Inc.).

5.14 Hormone Assays

Total thyroxine and testosterone were determined using the TOSOH® Bioscience AIA®-360 Automated Enzyme Immunoassay System. (TOSOH® and AIA® are registered trademarks of Tosoh Corporation).

Analysis of TSH was conducted using a commercially available rat TSH Enzyme linked Immunosorbant Assay kit purchased from ALPCO™ Immunoassays. Assays were conducted according to the manufacturer's instructions as follows. Assay materials were equilibrated to room temperature prior to use in the assay. Twenty-five µl of standard (Lot 001), blank (Lot 002) or sample was added, in duplicate, to the appropriate wells of the 96-well plate (Lot 008) pre-coated with TSH monoclonal antibodies. Two hundred µl of enzyme-labeled anti-rat TSH-antibody (Lot 002) was then added to all wells, the plate covered with the adhesive strip, and the plate incubated for 18-20 hours at 4±2 °C. The plate contents were discarded and the plate was washed four times with 300 µl of diluted wash solution (Lot 027). Tetra-Methyl-Benzidine substrate solution (Lot 011) (200 µl) was added to each well and the plate incubated in the dark for 30±1 minutes at room temperature. Stop solution (Lot 014) (50 µl) was added to each well, the plate gently mixed to ensure completion of color change, and the plate read within 15 minutes. The optical density of each well was determined at 450 nanometers (nm) and 630 nm using a Biotek® Synergy HT Multi-Mode microplate reader with Gen5™ data analysis software. Mean absorbance for each sample was calculated after adjustment for the absorbance at 630 nm. The TSH values were calculated from the calibration curve for each assay using ReaderFit© software. The external quality control standard (Rat Control 2 Lot 001) was within 106% of the reference point. The intra-and inter-assay coefficients of variation were 8.2% and 9.3%, respectively. (ALPCO™ is a registered trademark of ALPCO Diagnostics, BioTek® and Gen5™ are registered trademarks of BioTek Instruments, Inc., and ReaderFit is copyrighted by Hitachi Solutions America, Ltd.)

5.15 Data Collection and Statistical Analyses

Experimental data generated during the course of this study were recorded by hand and tabulated, summarized, and/or statistically analyzed using Microsoft® Excel and SPSS® 16.0. Environmental data were automatically recorded using MetaSys® Building Management System. (Microsoft® is a registered trademark of Microsoft® Corporation, SPSS® is a registered trademark of IBM Corp., and MetaSys® is a registered trademark of Johnson Controls).

The overall estrous cyclicity pattern of each female was characterized as regularly cycling (having recurring 4- to 5-day cycles), irregularly cycling (having cycles with a period of diestrus longer than 3 days or a period of estrus longer than 2 days), or not cycling (having prolonged periods of either estrus or diestrus). The default assumption was that animals were cycling regularly if the partial data fit the definition, and irregularly cycling if not enough data were available to distinguish between irregularly cycling and not-cycling. Cycle length was calculated as number of days from one diestrus to the next diestrus. Mean cycle length for each animal was calculated first, and the mean of these means was then calculated to represent the group.

All data, except histology and estrous cyclicity evaluations, were analyzed using a two-way Analysis of Variance (ANOVA) with Block (necropsy day) and Experimental Group as main effects. Age and body weight at VO, and all organ weights were also analyzed by Analysis of Covariance (ANCOVA) using the body weight at PND 21 as the covariate. Uterus weight was analyzed by ANOVA, including estrus stage at necropsy as a blocking factor. Thyroid scores and ovarian follicular development data were analyzed using separate Multivariate Analyses of Variance (MANOVAs). When statistically significant effects were observed ($p \leq 0.05$), appropriate post hoc tests were used to compare dose groups to the control group [e.g., Tukey's multiple comparison test or Sidak (for ANCOVA)]. Data were evaluated for homogeneity of variance by Levene's test. There were no cases requiring the data to be transformed or analyzed using nonparametric tests. Organ weights were also analyzed for linear trend with dose level. Chi-square analysis was used to determine significant differences between the cycling status (cycling vs. not cycling) and percent of animals cycling regularly between treated and control groups.

6 Results

6.1 Analytical Chemistry

The analytical chemistry results are summarized in Table 2. All results were within the 70-130% recovery limits for the analysis. As such, all results were reported using the nominal concentrations. Stability and homogeneity analyses indicated that storage and mixing conditions were acceptable.

Table 2. Analytical Results

Nominal Concentration milligrams per milliliter (mg/ml)	Analytical Concentration (mg/ml)	% of Nominal	% of Initial	% CV
purity	100%			
stability week 1	6.3	105	100	
stability week 2	6.1	102	97	
stability week 3	6.0	100	95	
stability week 4	6.3	105	100	
stability week 5	6.3	105	100	
stability week 6	6.3	105	100	
200 top (homogeneity)	160	80		3.5
200 middle (homogeneity)	170	85		
200 bottom (homogeneity)	160	80		
200	160	80		
100	88	88		
50	43	86		

6.2 Clinical Observations and Mortality

Bright yellow urine was observed in the cages of all rats in the 250, 500, and 1000 mg/kg-d dose groups throughout the treatment period. Congested breathing was observed in one male rat in each of the 250 and 500 mg/kg-day and one female rat in the 1000 mg/kg-day groups. Dried red perinasal material was observed in one male rat treated with 500 mg/kg-day NTO. Chromodacryorrhea was observed in one female rat in each of the 500 and 1000 mg/kg-day NTO groups. Nine male and three female rats were observed with abrasions, scabs, and/or hair loss in the dorsal cervical or neck region. These observations were not considered treatment related as they occurred in all dose groups (males: three control, one low dose, four intermediate dose; females: one control, one intermediate dose, one high dose). All rats survived until scheduled euthanasia (see Appendix D).

6.3 Body Mass and Food Consumption

The initial mean body mass of the corn oil control rats was 59.9 and 53.7 g for males and females, respectively (coefficient of variation of 6.9% and 6.2%). In male rats treated with NTO, initial body mass was 60.5 and 61.0 g and increased to 318.8 and 317.2 g in the 250 and 500 mg/kg-day groups, respectively. There were no statistical differences in body mass between treated and control males at any time during the study. In female rats treated with NTO, initial body mass was 53.0 and 54.6 g and increased to 168.1 and 162.1 g in the 500 and 1000 mg/kg-day groups, respectively. Body mass of female rats was significantly reduced ($p=0.012$) on PND 33 in the 1000 mg/kg-day group. Final body mass and body mass gain were significantly reduced in the 1000 mg/kg-day NTO group relative to the control group (7% and 10%, respectively) when analyzed by

the ANCOVA (p=0.031 and p=0.022, respectively) using body mass on PND 21 as the covariate. There were no statistical differences in body mass for females treated with 500 mg/kg-day NTO (see Appendices E and F).

Food consumption did not differ significantly between treated and control groups at any time during the study for male or female rats (see Appendix G).

6.4 Sexual Maturation Evaluation – Female

6.4.1 Vaginal Opening (VO)

Age of VO for the female rats was 34.1, 35.1, and 35.1 days in the corn oil control, 500, and 100 mg/kg-day NTO groups, respectively. Body mass at time of VO for female rats was 125.0, 129.2, and 123.7 g in the corn oil control, 500, and 100 mg/kg-day NTO groups, respectively. There were no statistical differences in age and body mass at time of VO between the control group and the NTO treated groups. Age and body mass at time of VO were unaffected by treatment with NTO at 500 and 1000 mg/kg-day. The linear trend analysis did not demonstrate a linear relationship between dose and age of VO or body weight at time of VO (see Appendix H).

6.4.2 Estrous Cyclicity

The mean age at first vaginal estrus for the female rats was 36.1 days in the corn oil control group and 36.3 and 36.2 days in the 500 and 1000 mg/kg-day NTO groups, respectively. The mean cycle length for female rats was 4.4 days in the corn oil control group and 4.2 and 4.6 days in the 500 and 1000 mg/kg-day NTO groups, respectively. All (100%) of the females were cycling in the control and 1000 mg/kg-day NTO group; 93.3% of the females in the 500 mg/kg-day group were cycling. In the control and 1000 mg/kg-day NTO groups, 80% of the females were cycling regularly, while in the 500 mg/kg-day group 86.7% were cycling regularly. There were no statistical differences in age at first vaginal estrus, cycle length, percent of rats cycling, or percent cycling regularly between the control group and the NTO treated groups. Estrous cyclicity was unaffected by treatment with NTO at 500 and 1000 mg/kg-day. The linear trend analysis did not demonstrate a linear relationship between dose and age at first estrus or cycle length (see Appendix I).

6.5 Sexual Maturation Evaluation – Male

6.5.1 Preputial Separation (PPS)

Age at PPS for the male rats was 43.8, 43.9, and 44.3 days in the corn oil control, 250, and 500 mg/kg-day NTO groups, respectively. Body mass at time of PPS for the male rats was 230.4, 235.3, and 239.4 g in the corn oil control, 250, and 500 mg/kg-day NTO groups, respectively. There were no statistical differences in age and body mass at time of PPS between the control group and NTO treated groups. The linear trend analysis did not demonstrate a linear relationship between dose and age at PPS or body mass at time of preputial separation (see Appendix J).

6.6 Pathology

Both testes were noted to be small in six of 15 males in the 250 mg/kg-day group and 15 of 15 males in the 500 mg/kg-day NTO. The seminal vesicles were noted to be small in one rat in the control group, two rats in the 250 mg/kg-day group, and one rat in the 500 mg/kg-day group.

Hydronephrosis was observed in two of 15 males in the 250 mg/kg-day group and in one of 15 males and one of 15 females in the 500 mg/kg-day NTO group. Pale kidney(s) was observed in three control females, three females at 500 mg/kg-day, and two females at 1000 mg/kg-day NTO. Diffuse red mottling of the lungs was noted in two controls and one male rat treated with 500 mg/kg-day NTO. Pale liver was observed in six females (three control, one 500 mg/kg-day, two 1000 mg/kg-day). Dilated or fluid-filled uterus was noted in eight females (four controls, three 500 mg/kg-day, one 1000 mg/kg-day). Pale small intestines were observed in nine males (four control, three 250 mg/kg-day, and two 500 mg/kg-day NTO) and 33 females (10 controls, 15 at 500 mg/kg-day, and 8 at 1000 mg/kg-day). Bedding was found in the stomach of 21 males (nine controls, five 250 mg/kg-day, seven 500 mg/kg-day) and three females (1000 mg/kg-day). No additional gross lesions were noted at the time of necropsy.

6.7 Organ Mass

6.7.1 Females

The mass of the liver, kidneys (paired), pituitary, adrenals (paired), and thyroid (fixed) were unaffected by treatment with NTO at 500 and 1000 mg/kg-day. Although not statistically significant, unadjusted and adjusted ovarian mass were slightly reduced in the 500 (95% and 94%, respectively) and 1000 mg/kg-day (91% and 90%, respectively) groups compared to the controls. Uterine mass (wet and blotted) was reduced only in the 1000 mg/kg-day group (85% and 88%, respectively). The linear trend analysis demonstrated a linear decrease in liver mass with increasing dose. No other organs demonstrated linear trends with dose (see Appendix K).

6.7.2 Males

Testes mass (left and right) were reduced to 70% and 35% of the control mass in rats treated with 250 and 500 mg/kg-day NTO, respectively, when analyzed by ANOVA ($p<0.001$ and $p<0.001$; $p<0.001$ and $p<0.001$, respectively) and by ANCOVA ($p<0.001$ and $p<0.001$; $p<0.001$ and $p<0.001$, respectively) using the body weight on PND 21 as the covariate.

Mass of the right epididymis was reduced in rats in the 500 mg/kg-day NTO group to 75% of control when analyzed by ANOVA ($p\leq0.001$) and by ANCOVA ($p\leq0.001$) using the body weight on PND 21 as the covariate. Although the mass of the left epididymis was similarly reduced (76%) in the 500 mg/kg-day group, this difference was not statistically significant. Treatment with 250 mg/kg-day NTO did not affect the mass of the epididymides.

Although not statistically significant, the 250 and 500 mg/kg-day groups demonstrated reduced mass of seminal vesicles and coagulating gland (88% and 81%, respectively), dorsolateral prostate (92% and 76%, respectively), and LABC (95% and 89%, respectively) compared to the controls. Mass of the ventral prostrate was reduced (91%) only in the 500 mg/kg-day group. The mass of the liver, kidneys (paired), pituitary, adrenals (paired), and thyroid (fixed) were unaffected by treatment with NTO at 250 and 500 mg/kg-day. The linear trend analysis demonstrated linear decreases in seminal vesicle (with and without fluid), dorsolateral prostate, LABC, epididymides, and testes mass with increasing dose. Other organs did not demonstrate linear trends with dose (see Appendix K).

6.8 Histopathology

6.8.1 Thyroid

Ultimobranchial cysts were noted in six males (three 250 mg/kg-day and three 500mg/kg-day) and five females (one control, three 500mg/kg-day, and one 1000 mg/kg-day). No other significant findings were appreciated. In females, mean follicle height score was 1.9, 2.3, and 2.1 in control, 500, and 1000 mg/kg-day groups, respectively. In males, mean follicle height score was 2.4, 2.3, and 2.3 in control, 250 and 500 mg/kg-day groups, respectively. Colloid scores were 3.9, 3.5, and 3.6 and 3.5, 3.7, and 3.9 in females and males, respectively, in the control, low and high dose groups. Neither follicle nor colloid score differed between treated and control groups for males or females. A follicle score of one and colloid score of five is defined as normal in the scoring scheme (USEPA 2009, 2009) (see Appendix L).

6.8.2 Male reproductive

Degeneration and atrophy of testicular seminiferous tubules (mild to moderate) was present in 15 of 15 males examined in the 250 mg/kg-d group. In tubules with mild to moderate testicular degeneration/atrophy, sertoli cells, spermatogonia, preleptotene, leptotene, zygotene and early pachytene spermatocytes were present and intact. Late pachytene and diplotene spermatocytes were often degenerative. Round spermatids 1-6 were generally present and intact. Elongating spermatids 7-10 were degenerative or necrotic while spermatids 11-19 were absent.

Degeneration and atrophy of testicular seminiferous tubules (marked to severe) was present in 15 of 15 males examined in the 500 mg/kg-day group. In tubules with marked to severe degeneration/atrophy, only Sertoli cells and spermatogonia remained; all other germ cells were absent. Sertoli cells and spermatogonia were consistently present with most other first layer germ cells, preleptotene, leptotene, and zygotene spermatocytes. Second layer pachytene spermatocytes, stages I-VI, were present but disassociated within lumen and often degenerate or necrotic.

No test article related changes were noted in the testes of the control males.

Severe hypospermia/aspermia was present in the epididymis of 15 of 15 males examined in the 250 mg/kg-d group and 15 of 15 males examined in the 500 mg/kg-d group. Moderate hypospermia is defined as an absence of mature spermatids in the head and body of the epididymis with mature spermatids evident in the tail section. Mature spermatids were absent in all portions of the epididymis in severe hypospermia/aspermia animals. No test article related changes were noted in the epididymides of the control males.

Epididymal clear cell quantity was evaluated and considered consistent across controls and all treated groups. There was no absence of clear cells (see Appendix L).

6.8.3 Female reproductive

Histology of vagina, uterus, and ovary at necropsy generally correlated with the final cytological swab just prior to necropsy. No true uterine hypertrophy or hypotrophy were noted histologically and all instances of increased or decreased luminal diameter correlated with estrus stage. Myometrial, stromal, and endometrial gland development appeared normal. All organ reproductive stages at necropsy correlated with the vaginal cytologic stage at necropsy (see Appendix L).

6.8.4 Kidney

All renal findings, including basophilic tubules, pelvic dilatation (hydronephrosis), and lymphocytic interstitial lymphocytic infiltrates occurred at similar rates in control and treated animals and were considered background or incidental findings unrelated to treatment (see Appendix L).

6.9 Clinical Chemistry

Female rats in the 1000 mg/kg-day NTO dose group had elevated chloride levels compared to the control group (p=0.018). This difference was not considered biologically significant as the chloride levels were within normal and historical control ranges (Giknis and Clifford 2006). There were no statistical differences in ALKP, ALT, AST, BUN, CHOL, CREA, GLU (non-fasting), TBIL, TP, Na, and K between control and NTO treated groups for female rats and all parameters were within either normal or historical control ranges.

The AST levels were significantly (p=0.025) elevated in male rats in the 500 mg/kg-day dose group relative to the control group. The AST levels of both controls and NTO treated groups were above normal ranges (Giknis and Clifford 2006); however, the values were within the historical control range for the performing laboratory. The TBIL levels were significantly reduced (p=0.011) in male rats in the 500 mg/kg-day NTO dose group relative to the control group. The TBIL levels in male rats in both the 250 and 500 mg/kg-day NTO groups were below both normal and historical control ranges (Giknis and Clifford 2006). There were no statistical differences in ALKP, ALT, BUN, CHOL, CREA, GLU (non-fasting), TP, Na, K, and Cl between control and NTO treated male rats and all parameters were within either normal or historical control ranges (see Appendix M).

6.10 Hormone Analyses

Serum T4 levels were 3.31, 2.93, and 2.97 micrograms per deciliter (µg/dl), and serum TSH levels were 1.87, 1.77, and 1.56 nanograms per milliliter (ng/ml) in female rats in the corn oil control, 500, and 1000 mg/kg-day NTO groups, respectively. There were no statistical differences in T4 or TSH levels between the control group and NTO treatment groups for female rats.

Serum testosterone levels were 2.22, 2.54, and 1.95 ng/ml in male rats in the corn oil control, 250, and 500 mg/kg-day NTO groups, respectively. Serum T4 levels were 3.91, 4.02, and 4.15 µg/dl, and serum TSH levels were 2.97, 2.03, and 3.63 ng/ml in male rats in the corn oil control, 250, and 500 mg/kg-day NTO groups, respectively. There were no statistical differences in testosterone, T4, or TSH levels between the control group and NTO treatment groups for male rats (see Appendix N).

6.11 Standing Operating Procedure and Protocol Deviations

The following deviations occurred during the study but were not considered to have compromised the integrity or validity of the study results:

As per the protocol and SOP 004, animal room temperature was to be maintained between 64 and 79 ºF and humidity between 30 and 70%. However, on 3/6/2012 due to a boiler failure, the animal room temperature dropped to 61 ºF and was out of range for approximately two hours. On 4/5/2012, due to damage to the humidifier system, humidity in the animal room was 24%from approximately 0600 to 1100 hours.

As per SOP 90, when the Johnson Controls system alarms after duty hours, the staff duty officer (SDO) is to notify the Attending Veterinarian (AV) or Animal Health Technicians (AHT) on duty. On 3/6/12 the temperature went out of range and the Johnson Control system alarmed to the SDO; however, the SDO contacted the contracted maintenance personnel on call instead of the AV or the AHT on duty.

The estrus stage of the female rats at the time of necropsy was also determined based on histological assessment of the vagina, ovary, and uterus. This data was collected in addition to the vaginal lavage data for determining estrus stage and serves to confirm the estrus stage at necropsy. These data improved the quality of the study.

Thyroids from 21 females were incompletely dissected, resulting in low thyroid masses (outside of expected range) and notations of missing thyroid (complete and partial). Tissue was re-examined by a pathologist and thyroid tissue was located for all except one high dose female. Tissues were re-weighed and the second data point represents the mass of the complete thyroid. Thyroid tissues for males were similarly re-examined and re-weighed for consistency. Only a representative follicular cell height and colloid area score was determined for the two sections from each of the two lobes of the thyroid.

7 Discussion

NTO has been shown to be a testicular and epididymal toxicant in adult rats (Crouse et al. 2010); however, it was not determined if NTO was acting directly or via an endocrine mediated mode of action. The main objective of this study was to investigate the potential endocrine disrupting effects of NTO in male and female rats exposed from weaning through puberty. PPS and VO, markers of the onset of puberty, are more sensitive markers of estrogenic disruption than fertility or ovarian/testicular histopathology (Biegel et al. 1998). In males, estrogenic compounds delay preputial separation, typically by 3 to 5 days. In females, estrogenic compounds accelerate VO and disrupt estrous cycles, leading to prolonged estrus. Females are generally more sensitive than males to estrogenic disruption. Estrogens inhibit food consumption and retard growth. In the current study, pubertal administration of NTO via oral gavage did not alter the age or body mass at puberty for male or female rats and did not induce changes in any reproductive endpoints in females (estrus cycling, organ mass). Although the high dose females did exhibit reductions in body mass, food consumption was not affected. NTO also failed to demonstrate estrogenic activity in the uterotrophic assay in ovariectomized rats (Quinn et al. 2013). These results provide no evidence for estrogenic or anti-estrogenic effects of NTO or effects mediated via alterations in hypothalamic/pituitary function.

In contrast, administration of NTO via oral gavage reduced the mass of androgen-dependent organs in male rats. Testis mass was reduced at 250 and 500 mg/kg-day. The reduction in testis mass was associated with tubular degeneration/atrophy. Less pronounced reductions were also seen in the mass of the epididymis and the accessory sex organs (e.g., seminal vesicles and dorsolateral prostate) at 500 mg/kg-day NTO. The reductions observed in androgen-dependent organ masses suggest a reduction in testosterone levels; however, serum testosterone levels were highly variable and were not obviously affected by NTO treatment. The variability in the testosterone levels may be attributable to the pulsatility of its release (Steiner et al. 1984). As many of the reproductive tissues are dependent on locally produced dihydrotestosterone (DHT) (Blohm et al. 1986; Blystone et al. 2007; George, Johnson, and Wilson 1989), the effects may be due to impaired ability to convert the available testosterone to DHT. Disturbance of key enzymes involved in steroidogenesis, including 5α-reductase, aromatase, and CYP17, has been identified as a

common mode of action for azole containing compounds (Blystone et al. 2007; Chapin and Williams 1989; Goetz et al. 2007; Kjaerstad and Andersen 2007).

Azole fungicides are designed to inhibit CYP51 in yeast and fungi to block biosynthesis of ergosterol. In humans, CYP51 is important for synthesis of cholesterol, inhibition of which may broadly inhibit steroid synthesis. Azoles also demonstrate cross-reactivity to various hepatic metabolic CYP enzymes and steroidogenic CYP enzymes (e.g., CYP17, CYP11β and aromatase) and have been shown to inhibit specific steroidogenic pathways. *In vitro* studies have shown imidazole fungicides to be potent mixed-type inhibitors of aromatase, androgen receptor antagonists, and inhibitors of testicular CYP17 (only compounds with aromatic ring side chains) (Ayub and Levell 1987; Sanderson 2006; Sanderson et al. 2002). Triazoles have been shown to be less potent competitive inhibitors of aromatase, and weak androgen and estrogen receptor antagonists (Kjaerstad and Andersen 2007; Sanderson 2006; Sanderson et al. 2002). Unlike the azole fungicides, NTO has shown no activity in estrogen and androgen receptor binding assays, estrogen transactivation assays, and has not demonstrated inhibition of *in vitro* aromatase activity or testosterone steroidogenesis in BLTK1 or H295R cell lines (Adams 2012). NTO also failed to demonstrate anti-androgenic effects *in vivo* in the Hershberger assay using castrated rats (Quinn et al. 2013). Lack of effects of NTO on serum testosterone and *in vitro* testosterone steroidogenesis combined with reduced androgen-dependent tissue masses suggest either disruption of 5α-reductase function, or direct tissue toxicity, or both.

Compounds can act on testis either via direct chemical reactivity and germ-cell toxicity or indirect toxicity mediated via non-germ cell sites and altered hormonal control of the testis (Working 1989). Indirect testicular toxicants act on Leydig cells, impacting testosterone production and in turn androgen dependent tissues and PPS (Noriega et al. 2009). NTO did not impact serum testosterone, however, ex *vivo* testicular testosterone production, a more precise measure of the steroidogenic capacity of the testis as it is not impacted by the variability inherent in the intact hypothalamic-pituitary-gonadal axis, was not measured in this study. Although androgen-dependent tissue masses were reduced, PPS was not delayed. Preputial separation, however, is not a sensitive indicator of endocrine disruption by steroid biosynthesis inhibitors (Marty, Crissman, and Carney 2001).

Interruption of hormonal regulation of the testis also disrupts spermatogenesis, resulting in the presence of degenerating pachytene spermatocytes and step 19 spermatids, a morphological pattern characteristic of androgen deficiency (Chapin and Williams 1989; Russell, Malone, and Karpas 1981). In the 250 mg/kg-day dose group, the initial set of cells with degenerative changes was late pachytene spermatocytes. Progressive maturation resulted in loss of subsequent developmental stages and complete absence of elongating spermatids. In the 500 mg/kg-day group, there was necrosis or loss of all cells developing past first layer spermatogonia, preleptotene, leptotene, and zygotene spermatocytes. Identification of pachytene spermatocytes as the initial stage with injury suggests that the pattern of injury may be in line with that characteristic of androgen deficiency. However, a time-course study may be useful to determine stage specific effects or identify a characteristic pattern of injury (Creasy 1997).

Sertoli cells, responsible for the maintenance and support of germ cells, are the target of direct testicular toxicants (Noriega et al. 2009). Benzimidazole fungicides induce characteristic vacuolization of Sertoli cells and stage-specific apoptosis of spermatocytes (Okamura et al. 2004; Hess and Nakai 2000). Benzimidazoles bind to tubulin and inhibit the polymerization of microtubules (Lacey 1990), disrupting spermatocytic meiosis and spermatogonial mitosis, leading to apoptosis (Okamura et al. 2004). The benzimidazole carbendazim induces testicular toxicity through microtubule inhibition mediated sloughing of immature spermatids at low doses and

seminiferous tubule atrophy secondary to occlusion of efferent ductules at higher doses (Nakai et al. 1992). Testicular toxicity of carbendazim is ameliorated by the antioxidant and/or anti-inflammatory properties of vitamin E and linseed oil (Metwally et al. 2011). Although Sertoli cells were present and intact at both NTO dose levels, direct germ cell toxicity as seen with other azoles could be responsible for the observed testicular toxicity. Additional studies would be necessary to determine the mechanism of NTO testicular toxicity.

Azoles that have demonstrated weak *in vivo* effects on androgen-dependent tissues in young rats (i.e., prochloraz) similar to the effects of NTO in the present study have feminized male offspring (i.e., reduced testosterone, nipple retention) and virilized female offspring (i.e., increased ano-genital distance) exposed in utero (Kjaerstad and Andersen 2007; Vinggaard et al. 2005). The triazoles tebuconazole and epoxiconazole similarly virilized female offspring after exposure in utero; however, only tebuconazole feminized male offspring (Taxvig et al. 2007). Epoxiconazole was also fetotoxic at high doses and increased birth weights and gestational length at lower doses. Tebuconazole increased progesterone and decreased testosterone concentrations in the testis of male fetuses whereas epoxiconazole increased serum progesterone and testosterone levels in dams. NTO did not demonstrate effects on reproductive success or cause developmental effects in a reproductive screening study (Crouse and Lent 2013). Offspring were sacrificed at PND 4 in the screening study and endocrine sensitive endpoints such as nipple retention, ano-genital distance, hormone levels, and onset of puberty were not measured. Exposure of offspring to NTO during gestation through puberty and examination of endocrine sensitive endpoints may reveal additional effects of NTO reproductive and endocrine development and function.

Anti-thyroid compounds disrupt function by inhibiting iodide transport into the thyroid (e.g., perchlorate and thiocyanate), by inhibiting iodination of tyrosine, by increasing peripheral clearance (induction of hepatic enzymes), and by inhibiting thyroperoxidase (e.g., sulfamethazine, thiourea, methimazole and aminotriazole) (Boas et al. 2006). Anti-thyroid compounds typically induce decreased serum T3/T4 and increase TSH levels. Increased TSH levels result in shrinkage of thyroid follicle colloid area and increased cell height. If sustained, high TSH levels result in follicular cell hypertrophy/hyperplasia (Capen 1997; Capen and Martin 1989). Thyroid histopathology is the most reliable indicator of disruption of thyroid function by exogenous compounds due to the variability and pulsatility of hormone levels (DeVito et al. 1999). Although follicle cell height was slightly increased (females: 2.1; males: 2.33; normal: 1.0) and colloid decreased (females: 3.66; males: 3.7; normal: 5.0) compared to normal (USEPA 2009, 2009), the scores did not differ with NTO treatment. The increased follicular cell height might suggest sustained, elevated TSH levels in all rats. This, however, was not the case as TSH, as well as T4, levels were within normal ranges (Christian and Trenton 2003). Thyroid hormone levels were also unaffected by NTO treatment. Thus, there were no apparent thyroid-receptor mediated effects of NTO.

8 Conclusions

In the current study, pubertal administration of NTO via oral gavage did not alter the age or body mass at puberty for male or female rats and did not induce changes in any reproductive endpoints in females (estrus cycling, organ mass). In contrast, administration of NTO via oral gavage reduced the mass of testes and accessory organs in male rats. The reduction in testis mass was associated with histological changes. Serum testosterone levels were highly variable and were not obviously affected by NTO treatment. Thyroid hormone levels and histopathology did not differ between NTO treated and control groups. These results provide no evidence for estrogenic, anti-estrogenic, or anti-thyroid effects of NTO or effects mediated via alterations in hypothalamic/pituitary function. The effects observed in the male reproductive system suggest that NTO may exhibit endocrine

The effects observed in the male reproductive system suggest that NTO may exhibit endocrine disrupting activity, potentially acting via inhibition of steroidogenesis or may be due to direct testicular toxicity and subsequent impairment of steroidogenesis.

9 Point of Contact

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MARK S. JOHNSON
Director, Toxicology Portfolio

2/26/2014
Date

Appendix A

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Appendix B

Quality Assurance Statement

Appendix B

Quality Assurance Statement

For: Toxicology Study No. 85-XC-0FP3-12, Protocol No. 0FP3-95-12-02-01, Pubertal Development and Thyroid Function in Intact Juvenile Rats Exposed to NTO, February - June 2012", the following critical phases were audited by the Quality Systems Office:

PRE IN-LIFE PHASE OF THE STUDY

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
Study Protocol Good Laboratory Practice Standards and Animal Care Review	12/23/2011	12/23/2011

IN-LIFE PHASE OF THE STUDY

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
Pre-Study Procedures, Pup/litter Observations and Weighing	03/21/2012	03/30/2012
Pre-Study Special Husbandry Considerations	03/21/2012	03/30/2012
Test Substance Receipt, Control, and Weighing Procedures	04/04/2012	04/10/2012
Test System - Special Husbandry and Food and Water Requirements	04/05/2012	04/10/2012
Administration of Test Substance by Oral Gavage	04/05/2012	04/10/2012
Test System - Identification and Vaginal Opening Observations	04/05/2012	04/12/2012
Esctrous Cyclicity Sample Collection Procedures	04/19/2012	05/03/2012
Preputial Separation Examination Procedures	04/19/2012	05/03/2012
Necropsy, Organ & Tissue Collection Procedures	04/24/2012	05/04/2012
Blood Collection for Clinical Chemistry and Hormonal Measurement	04/24/2012	05/04/2012

For: Toxicology Study No. 85-XC-0FP3-12, Protocol No. 0FP3-95-12-02-01, Pubertal Development and Thyroid Function in Intact Juvenile Rats Exposed to NTO, February - June 2012", the following critical phases were audited by the Quality Systems Office:

POST IN-LIFE PHASE OF THE STUDY

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
Thyroid-Stimulating Hormone Assay Procedures	06/08/2012	06/15/2012
MRICD Collaborator Tissue Processing Support Procedures	06/12/2012	06/15/2012
Pathology Contributing Scientist Report Review	07/22/2013	07/22/2013
Final Study Report Review	09/16/2013	09/17/2013
Study Raw Data Review	09/16/2013	09/17/2013

Note 1 All findings were made known to the Study Director and the Program Manager at the time of the audit/inspection. If there were no findings during the inspection, the inspection was reported to Management and the Study Director on the date shown in the table.

Note 2 In addition to the study specific critical phase inspections listed here, general facility and process based inspection not specifically related to this study are done monthly or annually in accordance with QA Standard Operating Procedure.

Note 3 This report has been audited by the Quality Assurance Unit (QSO), and is considered to be an accurate account of the data generated and of the procedures followed

Michael P. Kefauver
Quality Assurance Specialist, QSO

02/12/2014
Date

Appendix C

Archives and Study Personnel

C-1 Archives

All raw data, documentation, records, protocol, and a copy of the final report generated as a result of this study will be archived in room 1026, building E-2100, USAPHC, for a minimum of five (5) years following submission of the final report to the Sponsor. If the report is used to support a regulatory action, it shall, along with all supporting data, be retained indefinitely.

Records on animal receipt, diet, and facility environmental parameters will be archived by the Veterinary Medical Division, Toxicology Portfolio, for a minimum of five (5) years following submission of the final report to the Sponsor.

Some ancillary records pertaining to this study, such as instrument maintenance logs, animal room observation logs, etc., will not be archived until those logbooks have been completed. Once complete they will be archived in room 1026, building E-2100, USAPHC.

Wet tissues, histology slides, and paraffin blocks are stored in building E-5158.

C-2 Personnel

Management: Chris E. Hanson, COL, VC, Portfolio Director, Toxicology (succeeded by Mark S. Johnson, July 2012); Dr. Glenn Leach, Ph.D., Manager, Toxicity Evaluation Program (TEP) (succeeded by Shannon M. Wallace, LTC, VC, March 2012; succeeded by Arthur J. O'Neill, January 2013); Dr. Mark S. Johnson, Ph.D., Manager, Health Effects Research Program (HERP) (succeeded by Michael J. Quinn, January 2013).

Study Director: Dr. Emily May Lent, Ph.D., Toxicologist, Toxicity Evaluation Program (TEP)

Quality Assurance: Michael P. Kefauver, Quality Assurance Specialist, Quality Systems Office.

Veterinary Support and Animal Care: Dawn C. Fitzhugh, DVM, MAJ, VC; Robert Sunderland, Animal Health Technician; Rebecca Kilby, Animal Health Technician; Jason Williams, Animal Health Technician.

Pathology Lab Coordinator: Patricia A. Beall, Biologist, TEP

Histopathology: Shannon M. Wallace, DVM, DACVP, LTC, VC, Pathologist, VMD

In-Life Support: Lee C.B. Crouse, Biologist, TEP; Theresa L. Hanna, Biological Technician, TEP.

Clinical Chemistry: Matthew A. Bazar, Biologist, TEP; Mark R. Way, Biologist, TEP.

Archivist: Martha L. Thompson, Data Acquisition Specialist, TEP

Appendix D

Clinical Observations

APPENDIX D-1
CLINICAL OBSERVATIONS

Chemical Substance: NTO
Species: Crl:CD (SD)
Concentration: 50 mg/ml[A], 100 mg/ml[B], 200 mg/ml[C]
Route: Oral
Sex: Female
Diluent: corn oil

INDIVIDUAL ANIMAL EFFECTS

Animal No.	Dose Group	Clinical Sign	Day of First Appearance	Day of Last Appearance
12-0182	Corn Oil Control			
12-0192	Corn Oil Control			
12-0202	Corn Oil Control			
12-0219	Corn Oil Control			
12-0238	Corn Oil Control			
12-0248	Corn Oil Control			
12-0268	Corn Oil Control			
12-0280	Corn Oil Control			
12-0288	Corn Oil Control			
12-0319	Corn Oil Control			
12-0329	Corn Oil Control	hair loss dorsal cervical neck area	41	42
12-0337	Corn Oil Control			
12-0350	Corn Oil Control			
12-0357	Corn Oil Control			
12-0368	Corn Oil Control			
12-0181	500 mg/kg-day[B]			
12-0190	500 mg/kg-day[B]			
12-0197	500 mg/kg-day[B]			
12-0217	500 mg/kg-day[B]			
12-0241	500 mg/kg-day[B]			
12-0250	500 mg/kg-day[B]			
12-0271	500 mg/kg-day[B]			
12-0277	500 mg/kg-day[B]			
12-0290	500 mg/kg-day[B]	hair loss dorsal cervical neck area	41	42
12-0290	500 mg/kg-day[B]	chromodacryorrhea L eye	42	
12-0318	500 mg/kg-day[B]			
12-0326	500 mg/kg-day[B]			
12-0338	500 mg/kg-day[B]			
12-0349	500 mg/kg-day[B]			
12-0358	500 mg/kg-day[B]			
12-0370	500 mg/kg-day[B]			
12-0180	1000 mg/kg-day[C]			
12-0191	1000 mg/kg-day[C]			
12-0199	1000 mg/kg-day[C]			
12-0218	1000 mg/kg-day[C]			
12-0239	1000 mg/kg-day[C]			
12-0251	1000 mg/kg-day[C]			
12-0270	1000 mg/kg-day[C]			
12-0281	1000 mg/kg-day[C]			
12-0287	1000 mg/kg-day[C]	chromodacryorrhea L eye	41	43
12-0316	1000 mg/kg-day[C]			
12-0328	1000 mg/kg-day[C]	congested breathing	22	
12-0335	1000 mg/kg-day[C]			
12-0347	1000 mg/kg-day[C]	hair loss dorsal cervical neck area	39	
12-0347	1000 mg/kg-day[C]	hair loss and abrasions dorsal cervical neck area	40	42
12-0347	1000 mg/kg-day[C]	scab dorsal cervical neck area	43	43
12-0359	1000 mg/kg-day[C]			
12-0366	1000 mg/kg-day[C]			

APPENDIX D-2
CLINICAL OBSERVATIONS

Chemical Substance: NTO
Species: Crl:CD (SD)
Concentration: 50 mg/ml[A], 100 mg/ml[B], 200 mg/ml[C]
Route: Oral
Sex: Male
Diluent: corn oil

INDIVIDUAL ANIMAL EFFECTS

Animal No.	Dose Group	Clinical Sign	Day of First Appearance	Day of Last Appearance
12-0178	Corn Oil Control			
12-0184	Corn Oil Control	hair loss dorsal cervical neck area	41	49
12-0193	Corn Oil Control			
12-0213	Corn Oil Control			
12-0233	Corn Oil Control	scab dorsal cervical neck area	41	47
12-0233	Corn Oil Control	hair loss dorsal cervical neck area	48	53
12-0247	Corn Oil Control			
12-0266	Corn Oil Control			
12-0273	Corn Oil Control	hair loss dorsal cervical neck area	46	48
12-0285	Corn Oil Control			
12-0315	Corn Oil Control			
12-0320	Corn Oil Control			
12-0330	Corn Oil Control			
12-0342	Corn Oil Control			
12-0352	Corn Oil Control			
12-0362	Corn Oil Control			
12-0173	250 mg/kg-day[A]			
12-0188	250 mg/kg-day[A]			
12-0195	250 mg/kg-day[A]			
12-0214	250 mg/kg-day[A]			
12-0232	250 mg/kg-day[A]			
12-0246	250 mg/kg-day[A]			
12-0265	250 mg/kg-day[A]	congested breathing	29	29
12-0276	250 mg/kg-day[A]			
12-0286	250 mg/kg-day[A]			
12-0312	250 mg/kg-day[A]			
12-0325	250 mg/kg-day[A]			
12-0334	250 mg/kg-day[A]	scab dorsal cervical neck area	46	46
12-0344	250 mg/kg-day[A]			
12-0353	250 mg/kg-day[A]			
12-0361	250 mg/kg-day[A]			
12-0175	500 mg/kg-day[B]			
12-0183	500 mg/kg-day[B]	hair loss dorsal cervical neck area	43	46
12-0196	500 mg/kg-day[B]	dried red material around nose	31	31
12-0196	500 mg/kg-day[B]	congested breathing	49, 51	49, 51
12-0216	500 mg/kg-day[B]	hair loss and abrasions dorsal cervical neck area	40	46
12-0216	500 mg/kg-day[B]	hair loss and scab dorsal cervical neck area	47	49
12-0237	500 mg/kg-day[B]			
12-0242	500 mg/kg-day[B]	scab dorsal cervical neck area	44	47
12-0242	500 mg/kg-day[B]	hair loss dorsal cervical neck area	48	49
12-0264	500 mg/kg-day[B]			
12-0274	500 mg/kg-day[B]	scab dorsal cervical neck area	44	49
12-0274	500 mg/kg-day[B]	hair loss dorsal cervical neck area	50	52
12-0282	500 mg/kg-day[B]			
12-0311	500 mg/kg-day[B]			
12-0321	500 mg/kg-day[B]	scab dorsal cervical neck area	44	46
12-0321	500 mg/kg-day[B]	hair loss dorsal cervical neck area	47	47
12-0331	500 mg/kg-day[B]			
12-0343	500 mg/kg-day[B]			
12-0356	500 mg/kg-day[B]			
12-0364	500 mg/kg-day[B]			

Appendix E

Individual and Summary of Body Mass Data

Table E-1
Protocol No. 0FP3-95-12-02-01
Pubertal Deveopment and Thyroid Function in Intact Juvenile Rats Exposed to NTO

Individual Body Mass (grams)
Female Rats

		Day																					
Group	Animal ID	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	final (%control)
Corn Oil Control	12-0182	53.1	56.7	62.1	66.9	71.4	77.1	82.1	85.2	92.5	97.0	103.9	109.7	116.6	120.9	128.5	133.0	139.4	144.3	150.3	155.8	161.5		
	12-0192	58.4	60.5	66.6	73.1	78.0	84.2	90.4	96.3	103.2	109.4	119.3	127.2	133.8	143.3	152.7	153.4	162.8	170.8	175.7	179.3	185.8		
	12-0202	54.2	58.3	64.8	69.7	73.7	78.9	83.0	87.2	94.6	100.6	109.6	114.4	118.7	114.0	127.6	134.9	141.4	144.7	150.8	154.7	161.6		
	12-0219	55.4	58.2	64.4	69.6	73.1	78.7	84.6	89.8	95.6	101.1	107.1	113.6	120.1	116.2	129.4	134.8	142.2	148.2	153.8	156.9	162.5		
	12-0238	54.2	59.6	64.5	70.5	77.1	83.8	90.3	97.4	105.3	111.8	119.6	126.6	135.5	142.8	148.9	155.3	157.4	167.8	173.1	181.0	182.8		
	12-0248	49.2	53.7	58.2	64.5	68.2	73.5	78.1	83.2	90.3	95.2	100.3	106.7	113.6	120.5	121.6	128.0	127.7	134.7	139.5	145.2	145.6		
	12-0268	56.7	61.6	68.0	73.4	78.7	84.4	89.4	92.4	97.9	105.4	112.9	118.5	124.7	131.6	139.6	144.9	153.2	159.2	165.6	169.4	177.1		
	12-0280	55.1	60.3	65.6	72.3	77.6	83.1	88.4	92.8	101.2	107.6	112.1	121.2	127.1	136.2	142.7	147.5	153.6	161.6	167.1	166.3	175.6		
	12-0288	50.3	52.5	58.3	64.7	68.4	75.2	81.9	89.3	95.1	102.4	110.9	117.6	125.4	133.3	139.6	149.0	156.2	160.6	164.8	171.7	174.9	181.7	
	12-0319	57.0	60.6	65.8	71.0	77.2	83.3	88.3	94.2	101.4	108.7	116.6	123.4	132.7	142.0	147.1	156.8	163.6	171.9	173.1	173.4	182.0	184.1	
	12-0329	54.7	60.6	66.7	70.7	76.1	82.7	88.8	94.6	101.7	106.4	101.6	122.0	130.9	140.2	145.7	157.3	167.1	172.6	174.3	183.6	187.3	195.6	
	12-0337	45.7	49.6	55.5	60.4	64.9	70.3	76.2	81.6	87.8	94.0	88.9	104.3	112.5	117.4	125.1	132.7	135.1	134.8	143.0	148.4	153.6	157.1	
	12-0350	54.4	59.2	65.2	71.6	78.5	85.0	92.3	98.8	106.9	114.2	123.8	131.8	137.2	146.9	155.8	159.0	167.8	173.3	180.8	183.0	190.8	199.1	
	12-0357	56.0	59.7	63.9	70.2	75.8	82.2	87.4	94.3	102.2	107.8	116.5	123.3	129.3	137.4	146.0	151.1	153.7	163.3	170.0	174.7	181.2	181.5	
	12-0368	51.4	55.3	59.3	64.6	70.1	76.2	81.6	88.8	94.2	100.1	108.0	111.4	119.9	127.2	130.6	133.6	133.9	141.2	146.2	149.1	147.7	154.4	
	Mean	**53.7**	**57.8**	**63.3**	**68.9**	**73.9**	**79.9**	**85.5**	**91.1**	**98.0**	**104.1**	**110.1**	**118.1**	**125.2**	**131.3**	**138.7**	**144.8**	**150.3**	**156.6**	**161.9**	**166.2**	**171.3**	**179.1**	
	SD	**3.3**	**3.5**	**3.7**	**3.8**	**4.4**	**4.6**	**4.8**	**5.2**	**5.6**	**6.1**	**9.0**	**8.0**	**8.0**	**11.2**	**10.8**	**10.8**	**12.9**	**14.0**	**13.4**	**13.4**	**14.8**	**17.3**	
500 mg/kg-day	12-0181	60.8	65.1	71.9	77.1	82.3	88.5	94.6	100.6	108.5	116.4	126.4	133.1	142.6	149.8	156.1	163.8	165.7	175.3	182.2	190.3	188.1		109.8
	12-0190	60.5	65.4	72.3	78.2	84.3	91.0	97.4	103.5	110.9	118.3	126.0	135.7	142.8	149.3	155.0	160.1	168.1	173.1	179.8	184.7	190.1		111.0
	12-0197	52.1	54.6	62.1	69.0	74.5	80.8	85.8	91.9	98.3	106.5	115.0	123.0	130.0	139.3	146.6	153.2	159.9	162.8	170.4	181.4	184.9		107.9
	12-0217	54.4	55.9	59.8	63.5	67.5	73.4	77.6	83.5	89.3	94.8	101.8	107.4	114.7	122.3	128.4	133.6	141.7	147.1	153.9	158.5	164.5		96.0
	12-0241	52.7	56.7	62.1	67.3	73.8	79.7	85.8	91.4	98.7	107.0	112.9	121.5	127.9	132.5	139.4	147.1	154.1	158.8	159.9	166.7	169.9		99.2
	12-0250	48.0	52.6	58.7	64.1	68.6	73.4	79.8	85.1	90.6	95.1	100.3	107.7	112.6	116.2	124.4	128.9	133.8	133.3	142.0	146.4	149.4		87.2
	12-0271	55.6	59.5	65.5	70.3	76.0	81.1	86.8	92.2	97.4	103.8	112.4	119.7	127.0	134.6	137.6	146.4	153.4	158.7	164.5	166.8	175.2	181.3	102.3
	12-0277	53.4	57.6	63.4	68.0	73.6	79.8	84.3	91.6	97.4	103.8	110.4	118.4	125.6	130.6	138.0	144.1	148.5	154.3	156.4	161.2	167.6	172.4	97.8
	12-0290	45.8	51.5	56.7	62.2	67.1	72.4	78.4	84.9	91.5	99.7	104.8	113.6	122.2	127.5	135.4	143.9	149.6	154.9	159.1	164.7	170.7	177.5	99.6
	12-0318	50.3	58.3	63.9	69.8	74.7	80.3	86.5	92.2	97.1	104.5	107.1	115.3	120.4	126.2	132.2	138.6	144.7	149.4	155.4	157.1	161.4	167.0	94.2
	12-0326	52.2	56.1	62.0	67.4	71.9	77.6	83.9	89.4	96.5	101.3	108.9	115.5	126.0	130.3	142.2	148.5	155.0	154.3	169.0	173.8	175.6	179.8	102.5
	12-0338	48.2	49.5	55.4	60.3	65.6	69.1	74.8	79.3	83.1	88.5	95.1	99.3	104.3	110.5	114.6	118.3	124.4	128.2	131.8	132.6	137.6	142.6	80.3
	12-0349	53.5	56.5	60.4	64.6	68.9	73.9	78.1	83.7	90.8	96.0	103.0	110.5	117.1	115.0	127.1	132.1	130.6	132.3	142.4	146.6	151.0	150.5	88.1
	12-0358	58.1	62.9	67.1	71.3	74.9	80.6	86.8	93.9	100.0	106.8	113.9	122.0	130.3	137.1	142.3	148.1	151.7	158.2	162.5	166.7	172.7	181.8	100.8
	12-0370	49.6	52.9	57.7	62.8	68.0	72.4	77.8	83.8	89.8	96.5	104.3	111.1	119.8	125.2	128.9	136.8	136.9	146.7	152.0	155.4	162.6	171.4	94.9
	Mean	**53.0**	**57.0**	**62.6**	**67.7**	**72.8**	**78.3**	**83.9**	**89.8**	**96.0**	**102.6**	**109.5**	**116.9**	**124.2**	**129.8**	**136.5**	**142.9**	**147.9**	**152.5**	**158.8**	**163.5**	**168.1**	**169.4**	**98.1**
	SD	**4.4**	**4.7**	**5.0**	**5.2**	**5.4**	**6.1**	**6.4**	**6.6**	**7.3**	**8.0**	**8.8**	**9.6**	**10.3**	**11.4**	**11.2**	**11.9**	**12.6**	**13.7**	**13.7**	**15.3**	**14.6**	**14.0**	**8.5**
1000 mg/kg-day	12-0180	62.6	66.9	74.2	79.0	84.5	90.9	97.6	102.2	109.2	115.4	122.5	123.7	136.8	143.1	150.6	156.4	162.2	166.9	174.6	176.5	184.3		107.6
	12-0191	59.9	63.5	69.6	72.2	77.6	83.5	90.0	95.6	102.4	110.4	114.8	113.8	127.6	135.3	141.2	145.6	143.8	154.6	162.2	172.3	163.8		95.6
	12-0199	57.0	60.1	67.6	72.8	78.8	84.9	91.9	99.1	106.7	113.6	121.8	126.8	140.1	149.2	153.4	161.5	168.0	171.4	177.1	183.4	189.2		110.4
	12-0218	54.9	57.6	62.2	66.5	72.5	77.5	83.3	89.9	93.0	98.5	104.8	104.1	116.0	122.9	126.7	135.2	141.3	143.8	151.1	153.7	157.9		92.2
	12-0239	53.9	55.9	61.3	66.2	70.9	77.1	82.8	89.8	96.9	103.3	109.4	112.3	123.0	131.7	137.9	146.1	154.1	158.3	160.5	165.9	173.2		101.1
	12-0251	50.2	53.6	58.3	63.6	67.7	73.8	78.0	83.8	92.0	96.5	101.0	103.6	113.4	120.2	127.7	132.6	131.9	143.1	146.4	151.2	153.4		89.5
	12-0270	54.9	56.5	62.7	66.5	70.4	75.6	80.1	84.9	90.2	94.4	101.4	104.4	115.4	121.8	127.9	135.2	141.7	147.2	154.3	158.6	163.8		95.6
	12-0281	53.9	56.5	62.3	66.8	70.9	78.3	82.9	87.6	91.2	98.8	105.5	105.3	117.7	123.4	128.8	136.2	138.5	145.9	150.3	154.7	162.2		94.7
	12-0287	52.2	54.8	61.6	65.6	69.1	73.9	78.8	84.3	87.2	92.8	98.5	99.5	111.6	116.6	123.4	127.6	132.1	137.9	143.0	144.8	149.1	156.9	87.0
	12-0316	53.6	56.4	62.8	66.2	70.1	76.3	80.6	86.0	92.3	98.2	104.6	105.7	119.3	125.7	132.9	137.9	141.0	146.5	153.7	155.4	159.7	158.5	93.2
	12-0328	53.3	51.1	59.4	65.2	68.9	74.5	78.9	82.3	89.6	94.5	101.2	103.9	115.7	121.6	128.9	135.9	143.1	148.3	153.6	161.3	166.2	166.7	97.0
	12-0335	43.4	45.4	51.9	57.2	59.4	62.9	66.8	70.3	73.7	79.8	83.9	83.9	92.7	97.3	102.3	105.8	111.2	113.6	117.9	121.3	123.8	127.5	72.3
	12-0347	57.6	61.4	65.7	72.4	77.2	82.8	88.2	93.8	100.1	106.0	114.1	113.0	125.8	130.0	135.7	139.3	136.0	141.4	148.7	152.6	154.8	151.4	90.4
	12-0359	57.9	61.1	64.1	69.5	74.3	78.4	83.7	89.0	93.5	100.4	107.1	108.8	120.5	129.1	132.9	137.8	142.9	150.7	154.1	162.4	164.1	172.8	95.8
	12-0366	53.4	58.3	62.6	66.4	69.9	76.7	81.4	87.2	92.4	101.1	105.1	109.9	121.9	128.2	132.4	139.9	145.4	151.2	156.7	159.5	166.4	172.2	97.1
	Mean	**54.6**	**57.3**	**63.1**	**67.7**	**72.1**	**77.8**	**83.0**	**88.4**	**94.0**	**100.2**	**106.4**	**107.9**	**119.8**	**126.4**	**132.2**	**138.2**	**142.2**	**148.1**	**153.6**	**158.2**	**162.1**	**158.0**	**94.6**
	SD	**4.4**	**5.2**	**5.1**	**5.0**	**5.8**	**6.3**	**7.1**	**7.6**	**8.5**	**8.9**	**9.6**	**10.0**	**11.1**	**11.9**	**11.9**	**12.6**	**13.2**	**13.2**	**13.6**	**14.4**	**15.1**	**15.7**	**8.8**

Table E-2
Protocol No. 0FP3-95-12-02-01
Pubertal Deveopment and Thyroid Function in Intact Juvenile Rats Exposed to NTO

Individual Body Mass (grams)
Male Rats

Group	Animal ID	Post Natal Day 23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	final (%control)
Corn Oil Control	12-0178	64.1	68.2	74.0	79.0	84.5	91.4	97.4	104.9	111.6	119.4	126.5	134.9	142.8	150.9	161.0	169.2	176.6	185.1	195.4	203.0	208.3	216.9	225.8	238.7	246.2	257.0	268.6	271.7	279.8	288.6	297.8		
	12-0184	63.6	69.2	74.9	81.1	87.4	94.9	100.6	108.9	115.8	124.2	132.8	140.7	149.8	158.8	168.0	177.9	187.7	197.2	209.1	217.5	225.5	235.3	245.6	253.6	264.4	273.1	284.8	293.5	299.0	308.7	319.9		
	12-0193	54.8	61.4	65.5	71.3	77.4	83.5	90.3	97.4	105.3	114.5	120.4	132.6	140.6	148.3	156.2	166.0	174.9	185.1	196.8	204.8	215.7	228.5	234.7	240.9	252.4	260.8	270.6	282.0	293.5	296.2	306.3		
	12-0213	57.0	62.2	66.7	72.1	76.1	81.7	89.2	96.1	102.7	109.4	119.3	127.9	137.2	146.3	155.5	165.1	174.0	181.5	192.5	201.1	210.0	219.6	227.4	236.6	243.2	253.7	263.0	266.8	275.6	282.5	289.9		
	12-0233	60.5	63.7	70.2	76.1	83.8	93.5	99.7	107.5	115.3	123.1	132.2	142.8	152.6	160.5	170.1	178.8	190.2	199.4	211.9	220.0	231.9	242.4	250.9	260.1	271.5	279.4	289.9	300.3	311.0	319.6	331.3		
	12-0247	59.1	64.3	69.7	76.1	84.1	90.4	96.8	105.7	111.6	119.4	127.8	136.7	146.2	153.5	162.7	171.1	178.3	187.1	196.8	204.2	212.7	222.0	228.0	238.8	247.5	258.5	265.4	272.3	279.9	284.8	297.3		
	12-0266	63.1	67.1	73.1	78.4	84.8	89.8	94.9	101.4	109.1	104.1	122.5	130.5	140.9	147.5	156.4	163.1	173.7	182.6	192.4	200.4	209.4	219.0	226.7	235.2	245.0	253.2	262.5	272.4	283.4	291.6	306.1	314.5	
	12-0273	63.1	70.3	76.7	83.3	90.6	97.5	105.0	112.9	124.1	117.5	140.1	148.4	160.8	168.2	178.0	188.9	198.3	208.1	219.4	226.7	233.8	243.7	253.2	261.3	270.9	283.4	293.5	298.4	309.3	318.2	328.8	336.7	
	12-0285	58.7	64.4	71.7	78.9	86.1	93.5	100.6	109.4	116.6	127.1	135.7	146.9	157.9	168.5	178.8	187.6	197.1	210.5	219.6	229.3	237.2	246.9	254.9	269.6	277.1	285.7	296.9	305.7	318.9	323.4	338.5	346.1	
	12-0315	59.7	64.9	73.9	79.0	87.6	94.6	101.8	109.5	118.2	126.8	137.1	145.6	156.3	164.7	176.7	184.3	191.3	203.4	213.4	220.9	227.1	241.8	248.0	260.7	275.2	285.7	294.6	304.2	318.2	328.8	337.6	346.6	
	12-0320	65.4	71.0	74.6	82.1	89.9	96.7	104.1	113.6	124.3	134.1	144.2	157.1	168.3	181.0	188.8	201.8	214.9	228.5	235.7	250.8	260.7	270.3	279.2	291.5	301.5	316.1	323.8	333.7	343.5	354.6	378.1	376.6	
	12-0330	51.3	57.1	61.1	66.9	72.7	79.0	86.1	89.7	96.2	104.0	110.1	118.4	127.8	134.9	142.6	153.4	160.5	168.6	177.5	185.2	194.1	204.9	210.1	217.1	225.2	233.8	245.1	250.4	259.0	265.3	276.4	279.9	
	12-0342	59.2	64.3	70.1	76.5	83.2	89.7	99.9	103.9	112.4	120.5	127.9	138.5	147.1	154.0	166.1	172.8	181.5	189.9	201.3	207.3	221.4	225.0	232.2	246.7	253.1	264.0	274.4	282.6	290.5	299.2	308.8	321.0	
	12-0352	64.4	69.3	75.4	82.1	88.3	95.6	104.4	110.8	117.1	124.8	135.5	146.6	153.8	162.0	176.2	185.9	196.8	204.3	215.9	223.5	235.5	241.7	251.6	264.5	274.4	287.0	297.2	307.6	320.0	329.2	342.2	358.3	
	12-0362	55.1	59.8	66.4	71.5	78.8	85.5	93.6	99.9	108.6	117.5	128.8	137.4	148.4	156.8	169.0	179.1	187.1	196.7	206.4	214.6	225.4	227.9	242.2	255.5	263.2	275.2	280.9	293.3	301.5	306.5	310.6	324.8	
	Mean	**59.9**	**65.1**	**70.9**	**77.0**	**83.7**	**90.5**	**97.6**	**104.8**	**112.6**	**119.1**	**129.4**	**139.0**	**148.7**	**157.1**	**167.1**	**176.3**	**185.5**	**195.2**	**205.6**	**214.0**	**223.2**	**232.4**	**240.7**	**251.4**	**260.7**	**271.1**	**280.7**	**289.0**	**298.9**	**306.5**	**318.0**	**333.8**	
	SD	**4.1**	**4.0**	**4.4**	**4.7**	**5.3**	**5.7**	**5.8**	**6.8**	**7.6**	**8.4**	**8.9**	**9.6**	**10.2**	**11.2**	**11.8**	**12.3**	**13.3**	**14.7**	**14.5**	**15.7**	**16.0**	**16.0**	**16.8**	**17.9**	**18.7**	**19.8**	**19.4**	**20.8**	**21.8**	**22.9**	**25.5**	**28.0**	
250 mg/kg-day	12-0173	58.5	64.0	68.3	76.3	77.3	82.6	87.9	94.6	101.9	106.6	114.0	121.7	128.5	137.2	145.1	153.2	162.0	169.2	179.1	187.9	195.3	202.8	209.8	220.2	227.5	237.1	244.9	251.9	259.4	267.2	267.0		84.0
	12-0188	64.1	69.6	75.7	81.2	87.1	92.3	99.9	106.7	116.1	123.1	134.1	141.5	153.8	163.1	174.6	186.1	197.6	206.0	221.5	230.9	240.4	247.1	252.9	263.9	274.9	285.2	293.2	306.1	319.0	326.4	338.8		106.5
	12-0195	61.8	68.1	74.9	79.8	84.4	89.1	99.0	106.3	114.9	122.2	133.4	139.7	135.1	156.2	167.3	179.4	190.7	200.3	210.2	213.5	220.2	229.9	241.3	247.1	256.8	265.5	274.8	284.5	293.1	299.4	309.2		97.2
	12-0214	66.0	70.5	75.6	81.2	88.9	95.8	102.3	112.8	120.5	129.7	139.7	149.5	144.3	164.1	178.6	191.2	213.3	213.2	223.8	232.8	243.6	251.8	263.8	272.9	283.0	287.2	302.6	311.4	320.1	331.3	341.1		107.3
	12-0232	57.6	63.6	69.3	75.8	84.1	90.5	97.9	105.4	113.6	123.3	133.6	141.4	153.1	162.2	172.9	181.1	193.1	205.1	216.4	226.5	236.2	245.6	253.2	261.9	272.9	280.2	290.9	300.3	307.5	312.8	329.1		103.5
	12-0246	62.4	68.4	72.6	79.3	86.8	94.3	101.5	109.6	116.4	125.5	133.8	143.5	152.3	160.1	171.3	179.6	189.1	197.3	206.0	209.0	215.4	223.5	229.8	239.9	248.8	259.6	268.1	275.7	285.5	289.6	302.5		95.1
	12-0265	62.1	68.2	72.4	79.2	84.9	92.3	99.3	104.4	113.2	120.5	128.9	137.8	149.8	157.1	167.0	177.1	186.3	196.3	207.4	214.9	227.1	233.7	243.5	251.9	264.9	274.3	284.8	294.4	305.3	310.2	320.8		100.9
	12-0276	57.1	64.2	69.6	76.6	81.6	91.5	98.1	106.8	113.4	122.2	132.6	139.8	152.8	160.7	170.4	182.3	192.3	203.0	214.8	224.3	235.5	247.0	254.7	263.3	276.0	283.9	292.9	299.5	308.9	317.2	331.8		104.3
	12-0286	60.5	66.3	72.4	77.7	86.1	91.5	99.5	107.7	114.7	125.6	133.5	143.4	155.5	164.8	175.5	185.3	195.6	205.1	214.0	221.7	234.5	242.0	251.3	261.3	271.6	280.6	290.8	299.9	308.3	316.8	325.5	333.5	102.4
	12-0312	62.8	68.0	74.7	81.5	87.8	95.4	102.0	108.9	118.4	129.4	136.8	146.3	155.9	164.8	174.9	188.0	195.0	206.6	217.9	228.6	236.2	247.8	253.8	267.3	277.4	287.7	299.5	305.6	317.3	328.3	338.7	350.3	106.5
	12-0325	62.1	68.9	75.4	80.5	88.1	94.8	100.8	108.1	114.7	124.1	133.3	137.1	149.9	157.5	166.5	179.7	190.4	199.6	212.2	217.6	227.3	235.8	245.5	252.0	261.4	273.3	282.3	285.9	299.5	305.5	313.9	319.1	98.7
	12-0334	48.0	55.1	61.3	66.1	72.9	79.0	86.6	91.4	97.6	106.6	112.0	118.6	128.4	134.5	142.9	150.7	158.1	166.1	177.4	183.9	189.2	198.5	207.5	214.2	223.3	228.4	235.2	242.7	250.1	253.8	261.3	272.1	82.2
	12-0344	63.3	70.4	77.1	83.8	91.2	97.6	105.8	113.8	124.1	131.4	143.2	154.3	164.4	172.7	185.2	193.9	207.1	215.2	224.6	233.3	243.4	248.6	259.3	267.6	279.3	289.9	299.4	308.9	321.1	326.4	336.6	346.3	105.9
	12-0353	63.8	70.1	75.2	80.6	86.5	93.7	101.1	109.5	118.1	125.3	135.5	143.3	154.8	164.9	174.6	184.0	192.3	204.1	211.4	223.0	234.0	242.8	251.6	260.2	269.6	279.9	288.1	297.9	309.3	312.7	323.1	331.8	101.6
	12-0361	57.6	63.5	69.7	76.1	82.8	91.8	98.2	105.8	116.5	121.4	133.2	142.7	154.4	164.5	175.3	185.9	192.9	204.4	213.3	225.3	235.4	243.6	254.4	263.9	276.0	288.3	298.8	309.3	316.2	330.0	342.5	355.0	107.7
	Mean	**60.5**	**66.6**	**72.3**	**78.4**	**84.7**	**91.5**	**98.7**	**106.1**	**114.3**	**122.5**	**131.8**	**140.0**	**148.9**	**159.0**	**169.5**	**179.8**	**190.4**	**199.4**	**210.0**	**218.2**	**227.6**	**236.0**	**244.8**	**253.8**	**264.2**	**273.4**	**283.1**	**291.6**	**301.4**	**308.5**	**318.8**	**329.7**	**100.3**
	SD	**4.4**	**4.1**	**4.1**	**4.1**	**4.7**	**4.9**	**5.1**	**5.9**	**6.6**	**7.2**	**8.3**	**9.2**	**10.4**	**10.2**	**11.4**	**12.2**	**14.1**	**13.9**	**14.0**	**14.9**	**16.4**	**16.3**	**16.7**	**17.2**	**18.2**	**18.7**	**19.9**	**20.6**	**21.5**	**22.9**	**25.2**	**28.3**	**7.9**
500 mg/kg-day	12-0175	63.5	68.8	73.7	78.1	83.3	88.9	94.5	101.3	107.7	113.8	120.3	126.2	133.6	141.2	149.4	156.7	167.7	175.3	184.9	195.4	202.3	213.0	218.8	231.6	240.3	252.2	258.3	265.7	278.0	284.5	299.0		94.0
	12-0183	63.0	70.1	75.9	81.7	87.5	94.1	100.2	107.8	116.2	124.6	133.4	142.7	155.0	159.0	167.4	178.8	191.2	201.9	213.1	221.6	231.5	241.9	252.0	257.2	268.7	279.9	288.3	292.4	303.6	310.3	318.8		100.3
	12-0196	65.6	72.5	78.8	86.2	95.3	103.2	111.7	121.3	130.9	143.0	152.6	165.3	176.8	188.5	200.1	210.4	225.1	236.5	247.6	258.2	269.5	284.0	291.2	300.8	315.8	326.5	331.9	343.0	354.6	359.1	368.6		115.9
	12-0216	63.0	69.9	75.5	80.0	87.7	95.1	103.1	109.8	116.5	125.8	134.8	144.0	155.6	163.3	171.7	180.7	192.1	202.3	211.5	218.2	230.6	239.6	247.9	259.8	270.4	280.8	290.6	299.2	312.2	318.6	329.1		103.5
	12-0237	60.2	66.9	74.1	80.9	87.6	94.5	102.9	109.8	118.9	127.9	136.9	146.0	158.3	167.4	177.2	188.4	197.8	205.1	221.1	227.7	235.3	247.7	261.5	270.5	279.3	287.5	301.3	309.6	315.7	325.8	333.8		105.0
	12-0242	60.6	65.2	70.8	77.2	83.0	89.5	97.3	105.2	113.7	121.4	130.2	138.4	148.2	154.6	164.2	173.2	180.6	189.8	199.0	206.6	215.5	223.4	230.8	240.6	246.9	256.4	265.3	276.3	284.8	292.0	301.9		94.9
	12-0264	61.6	66.9	72.0	76.9	83.5	90.1	97.1	104.6	113.0	121.2	130.5	140.4	149.8	157.2	166.5	177.5	187.2	196.9	208.1	217.1	226.7	235.8	247.3	255.9	268.9	277.7	284.8	296.2	303.3	309.9	323.8		101.8
	12-0274	63.1	68.1	73.6	80.0	89.0	95.5	103.4	111.5	120.8	129.1	140.0	150.6	159.5	167.9	179.0	190.6	198.8	211.0	221.7	231.7	239.8	251.2	261.3	268.3	273.2	280.4	287.5	298.0	307.1	317.3	326.3		102.6
	12-0282	59.7	64.9	71.2	76.5	83.3	90.3	96.8	103.6	111.1	119.2	129.1	137.6	146.1	154.9	164.5	172.7	179.6	188.0	197.1	205.5	213.2	222.5	230.9	237.4	243.0	254.3	261.6	266.6	274.8	277.4	290.7	296.8	91.4
	12-0311	62.6	67.3	73.8	78.4	85.8	92.2	99.3	106.1	114.3	122.6	131.4	139.9	151.1	157.2	170.0	178.6	183.1	194.2	205.4	214.3	220.5	232.9	238.6	246.3	246.2	258.4	263.9	273.7	289.6	293.7	308.7	316.4	97.1
	12-0321	66.5	73.4	80.1	87.6	94.6	103.6	111.6	119.1	130.5	138.2	150.6	159.5	168.9	182.9	192.8	205.2	215.6	225.7	238.3	246.0	256.9	267.1	279.4	276.6	282.2	290.3	303.6	310.5	331.5	339.8	340.5	350.1	107.1
	12-0331	50.6	56.7	62.2	67.7	73.8	79.5	85.7	93.4	99.7	106.5	113.5	121.8	127.8	140.6	148.0	157.5	163.6	174.3	183.0	187.0	195.8	203.6	211.6	220.3	230.0	237.1	243.8	252.8	265.3	273.5	282.4	293.8	88.8
	12-0343	58.4	65.1	71.1	77.0	83.6	89.6	97.0	103.5	112.8	119.6	127.9	135.5	143.8	153.0	164.3	173.4	184.1	193.2	200.6	207.4	215.0	221.9	229.0	238.5	248.8	258.9	263.9	272.6	281.8	289.3	301.4	309.9	94.8
	12-0356	61.2	67.8	73.4	74.6	82.2	88.0	95.4	103.0	112.0	119.8	129.4	137.3	149.8	154.5	164.8	171.6	183.6	193.3	202.9	209.3	220.5	229.3	242.1	240.9	251.2	263.4	272.7	286.8	294.4	304.8	317.0	327.3	99.7
	12-0364	55.3	62.3	68.9	71.9	78.5	85.1	92.5	99.1	108.2	116.7	125.4	134.0	143.5	151.4	162.9	172.1	180.9	189.5	201.5	209.5	219.9	226.2	236.8	245.1	256.5	266.0	274.7	285.0	293.5	305.3	315.3	324.3	99.2
	Mean	**61.0**	**67.1**	**72.9**	**78.3**	**85.2**	**91.9**	**99.2**	**106.6**	**115.1**	**123.3**	**132.4**	**141.3**	**151.2**	**159.6**	**169.5**	**179.2**	**188.7**	**198.5**	**209.1**	**217.0**	**226.2**	**236.0**	**245.3**	**252.7**	**261.4**	**271.3**	**279.5**	**288.6**	**299.3**	**306.8**	**317.2**	**316.9**	**99.7**
	SD	**4.0**	**4.1**	**4.4**	**5.0**	**5.5**	**6.2**	**6.8**	**7.1**	**8.1**	**9.0**	**10.1**	**11.2**	**12.3**	**13.1**	**13.8**	**14.8**	**16.1**	**16.6**	**17.6**	**18.3**	**19.2**	**20.6**	**21.5**	**20.4**	**21.6**	**21.4**	**22.2**	**22.5**	**23.2**	**23.3**	**21.6**	**19.4**	**6.8**

Appendix F

Individual and Summary of Body Mass Gain Data

Table F-1
Protocol No. 0FP3-95-12-02-01
Pubertal Deveopment and Thyroid Function in Intact Juvenile Rats Exposed to NTO

Individual Body Mass Gain (grams)
Female Rats

		Days																				
Group	Animal ID	22-23	23-24	24-25	25-26	26-27	27-28	28-29	29-30	30-31	31-32	32-33	33-34	34-35	35-36	36-37	37-38	38-39	39-40	40-41	41-42	42-43	total
Corn Oil	12-0182	3.6	5.4	4.8	4.5	5.7	5.0	3.1	7.3	4.5	6.9	5.8	6.9	4.3	7.6	4.5	6.4	4.9	6.0	5.5	5.7		108.4
Control	12-0192	2.1	6.1	6.5	4.9	6.2	6.2	5.9	6.9	6.2	9.9	7.9	6.6	9.5	9.4	0.7	9.4	8.0	4.9	3.6	6.5		127.4
	12-0202	4.1	6.5	4.9	4.0	5.2	4.1	4.2	7.4	6.0	9.0	4.8	4.3	-4.7	13.6	7.3	6.5	3.3	6.1	3.9	6.9		107.4
	12-0219	2.8	6.2	5.2	3.5	5.6	5.9	5.2	5.8	5.5	6.0	6.5	6.5	-3.9	13.2	5.4	7.4	6.0	5.6	3.1	5.6		107.1
	12-0238	5.4	4.9	6.0	6.6	6.7	6.5	7.1	7.9	6.5	7.8	7.0	8.9	7.3	6.1	6.4	2.1	10.4	5.3	7.9	1.8		128.6
	12-0248	4.5	4.5	6.3	3.7	5.3	4.6	5.1	7.1	4.9	5.1	6.4	6.9	6.9	1.1	6.4	-0.3	7.0	4.8	5.7	0.4		96.4
	12-0268	4.9	6.4	5.4	5.3	5.7	5.0	3.0	5.5	7.5	7.5	5.6	6.2	6.9	8.0	5.3	8.3	6.0	6.4	3.8	7.7		120.4
	12-0280	5.2	5.3	6.7	5.3	5.5	5.3	4.4	8.4	6.4	4.5	9.1	5.9	9.1	6.5	4.8	6.1	8.0	5.5	-0.8	9.3		120.5
	12-0288	2.2	5.8	6.4	3.7	6.8	6.7	7.4	5.8	7.3	8.5	6.7	7.8	7.9	6.3	9.4	7.2	4.4	4.2	6.9	3.2	6.8	124.6
	12-0319	3.6	5.2	5.2	6.2	6.1	5.0	5.9	7.2	7.3	7.9	6.8	9.3	9.3	5.1	9.7	6.8	8.3	1.2	0.3	8.6	2.1	125.0
	12-0329	5.9	6.1	4.0	5.4	6.6	6.1	5.8	7.1	4.7	-4.8	20.4	8.9	9.3	5.5	11.6	9.8	5.5	1.7	9.3	3.7	8.3	132.6
	12-0337	3.9	5.9	4.9	4.5	5.4	5.9	5.4	6.2	6.2	-5.1	15.4	8.2	4.9	7.7	7.6	2.4	-0.3	8.2	5.4	5.2	3.5	107.9
	12-0350	4.8	6.0	6.4	6.9	6.5	7.3	6.5	8.1	7.3	9.6	8.0	5.4	9.7	8.9	3.2	8.8	5.5	7.5	2.2	7.8	8.3	136.4
	12-0357	3.7	4.2	6.3	5.6	6.4	5.2	6.9	7.9	5.6	8.7	6.8	6.0	8.1	8.6	5.1	2.6	9.6	6.7	4.7	6.5	0.3	125.2
	12-0368	3.9	4.0	5.3	5.5	6.1	5.4	7.2	5.4	5.9	7.9	3.4	8.5	7.3	3.4	3.0	0.3	7.3	5.0	2.9	-1.4	6.7	96.3
	Mean	**4.0**	**5.5**	**5.6**	**5.0**	**6.0**	**5.6**	**5.5**	**6.9**	**6.1**	**6.0**	**8.0**	**7.1**	**6.1**	**7.4**	**6.0**	**5.6**	**6.3**	**5.3**	**4.3**	**5.2**	**5.1**	**117.6**
	SD	**1.1**	**0.8**	**0.8**	**1.1**	**0.5**	**0.9**	**1.4**	**1.0**	**1.0**	**4.7**	**4.3**	**1.5**	**4.5**	**3.3**	**2.8**	**3.3**	**2.7**	**1.9**	**2.7**	**3.1**	**3.2**	**12.7**
500 mg/kg-day	12-0181	4.3	6.8	5.2	5.2	6.2	6.1	6.0	7.9	7.9	10.0	6.7	9.5	7.2	6.3	7.7	1.9	9.6	6.9	8.1	-2.2		127.3
	12-0190	4.9	6.9	5.9	6.1	6.7	6.4	6.1	7.4	7.4	7.7	9.7	7.1	6.5	5.7	5.1	8.0	5.0	6.7	4.9	5.4		129.6
	12-0197	2.5	7.5	6.9	5.5	6.3	5.0	6.1	6.4	8.2	8.5	8.0	7.0	9.3	7.3	6.6	6.7	2.9	7.6	11.0	3.5		132.8
	12-0217	1.5	3.9	3.7	4.0	5.9	4.2	5.9	5.8	5.5	7.0	5.6	7.3	7.6	6.1	5.2	8.1	5.4	6.8	4.6	6.0		110.1
	12-0241	4.0	5.4	5.2	6.5	5.9	6.1	5.6	7.3	8.3	5.9	8.6	6.4	4.6	6.9	7.7	7.0	4.7	1.1	6.8	3.2		117.2
	12-0250	4.6	6.1	5.4	4.5	4.8	6.4	5.3	5.5	4.5	5.2	7.4	4.9	3.6	8.2	4.5	4.9	-0.5	8.7	4.4	3.0		101.4
	12-0271	3.9	6.0	4.8	5.7	5.1	5.7	5.4	5.2	6.4	8.6	7.3	7.3	7.6	3.0	8.8	7.0	5.3	5.8	2.3	8.4	6.1	119.6
	12-0277	4.2	5.8	4.6	5.6	6.2	4.5	7.3	5.8	6.4	6.6	8.0	7.2	5.0	7.4	6.1	4.4	5.8	2.1	4.8	6.4	4.8	114.2
	12-0290	5.7	5.2	5.5	4.9	5.3	6.0	6.5	6.6	8.2	5.1	8.8	8.6	5.3	7.9	8.5	5.7	5.3	4.2	5.6	6.0	6.8	124.9
	12-0318	8.0	5.6	5.9	4.9	5.6	6.2	5.7	4.9	7.4	2.6	8.2	5.1	5.8	6.0	6.4	6.1	4.7	6.0	1.7	4.3	5.6	111.1
	12-0326	3.9	5.9	5.4	4.5	5.7	6.3	5.5	7.1	4.8	7.6	6.6	10.5	4.3	11.9	6.3	6.5	-0.7	14.7	4.8	1.8	4.2	123.4
	12-0338	1.3	5.9	4.9	5.3	3.5	5.7	4.5	3.8	5.4	6.6	4.2	5.0	6.2	4.1	3.7	6.1	3.8	3.6	0.8	5.0	5.0	89.4
	12-0349	3.0	3.9	4.2	4.3	5.0	4.2	5.6	7.1	5.2	7.0	7.5	6.6	-2.1	12.1	5.0	-1.5	1.7	10.1	4.2	4.4	-0.5	97.5
	12-0358	4.8	4.2	4.2	3.6	5.7	6.2	7.1	6.1	6.8	7.1	8.1	8.3	6.8	5.2	5.8	3.6	6.5	4.3	4.2	6.0	9.1	114.6
	12-0370	3.3	4.8	5.1	5.2	4.4	5.4	6.0	6.0	6.7	7.8	6.8	8.7	5.4	3.7	7.9	0.1	9.8	5.3	3.4	7.2	8.8	113.0
	Mean	**4.0**	**5.6**	**5.1**	**5.1**	**5.5**	**5.6**	**5.9**	**6.2**	**6.6**	**6.9**	**7.4**	**7.3**	**5.5**	**6.8**	**6.4**	**5.0**	**4.6**	**6.3**	**4.8**	**4.6**	**5.5**	**115.1**
	SD	**1.7**	**1.1**	**0.8**	**0.8**	**0.8**	**0.8**	**0.7**	**1.1**	**1.3**	**1.7**	**1.3**	**1.6**	**2.6**	**2.6**	**1.5**	**2.8**	**3.0**	**3.3**	**2.5**	**2.6**	**2.8**	**12.1**
1000 mg/kg-day	12-0180	4.3	7.3	4.8	5.5	6.4	6.7	4.6	7.0	6.2	7.1	1.2	13.1	6.3	7.5	5.8	5.8	4.7	7.7	1.9	7.8		121.7
	12-0191	3.6	6.1	2.6	5.4	5.9	6.5	5.6	6.8	8.0	4.4	-1.0	13.8	7.7	5.9	4.4	-1.8	10.8	7.6	10.1	-8.5		103.9
	12-0199	3.1	7.5	5.2	6.0	6.1	7.0	7.2	7.6	6.9	8.2	5.0	13.3	9.1	4.2	8.1	6.5	3.4	5.7	6.3	5.8		132.2
	12-0218	2.7	4.6	4.3	6.0	5.0	5.8	6.6	3.1	5.5	6.3	-0.7	11.9	6.9	3.8	8.5	6.1	2.5	7.3	2.6	4.2		103.0
	12-0239	2.0	5.4	4.9	4.7	6.2	5.7	7.0	7.1	6.4	6.1	2.9	10.7	8.7	6.2	8.2	8.0	4.2	2.2	5.4	7.3		119.3
	12-0251	3.4	4.7	5.3	4.1	6.1	4.2	5.8	8.2	4.5	4.5	2.6	9.8	6.8	7.5	4.9	-0.7	11.2	3.3	4.8	2.2		103.2
	12-0270	1.6	6.2	3.8	3.9	5.2	4.5	4.8	5.3	4.2	7.0	3.0	11.0	6.4	6.1	7.3	6.5	5.5	7.1	4.3	5.2		108.9
	12-0281	2.6	5.8	4.5	4.1	7.4	4.6	4.7	3.6	7.6	6.7	-0.2	12.4	5.7	5.4	7.4	2.3	7.4	4.4	4.4	7.5		108.3
	12-0287	2.6	6.8	4.0	3.5	4.8	4.9	5.5	2.9	5.6	5.7	1.0	12.1	5.0	6.8	4.2	4.5	5.8	5.1	1.8	4.3	7.8	96.9
	12-0316	2.8	6.4	3.4	3.9	6.2	4.3	5.4	6.3	5.9	6.4	1.1	13.6	6.4	7.2	5.0	3.1	5.5	7.2	1.7	4.3	-1.2	106.1
	12-0328	-2.2	8.3	5.8	3.7	5.6	4.4	3.4	7.3	4.9	6.7	2.7	11.8	5.9	7.3	7.0	7.2	5.2	5.3	7.7	4.9	0.5	112.9
	12-0335	2.0	6.5	5.3	2.2	3.5	3.9	3.5	3.4	6.1	4.1	0.0	8.8	4.6	5.0	3.5	5.4	2.4	4.3	3.4	2.5	3.7	80.4
	12-0347	3.8	4.3	6.7	4.8	5.6	5.4	5.6	6.3	5.9	8.1	-1.1	12.8	4.2	5.7	3.6	-3.3	5.4	7.3	3.9	2.2	-3.4	97.2
	12-0359	3.2	3.0	5.4	4.8	4.1	5.3	5.3	4.5	6.9	6.7	1.7	11.7	8.6	3.8	4.9	5.1	7.8	3.4	8.3	1.7	8.7	106.2
	12-0366	4.9	4.3	3.8	3.5	6.8	4.7	5.8	5.2	8.7	4.0	4.8	12.0	6.3	4.2	7.5	5.5	5.8	5.5	2.8	6.9	5.8	113.0
	Mean	**2.7**	**5.8**	**4.7**	**4.4**	**5.7**	**5.2**	**5.4**	**5.6**	**6.2**	**6.1**	**1.5**	**11.9**	**6.6**	**5.8**	**6.0**	**4.0**	**5.8**	**5.6**	**4.6**	**3.9**	**3.1**	**107.5**
	SD	**1.6**	**1.4**	**1.0**	**1.0**	**1.0**	**1.0**	**1.1**	**1.8**	**1.3**	**1.3**	**2.0**	**1.4**	**1.5**	**1.3**	**1.8**	**3.4**	**2.6**	**1.8**	**2.5**	**4.0**	**4.6**	**12.0**

Table F-2
Protocol No. 0FP3-85-12-02-01
Pubertal Deveopment and Thyroid Function in Intact Juvenile Rats Exposed to NTO

Individual Body Mass Gain (grams)
Male Rats
Post Natal Day

Group	Animal ID	23-24	24-25	25-26	26-27	27-28	28-29	29-30	30-31	31-32	32-33	33-34	34-35	35-36	36-37	37-38	38-39	39-40	40-41	41-42	42-43	43-44	44-45	45-46	46-47	47-48	48-49	49-50	50-51	51-52	52-53	total
Corn Oil	12-0178	4.1	5.8	5.0	5.5	6.9	6.0	7.5	6.7	7.8	7.1	8.4	7.9	8.1	10.1	8.2	7.4	8.5	10.3	7.6	5.3	8.6	8.9	12.9	7.5	10.8	11.6	3.1	8.1	8.8	9.2	233.7
Control	12-0184	5.6	5.7	6.2	6.3	7.5	5.7	8.3	6.9	8.4	8.6	7.9	9.1	9.0	9.2	9.9	9.8	9.5	11.9	8.4	8.0	9.8	10.3	8.0	10.8	8.7	11.7	8.7	5.5	9.7	11.2	256.3
	12-0193	6.6	4.1	5.8	6.1	6.1	6.8	7.1	7.9	9.2	5.9	12.2	8.0	7.7	7.9	9.8	8.9	10.2	11.7	8.0	10.9	12.8	6.2	6.2	11.5	8.4	9.8	11.4	11.5	2.7	10.1	251.5
	12-0213	5.2	4.5	5.4	4.0	5.6	7.5	6.9	6.6	6.7	9.9	8.6	9.3	9.1	9.2	9.6	8.9	7.5	11.0	8.6	8.9	9.6	7.8	9.2	6.6	10.5	9.3	3.8	8.8	6.9	7.4	232.9
	12-0233	3.2	6.5	5.9	7.7	9.7	6.2	7.8	7.8	7.8	9.1	10.6	9.8	7.9	9.6	8.7	11.4	9.2	12.5	8.1	11.9	10.5	8.5	9.2	11.4	7.9	10.5	10.4	10.7	8.6	11.7	270.8
	12-0247	5.2	5.4	6.4	8.0	6.3	6.4	8.9	5.9	7.8	8.4	8.9	9.5	7.3	9.2	6.4	7.2	8.8	9.7	7.4	8.5	9.3	6.0	10.8	8.7	11.0	6.9	6.9	7.6	4.9	12.5	238.2
	12-0266	4.0	6.0	5.3	6.4	5.0	5.1	6.5	7.7	-5.0	18.4	8.0	10.4	6.6	8.9	6.7	10.6	8.9	9.8	8.0	9.0	9.6	7.7	8.5	9.8	8.2	9.3	9.9	11.0	8.2	14.5	243.0
	12-0273	7.2	6.4	6.6	7.3	6.9	7.5	7.9	11.2	-6.6	22.6	8.3	12.4	7.4	9.8	10.9	9.4	9.8	11.3	7.3	7.1	9.9	9.5	8.1	9.6	12.5	10.1	4.9	10.9	8.9	10.6	265.7
	12-0285	5.7	7.3	7.2	7.2	7.4	7.1	8.8	7.2	10.5	8.6	11.2	11.0	10.6	10.3	8.8	9.5	13.4	9.1	9.7	7.9	9.7	8.0	14.7	7.5	8.6	11.2	8.8	13.2	4.5	15.1	279.8
	12-0315	5.2	9.0	5.1	8.6	7.0	7.2	7.7	8.7	8.6	10.3	8.5	10.7	8.4	12.0	7.6	7.0	12.1	10.0	7.5	6.2	14.7	6.2	12.7	14.5	10.5	8.9	9.6	14.0	10.6	8.8	277.9
	12-0320	5.6	3.6	7.5	7.8	6.8	7.4	9.5	10.7	9.8	10.1	12.9	11.2	12.7	7.8	13.0	13.1	13.6	7.2	15.1	9.9	9.6	8.9	12.3	10.0	14.6	7.7	9.9	9.8	11.1	23.5	312.7
	12-0330	5.8	4.0	5.8	5.8	6.3	7.1	3.6	6.5	7.8	6.1	8.3	9.4	7.1	7.7	10.8	7.1	8.1	8.9	7.7	8.9	10.8	5.2	7.0	8.1	8.6	11.3	5.3	8.6	6.3	11.1	225.1
	12-0342	5.1	5.8	6.4	6.7	6.5	10.2	4.0	8.5	8.1	7.4	10.6	8.6	6.9	12.1	6.7	8.7	8.4	11.4	6.0	14.1	3.6	7.2	14.5	6.4	10.9	10.4	8.2	7.9	8.7	9.6	249.6
	12-0352	4.9	6.1	6.7	6.2	7.3	8.8	6.4	6.3	7.7	10.7	11.1	7.2	8.2	14.2	9.7	9.9	8.5	11.6	7.6	12.0	6.2	9.9	12.9	9.9	12.6	10.2	10.4	12.4	9.2	13.0	277.8
	12-0362	4.7	6.6	5.1	7.3	6.7	8.1	6.3	8.7	8.9	11.3	8.6	11.0	8.4	12.2	10.1	8.0	9.6	9.7	8.2	10.8	2.5	14.3	13.3	7.7	12.0	5.7	12.4	8.2	5.0	4.1	255.5
	Mean	5.2	5.8	6.0	6.7	6.8	7.1	7.1	7.8	6.5	10.3	9.6	9.7	8.4	10.0	9.3	9.1	9.7	10.4	8.3	9.3	9.1	8.3	10.7	9.3	10.4	9.6	8.2	9.9	7.6	11.5	258.0
	SD	1.0	1.4	0.8	1.2	1.0	1.3	1.7	1.5	5.1	4.5	1.7	1.4	1.6	1.9	1.7	1.7	1.9	1.4	2.0	2.4	3.1	2.2	2.8	2.2	2.0	1.7	2.8	2.3	2.4	4.3	23.2
250	12-0173	5.5	4.3	8.0	1.0	5.3	5.3	6.7	7.3	4.7	7.4	7.7	6.8	8.7	7.9	8.1	8.8	7.2	9.9	8.8	7.4	7.5	7.0	10.4	7.3	9.6	7.8	7.0	7.5	7.8	-0.2	208.5
mg/kg-day	12-0188	5.5	6.1	5.5	5.9	5.2	7.6	6.8	9.4	7.0	11.0	7.4	12.3	9.3	11.5	11.5	11.5	8.4	15.5	9.4	9.5	6.7	5.8	11.0	11.0	10.3	8.0	12.9	12.9	7.4	12.4	274.7
	12-0195	6.3	6.8	4.9	4.6	4.7	9.9	7.3	8.6	7.3	11.2	6.3	-4.6	21.1	11.1	12.1	11.3	9.6	9.9	3.3	6.7	9.7	11.4	5.8	9.7	8.7	9.3	9.7	8.6	6.3	9.8	247.4
	12-0214	4.5	5.1	5.6	7.7	6.9	6.5	10.5	7.7	9.2	10.0	9.8	-5.2	19.8	14.5	12.6	22.1	-0.1	10.6	9.0	10.8	8.2	12.0	9.1	10.1	4.2	15.4	8.8	8.7	11.2	9.8	275.1
	12-0232	6.0	5.7	6.5	8.3	6.4	7.4	7.5	8.2	9.7	10.3	7.8	11.7	9.1	10.7	8.2	12.0	12.0	11.3	10.1	9.7	9.4	7.6	8.7	11.0	7.3	10.7	9.4	7.2	5.3	16.3	271.5
	12-0246	6.0	4.2	6.7	7.5	7.5	7.2	8.1	6.8	9.1	8.3	9.7	8.8	7.8	11.2	8.3	9.5	8.2	8.7	3.0	6.4	8.1	6.3	10.1	8.9	10.8	8.5	7.6	9.8	4.1	12.9	240.1
	12-0265	6.1	4.2	6.8	5.7	7.4	7.0	5.1	8.8	7.3	8.4	8.9	12.0	7.3	9.9	10.1	9.2	10.0	11.1	7.5	12.2	6.6	9.8	8.4	13.0	9.4	10.5	9.6	10.9	4.9	10.6	258.7
	12-0276	7.1	5.4	7.0	5.0	9.9	6.6	8.7	6.6	8.8	10.4	7.2	13.0	7.9	9.7	11.9	10.0	10.7	11.8	9.5	11.2	11.5	7.7	8.6	12.7	7.9	9.0	6.6	9.4	8.3	14.6	274.7
	12-0286	5.8	6.1	5.3	8.4	5.4	8.0	8.2	7.0	10.9	7.9	9.9	12.1	9.3	10.7	9.8	10.3	9.5	8.9	7.7	12.8	7.5	9.3	10.0	10.3	9.0	10.2	9.1	8.4	8.5	8.7	265.0
	12-0312	5.2	6.7	6.8	6.3	7.6	6.6	6.9	9.5	11.0	7.4	9.5	9.6	8.9	10.1	13.1	7.0	11.6	11.3	10.7	7.6	11.6	6.0	13.5	10.1	10.3	11.8	6.1	11.7	11.0	10.4	275.9
	12-0325	6.8	6.5	5.1	7.6	6.7	6.0	7.3	6.6	9.4	9.2	3.8	12.8	7.6	9.0	13.2	10.7	9.2	12.6	5.4	9.7	8.5	9.7	6.5	9.4	11.9	9.0	3.6	13.6	6.0	8.4	251.8
	12-0334	7.1	6.2	4.8	6.8	6.1	7.6	4.8	6.2	9.0	5.4	6.6	9.8	6.1	8.4	7.8	7.4	8.0	11.3	6.5	5.3	9.3	9.0	6.7	9.1	5.1	6.8	7.5	7.4	3.7	7.5	213.3
	12-0344	7.1	6.7	6.7	7.4	6.4	8.2	8.0	10.3	7.3	11.8	11.1	10.1	8.3	12.5	8.7	13.2	8.1	9.4	8.7	10.1	5.2	10.7	8.3	11.7	10.6	9.5	9.5	12.2	5.3	10.2	273.3
	12-0353	6.3	5.1	5.4	5.9	7.2	7.4	8.4	8.6	7.2	10.2	7.8	11.5	10.1	9.7	9.4	8.3	11.8	7.3	11.6	11.0	8.8	8.8	8.6	9.4	10.3	8.2	9.8	11.4	3.4	10.4	259.3
	12-0361	5.9	6.2	6.4	6.7	9.0	6.4	7.6	10.7	4.9	11.8	9.5	11.7	10.1	10.8	10.6	7.0	11.5	8.9	12.0	10.1	8.2	10.8	9.5	12.1	12.3	10.5	10.5	6.9	13.8	12.5	284.9
	Mean	6.1	5.7	6.1	6.3	6.8	7.2	7.5	8.2	8.2	9.4	8.2	8.8	10.1	10.5	10.4	10.6	9.0	10.6	8.2	9.4	8.5	8.8	9.0	10.4	9.2	9.7	8.5	9.8	7.1	10.3	258.3
	SD	0.7	0.9	0.9	1.9	1.4	1.1	1.4	1.4	1.9	1.9	1.8	5.8	4.3	1.6	1.9	3.7	3.0	2.0	2.7	2.2	1.7	2.0	1.9	1.5	2.3	2.1	2.2	2.2	3.0	3.7	22.8
500	12-0175	5.3	4.9	4.4	5.2	5.6	5.6	6.8	6.4	6.1	6.5	5.9	7.4	7.6	8.2	7.3	11.0	7.6	9.6	10.5	6.9	10.7	5.8	12.8	8.7	11.9	6.1	7.4	12.3	6.5	14.5	235.5
mg/kg-day	12-0183	7.1	5.8	5.8	5.8	6.6	6.1	7.6	8.4	8.4	8.8	9.3	12.3	4.0	8.4	11.4	12.4	10.7	11.2	8.5	9.9	10.4	10.1	5.2	11.5	11.2	8.4	4.1	11.2	6.7	8.5	255.8
	12-0196	6.9	6.3	7.4	9.1	7.9	8.5	9.6	9.6	12.1	9.6	12.7	11.5	11.7	11.6	10.3	14.7	11.4	11.1	10.6	11.3	14.5	7.2	9.6	15.0	10.7	5.4	11.1	11.6	4.5	9.5	303.0
	12-0216	6.9	5.6	4.5	7.7	7.4	8.0	6.7	6.7	9.3	9.0	9.2	11.6	7.7	8.4	9.0	11.4	10.2	9.2	6.7	12.4	9.0	8.3	11.9	10.6	10.4	9.8	8.6	13.0	6.4	10.5	266.1
	12-0237	6.7	7.2	6.8	6.7	6.9	8.4	6.9	9.1	9.0	9.0	9.1	12.3	9.1	9.8	11.2	9.4	7.3	16.0	6.6	7.6	12.4	13.8	9.0	8.8	8.2	13.8	8.3	6.1	10.1	8.0	273.6
	12-0242	4.6	5.6	6.4	5.8	6.5	7.8	7.9	8.5	7.7	8.8	8.2	9.8	6.4	9.6	9.0	7.4	9.2	9.2	7.6	8.9	7.9	7.4	9.8	6.3	9.5	8.9	11.0	8.5	7.2	9.9	241.3
	12-0264	5.3	5.1	4.9	6.6	6.6	7.0	7.5	8.4	8.2	9.3	9.9	9.4	7.4	9.3	11.0	9.7	9.7	11.2	9.0	9.6	9.1	11.5	8.6	13.0	8.8	7.1	11.4	7.1	6.6	13.9	262.2
	12-0274	5.0	5.5	6.4	9.0	6.5	7.9	8.1	9.3	8.3	10.9	10.6	8.9	8.4	11.1	11.6	8.2	12.2	10.7	10.0	8.1	11.4	10.1	7.0	4.9	7.2	7.1	10.5	9.1	10.2	9.0	263.2
	12-0282	5.2	6.3	5.3	6.8	7.0	6.5	6.8	7.5	8.1	9.9	8.5	8.5	8.8	9.6	8.2	6.9	8.4	9.1	8.4	7.7	9.3	8.4	6.5	5.6	11.3	7.3	5.0	8.2	2.6	13.3	231.0
	12-0311	4.7	6.5	4.6	7.4	6.4	7.1	6.8	8.2	8.3	8.8	8.5	11.2	6.1	12.8	8.6	4.5	11.1	11.2	8.9	6.2	12.4	5.7	7.7	-0.1	12.2	5.5	9.8	15.9	4.1	15.0	246.1
	12-0321	6.9	6.7	7.5	7.0	9.0	8.0	7.5	11.4	7.7	12.4	8.9	9.4	14.0	9.9	12.4	10.4	10.1	12.6	7.7	10.9	10.2	12.3	-2.8	5.6	8.1	13.3	6.9	21.0	8.3	0.7	274.0
	12-0331	6.1	5.5	5.5	6.1	5.7	6.2	7.7	6.3	6.8	7.0	8.3	6.0	12.8	7.4	9.5	6.1	10.7	8.7	4.0	8.8	7.8	8.0	8.7	9.7	7.1	6.7	9.0	12.5	8.2	8.9	231.8
	12-0343	6.7	6.0	5.9	6.6	6.0	7.4	6.5	9.3	6.8	8.3	7.6	8.3	9.2	11.3	9.1	10.7	9.1	7.4	6.8	7.6	6.9	7.1	9.5	10.3	10.1	5.0	8.7	9.2	7.5	12.1	243.0
	12-0356	6.6	5.6	1.2	7.6	5.8	7.4	7.6	9.0	7.8	9.6	7.9	12.5	4.7	10.3	6.8	12.0	9.7	9.6	6.4	11.2	8.8	12.8	-1.2	10.3	12.2	9.3	14.1	7.6	10.4	12.2	255.8
	12-0364	7.0	4.6	5.0	6.6	6.6	7.4	6.6	9.1	8.5	8.7	8.6	9.5	7.9	11.5	9.2	8.8	8.6	12.0	8.0	10.4	6.3	10.6	8.3	11.4	9.5	8.7	10.3	8.5	11.8	10.0	260.0
	Mean	6.1	5.8	5.4	6.9	6.7	7.3	7.4	8.5	8.2	9.1	8.9	9.9	8.4	9.9	9.6	9.6	9.7	10.6	8.0	9.2	9.8	9.3	7.4	8.8	9.9	8.2	9.1	10.8	7.4	10.4	256.2
	SD	0.9	0.7	1.5	1.1	0.9	0.9	0.8	1.3	1.4	1.4	1.5	1.9	2.8	1.5	1.6	2.7	1.4	2.0	1.8	1.8	2.2	2.5	4.3	3.8	1.7	2.6	2.6	3.9	2.5	3.5	19.2

Appendix G

Individual and Summary of Food Consumption Data

Table G-1
Protocol No. 0FP3-95-12-02-01
Pubertal Deveopment and Thyroid Function in Intact Juvenile Rats Exposed to NTO

Food Consumption Per Rat (grams)
Female Rats

		Days			
Group	**Animal ID**	**22-28**	**28-35**	**35-42**	**Total**
Corn Oil Control	12-0182/0192	61.0	96.0	113.7	270.7
	12-0202/0219	55.8	82.1	100.1	238.0
	12-0238/0248	58.7	94.4	102.6	255.6
	12-0268/0280	62.2	95.5	116.8	274.5
	12-0288/0319	59.6	98.0	115.6	273.2
	12-0329/0337	59.0	93.0	117.4	269.4
	12-0350/0357/0368	62.1	104.3	115.6	282.0
	Mean	**59.8**	**94.7**	**111.7**	**266.2**
	SD	**2.25**	**6.69**	**7.20**	**14.76**
500 mg/kg-day	12-0181/0190	66.2	107.3	124.0	297.4
	12-01970217	54.7	92.5	111.6	258.8
	12-0241/0250	59.6	91.5	103.6	254.6
	12-0271/0277	56.9	94.6	110.2	261.6
	12-0290/0318	57.6	92.9	109.2	259.7
	12-0326/0338	54.4	84.5	102.2	241.1
	12-0349/0358/0370	52.7	89.3	99.3	241.3
	Mean	**57.4**	**93.2**	**108.6**	**259.2**
	SD	**4.47**	**6.99**	**8.19**	**18.86**
1000 mg/kg-day	12-0180/0191	61.7	98.7	114.8	275.2
	12-0199/0218	59.8	95.2	111.6	266.5
	12-0239/0251	56.0	94.0	110.8	260.8
	12-0270/0281	52.6	85.3	104.9	242.7
	12-0287/0316	53.4	86.1	102.6	242.0
	12-0328/0335	47.3	79.3	96.5	223.1
	12-0347/0359/0366	54.8	91.6	101.6	248.1
	Mean	**55.1**	**90.0**	**106.1**	**251.2**
	SD	**4.77**	**6.77**	**6.49**	**17.61**

Table G-2
Protocol No. 0FP3-95-12-02-01

Pubertal Deveopment and Thyroid Function in Intact Juvenile Rats Exposed to NTO

Food Consumption Per Rat (grams)
Male Rats

		Days					
Group	Animal ID	23-29	29-36	36-43	43-50	50-53/54	Total
Corn Oil Control	12-0178/0184	66.6	108.8	142.2	212.6	73.9	603.9
	12-0193/0213	62.2	110.8	89.5	209.6	71.2	543.2
	12-0233/0247	70.4	115.2	107.4	218.7	73.3	584.9
	12-0266/0273	69.5	113.5	104.4	222.2	101.6	611.0
	12-0285/0315	73.5	123.2	157.1	245.3	104.0	703.0
	12-0320/0330	66.3	120.3	116.0	235.9	98.7	637.1
	12-0342/0352/0362	67.7	118.3	135.2	216.6	100.6	638.4
	Mean	**68.0**	**115.7**	**121.7**	**223.0**	**89.0**	**617.3**
	SD	**3.57**	**5.19**	**23.92**	**13.00**	**15.29**	**49.91**
250 mg/kg-day	12-0173/0188	60.8	99.5	80.3	214.5	75.4	530.5
	12-0195/0214	67.7	113.4	108.6	210.2	73.4	573.2
	12-0232/0246	69.2	116.8	129.7	214.4	73.9	603.9
	12-0265/0276	70.7	115.1	159.2	243.6	75.6	664.1
	12-0286/0312	72.0	121.1	159.4	242.0	105.5	699.9
	12-0325/0334	66.2	105.0	94.2	245.0	93.7	604.0
	12-0344/0353/0361	72.1	119.6	137.1	223.8	102.2	654.8
	Mean	**68.4**	**112.9**	**124.1**	**227.6**	**85.6**	**618.6**
	SD	**4.00**	**7.91**	**30.89**	**15.42**	**14.30**	**58.10**
500 mg/kg-day	12-0175/0183	66.4	102.1	142.3	224.6	78.1	613.4
	12-0196/0216	75.8	130.4	155.7	248.5	86.4	696.6
	12-0237/0242	70.4	117.3	135.7	89.1		412.3
	12-0264/0274	69.8	123.0	112.2	226.8	118.3	650.0
	12-0282/0311	68.5	115.7	144.4	219.4	93.0	640.9
	12-0321/0331	70.2	117.8	128.3	228.0	97.0	641.3
	12-0343/0356/0364	65.8	112.2	112.5	213.6	101.1	605.2
	Mean	**69.5**	**116.9**	**133.0**	**207.1**	**95.6**	**641.2**
	SD	**3.30**	**8.78**	**16.39**	**53.18**	**13.74**	**32.29**

Appendix H

Summary of Vaginal Opening Data

Table H
Protocol No. 0FP3-95-12-02-01
Pubertal Deveopment and Thyroid Function in Intact Juvenile Rats Exposed to NTO

Vaginal Opening and Summary of Body Mass Data in Female Rats

NTO	Effect	Tranform or nonparam	Pairwise test	p-value	Vehicle Control				500 mg/kg-day					1000 mg/kg-day				
					Mean	SD	CV	N	Mean	SD	CV	N	p-value	Mean	SD	CV	N	p-value
Age at Initiation of VO (PND)	U			0.204	33.0	1.323	4.01	9	34.2	0.837	2.45	5		34.0	1.414	4.16	2	
	A				32.9	2.178	6.61	9	34.2	2.122	6.20	5		34.0	2.121	6.24	2	
Age at VO (PND)	U			0.131	34.1	1.187	3.48	15	35.1	1.100	3.14	15		35.1	1.870	5.33	15	
	A			0.066	34.2	1.631	4.76	15	35.1	1.580	4.51	15		35.1	1.580	4.51	15	
Body mass at VO (g)	U			0.429	124.99	11.000	8.80	15	129.23	10.327	7.99	15		123.69	14.491	11.72	15	
	A			0.090	125.94	11.971	9.51	15	129.23	11.600	8.98	15		123.69	11.600	9.38	15	
Initial body mass (PND 22, g)	U			0.580	53.72	3.333	6.21	15	53.01	4.412	8.32	15		54.58	4.421	8.10	15	
	A			0.429	54.08	1.844	3.41	15	53.26	1.840	3.45	15		53.97	1.847	3.42	15	
Final body mass (g)	U			0.236	171.33	14.771	8.62	15	168.09	14.552	8.66	15		162.13	15.080	9.30	15	
	A*		Sidak	0.031	172.22	11.785	6.84	15	168.72	11.774	6.98	15		160.61	11.824	7.36	15	0.031
Final body mass (% of control)	U			0.280					98.11	8.494	8.66	15		94.63	8.802	9.30	15	
	A			0.058					98.74	6.565	6.65	15		93.99	6.565	6.98	15	
Body mass gain (final minus initial body mass) (g)	U			0.078	117.61	12.732	10.83	15	115.07	12.149	10.56	15		107.55	12.015	11.17	15	
	A*		Sidak	0.020	118.15	11.154	9.44	15	115.45	11.146	9.65	15		106.63	11.189	10.49	15	0.022

Appendix I

Summary of Female Estrus Cyclicity Data

Table I
Protocol No. 0FP3-95-12-02-01
Pubertal Deveopment and Thyroid Function in Intact Juvenile Rats Exposed to NTO

Summary of Estrus Cyclicity Data in Female Rats

NTO	Effect	Tranform or nonparam	Pairwise test	p-value	Vehicle Control				500 mg/kg-day					1000 mg/kg-day				
					Mean	SD	CV	N	Mean	SD	CV	N	p-value	Mean	SD	CV	N	p-value
Mean Age at First Vaginal Estrus (PND)				0.965	36.1	1.831	5.08	15	36.3	1.939	5.34	14		36.2	2.808	7.76	15	
Mean Cycle Length (days)				0.495	4.43	0.942	21.26	15	4.15	0.555	13.37	13		4.58	1.165	25.44	12	
Cycling (%)				0.360	100			15	93.3			14		100			15	
Regularly Cycling (%)				0.525	80.0			12	86.7			13		80.0			12	
Cycle Status at Necropsy (# Females)																		
Diestrus								4				4					4	
Proestrus								6				6					3	
Estrus								2				2					4	
Metestrus								3				3					4	

Appendix J

Summary of Preputial Separation Data

Table J
Protocol No. 0FP3-95-12-02-01
Pubertal Deveopment and Thyroid Function in Intact Juvenile Rats Exposed to NTO

Preputial Separation and Summary of Body Mass Data in Female Rats

NTO	Effect	Tranform or nonparam	Pairwise test	p-value	Vehicle Control				250 mg/kg-day					500 mg/kg-day				
					Mean	SD	CV	N	Mean	SD	CV	N	p-value	Mean	SD	CV	N	p-value
Age at Initiation of PPS (PND)	U			0.151	41.1	0.990	2.41	15	41.7	1.047	2.51	15		41.9	1.027	2.45	14	
	A			0.111	41.1	1.015	2.47	15	41.7	1.015	2.43	15		41.9	1.010	2.41	14	
Age at PPS (PND)	U			0.667	43.8	1.821	4.16	15	43.9	1.668	3.80	15		44.3	1.543	3.48	15	
	A			0.509	43.7	1.607	3.68	15	44.0	1.600	3.63	15		44.4	1.596	3.60	15	
Body mass at PPS (g)	U			0.383	230.42	21.614	9.38	15	235.34	17.753	7.54	15		239.44	12.348	5.16	15	
	A			0.430	230.76	17.916	7.76	15	235.11	17.851	7.59	15		239.34	17.800	7.44	15	
Initial body mass (PND 23, g)	U			0.787	59.94	4.112	6.86	15	60.51	4.361	7.21	15		60.99	3.990	6.54	15	
	A			0.586	60.66	2.188	3.61	15	60.01	2.180	3.63	15		60.78	2.173	3.57	15	
Final body mass (g)	U			0.983	317.97	25.458	8.01	15	318.79	25.193	7.90	15		317.15	21.605	6.81	15	
	A			0.887	320.20	21.902	6.84	15	317.23	21.820	6.88	15		316.49	21.758	6.87	15	
Final body mass (% of control)	U			0.850					100.26	7.923	7.90	15		99.74	6.795	6.81	15	
	A			0.916					100.13	6.859	6.85	15		99.87	6.859	6.87	15	
Body mass gain (final minus initial body mass) (g)	U			0.959	258.03	23.177	8.98	15	258.28	22.804	8.83	15		256.16	19.207	7.50	15	
	A			0.880	259.54	20.883	8.05	15	257.22	20.806	8.09	15		255.71	20.744	8.11	15	

Appendix K

Individual and Summary of Organ Mass Data

Table K-1
Protocol No. 0FP3-95-12-02-01
Pubertal Deveopment and Thyroid Function in Intact Juvenile Rats Exposed to NTO

Summary of Organ Mass Data in Female Rats

NTO	Effect	Tranform or nonparam	Pairwise test	p-value	Vehicle Control				500 mg/kg-day					1000 mg/kg-day					500 mg/kg-day		1000 mg/kg-day	
					Mean	SD	CV	N	Mean	SD	CV	N	p-value	Mean	SD	CV	N	p-value	Mean	%control	Mean	%control
Liver (g)	U			0.469	8.050	1.2034	14.95	15	7.716	0.8345	10.82	15		7.231	0.9534	13.18	15		7.716	0.9584	7.231	0.8983
	A			0.409	8.126	0.8908	10.96	15	7.740	0.9063	11.71	15		7.134	0.8908	12.49	15		7.740	0.9525	7.134	0.8779
	R			0.573	0.046	0.0037	7.93	15	0.045	0.0021	4.64	15		0.044	0.0031	7.13	15		0.045	0.9737	0.044	0.9550
Kidneys (g)	U			0.600	1.494	0.1936	12.96	15	1.512	0.1801	11.91	15		1.414	0.1248	8.82	15		1.512	1.0116	1.414	0.9467
	A			0.527	1.511	0.1472	9.74	15	1.510	0.1472	9.75	15		1.400	0.1472	10.51	15		1.510	0.9993	1.400	0.9265
	R			0.546	0.009	0.0006	7.43	15	0.009	0.0007	7.80	15		0.009	0.0006	6.53	15		0.009	1.0265	0.009	1.0090
Pituitary (mg)	U			0.903	8.59	2.934	34.17	15	9.17	4.628	50.45	15		9.15	5.504	60.15	14		9.17	1.0682	9.15	1.0656
	A			0.901	9.00	3.873	43.03	15	9.00	3.873	43.03	15		9.00	3.742	41.57	14		9.00	1.0000	9.00	1.0000
	R			0.754	49.92	17.922	35.90	15	54.83	31.915	58.21	15		57.59	36.439	63.27	14		54.83	1.0983	57.59	1.1536
Adrenals (mg)	U			0.980	39.33	6.057	15.40	15	38.89	9.501	24.43	15		38.86	5.362	13.80	15		38.89	0.9888	38.86	0.9881
	A			0.954	40.00	7.746	19.36	15	40.00	7.746	19.36	15		39.00	7.746	19.86	15		40.00	1.0000	39.00	0.9750
	R			0.796	227.22	35.197	15.49	15	226.82	49.477	21.81	15		238.02	30.376	12.76	15		226.82	0.9982	238.02	1.0475
Ovaries (mg)	U			0.242	97.96	14.140	14.43	15	93.30	15.004	16.08	15		89.57	10.965	12.24	15		93.30	0.9524	89.57	0.9144
	A			0.206	99.00	11.619	11.74	15	93.00	11.619	12.49	15		89.00	11.619	13.06	15		93.00	0.9394	89.00	0.8990
Uterus, wet (mg)	U			0.385	357.23	157.497	44.09	14	364.48	158.177	43.40	15		304.28	105.363	34.63	15		364.48	1.0203	304.28	0.8518
	A			0.390	356.00	142.183	39.94	14	379.00	143.300	37.81	15		303.00	143.300	47.29	15		379.00	1.0646	303.00	0.8511
Uterus, blotted (mg)	U			0.421	302.47	89.059	29.44	15	310.07	84.926	27.39	15		267.61	64.417	24.07	15		310.07	1.0251	267.61	0.8848
	A			0.429	302.00	81.333	26.93	15	317.00	81.333	25.66	15		267.00	81.333	30.46	15		317.00	1.0497	267.00	0.8841
Thyroid (mg)	U			0.862	14.218	2.5441	17.89	15	14.590	3.1701	21.73	15		15.048	3.0108	20.01	11		14.590	1.0261	15.048	1.0584
	A			0.829	14.168	2.7498	19.41	15	14.785	2.8002	18.94	15		14.887	3.2184	21.62	15		14.785	1.0435	14.887	1.0507

Table K-2
Protocol No. 0FP3-05-12-02-01
Pubertal Deveopment and Thyroid Function in Intact Juvenile Rats Exposed to NTO

Summary of Organ Mass Data in Male Rats

NTO	Effect	Tranform or nonparam	Pairwise test	p-value	Vehicle Control				250 mg/kg-day					500 mg/kg-day					250 mg/kg-day			500 mg/kg-day		
					Mean	SD	SE	N	Mean	SD	SE	N	p-value	Mean	SD	SE	N	p-value	Mean	%control		Mean	%control	
Liver (g)	U			0.986	15.628	1.7526	0.45	15	15.486	1.4777	0.38	15		15.698	1.3723	0.35	15		15.486	0.99		15.698	1.00	
	A			0.932	15.659	1.4446	0.37	15	15.417	1.4098	0.36	15		15.627	1.4059	0.36	15		15.417	0.98		15.627	1.00	
	R			0.725	0.048	0.0027	0.00	15	0.048	0.0023	0.00	15		0.049	0.0030	0.00	15		0.048	0.99		0.049	1.01	
Kidneys (g)	U			0.979	2.532	0.3206	0.08	15	2.524	0.1963	0.05	15		2.536	0.1726	0.04	15		2.524	1.00		2.536	1.00	
	A			0.953	2.533	0.2169	0.06	15	2.513	0.2091	0.05	15		2.530	0.2091	0.05	15		2.513	0.99		2.530	1.00	
	R			0.470	0.008	0.0005	0.00	15	0.008	0.0004	0.00	15		0.008	0.0004	0.00	15		0.008	1.00		0.008	1.01	
Pituitary (mg)	U			0.614	7.29	2.058	0.53	15	8.22	2.091	0.54	15		8.71	2.087	0.56	14		8.22	1.13		8.71	1.19	
	A			0.230	7.29	2.058	0.53	15	8.22	2.091	0.54	15		8.71	2.087	0.56	14		8.22	1.13		8.71	1.19	
	R			0.134	22.72	6.534	1.69	15	25.28	5.480	1.42	15		26.78	5.465	1.46	14		25.28	1.11		26.78	1.18	
Adrenals (mg)	U			0.307	52.87	7.298	1.88	15	54.32	7.159	1.85	15		54.51	6.849	1.77	15		54.32	1.03		54.51	1.03	
	A			NA	53.00	7.746	2.00	15	54.00	7.746	2.00	15		54.00	7.746	2.00	15		54.00	1.02		54.00	1.02	
	R			0.576	163.50	16.884	4.36	15	168.43	21.197	5.47	15		169.93	21.277	5.49	15		168.43	1.03		169.93	1.04	
Seminal vesicle + coagulating gland, with fluid (mg)	U			0.404	569.44	128.581	38.77	11	485.70	153.704	46.34	11		437.02	77.305	20.66	14		485.70	0.85		437.02	0.77	
	A			0.293	549.00	122.715	37.00	11	481.00	126.032	38.00	11		437.00	115.991	31.00	14		481.00	0.88		437.00	0.80	
Seminal vesicle + coagulating gland, without fluid (mg)	U			0.245	333.36	58.425	15.09	15	293.46	56.369	15.07	14		269.91	47.151	12.60	14		293.46	0.88		269.91	0.81	
	A			0.188	334.00	54.222	14.00	15	294.00	56.125	15.00	14		268.00	56.125	15.00	14		294.00	0.88		268.00	0.80	
Ventral prostate (mg)	U			0.472	238.47	50.050	12.92	15	250.14	55.723	14.39	15		217.93	40.527	10.46	15		250.14	1.05		217.93	0.91	
	A			0.454	236.00	50.349	13.00	15	251.00	50.349	13.00	15		217.00	50.349	13.00	15		251.00	1.06		217.00	0.92	
Dorsolateral prostate (mg)	U			0.057	178.59	46.906	13.54	12	164.31	23.594	6.31	14		134.85	23.325	6.02	15		164.31	0.92		134.85	0.76	
	A			0.078	180.00	34.641	10.00	12	165.00	33.675	9.00	14		135.00	30.984	8.00	15		165.00	0.92		135.00	0.75	
LABC (mg)	U			0.500	600.15	62.959	16.26	15	569.17	77.914	20.12	15		533.55	51.873	13.39	15		569.17	0.95		533.55	0.89	
	A			0.449	595.00	61.968	16.00	15	571.00	58.095	15.00	15		532.00	58.095	15.00	15		571.00	0.96		532.00	0.89	
Epididymis (left, mg)	U			0.107	244.11	32.933	8.50	15	246.30	29.417	7.60	15		185.54	25.651	6.62	15		246.30	1.01		185.54	0.76	
	A			0.109	240.00	30.984	8.00	15	247.00	27.111	7.00	15		185.00	27.111	7.00	15		247.00	1.03		185.00	0.77	
Epididymis (right, mg)	U*		Tukey	0.014	241.70	30.058	7.76	15	242.16	37.890	9.78	15		181.25	20.466	5.28	15	<0.001	242.16	1.00		181.25	0.75	
	A*		Sidak	0.030	238.00	30.984	8.00	15	243.00	30.984	8.00	15		182.00	30.984	8.00	15	<0.001	243.00	1.02		182.00	0.76	
Testis (left, mg)	U*		Tukey	0.008	1442.01	96.928	25.03	15	1013.13	173.206	44.72	15	<0.001	512.46	101.848	26.30	15	<0.001	1013.13	0.70		512.46	0.36	
	A*		Sidak	0.007	1446.00	123.935	32.00	15	1005.00	123.935	32.00	15	<0.001	509.00	120.062	31.00	15	<0.001	1005.00	0.70		509.00	0.35	
Testis (right, mg)	U*		Tukey	0.004	1446.75	104.884	27.08	15	1005.70	176.481	45.57	15	<0.001	509.93	91.295	23.57	15	<0.001	1005.70	0.70		509.93	0.35	
	A*		Sidak	0.004	1452.00	131.681	34.00	15	999.00	127.808	33.00	15	<0.001	506.00	127.808	33.00	15	<0.001	999.00	0.69		506.00	0.35	
Thyroid (mg)	U			0.635	18.494	4.1764	1.08	15	20.74	5.571	1.44	15		19.632	3.9405	1.02	15		20.74	1.12		19.632	1.06	
	A			0.719	18.925	4.5236	1.17	15	20.48	4.415	1.14	15		19.588	4.4036	1.14	15		20.48	1.08		19.588	1.04	

Appendix L

Histopathology Report

Pathology report for
0FP3-95-12-02-01
Pubertal Development and Thyroid Function in Intact Juvenile Rats Exposed to NTO

23 July 2013

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2. INTRODUCTION

In an initiative by the Department of Defense (DOD) to improve munitions safety, the US Army is developing insensitive munitions (IM) for incorporation into its inventory of conventional military munitions systems. 3-Nitro-1,2,4-triazol-5-one (NTO) is being investigated as a less sensitive direct replacement for traditional explosives such as TNT and RDX. NTO is a crystalline powder that is one of the components used in the formulation of an insensitive explosive referred to as IMX101. To support possible fielding of these PAX explosives, a Toxicity Clearance would have to be granted and occupational exposure guidelines developed. Consequently, toxicity data in a mammalian system need to be generated to assess occupational health hazards associated with the use and production of this material.

To assess the endocrine disrupting potential of NTO, two screening assays were conducted using juvenile male and female rats. Fifteen rats per sex were exposed to NTO in corn oil via oral gavage from post natal day (PND) 22/23 through a period approximating normal pubertal development (females: PND 42-43; males: PND 53-54). The treatment groups were control, 500mg/kg-day and 1000mg/kg-day for females and control, 250mg/kg-day and 500mg/kg-day for males. Females were examined daily (starting on PND22) for vaginal opening and, beginning at vaginal opening, vaginal smears were collected to monitor estrous cyclicity. The purpose of these assays is to determine whether NTO has the potential to interact with the endocrine system *in vivo* by identifying effects on pubertal development and thyroid function in the intact juvenile rat. These assays can detect anti-thyroid, estrogenic, anti-estrogenic, androgenic, and anti-androgenic activity as well as agents acting via gonadotropins, prolactin or hypothalamic function.

3. METHODS

Vaginal lavage cell suspensions were expelled onto a labeled glass slides and were cover-slipped. Cells were examined wet with no staining, under 10X and 20X power, by light micropscopy. Stage of the estrus cycle was determined and recorded daily as diestrus, proestus, or estrus based on the preponderance of cell types present (i.e. leukocytes, cornified epithelial cells, nucleated epithelial cells), see Table 5 (Marcondes et al. 2002; OECD 2009).

Necropsies were performed at US Army Public Health Command (USAPHC), Army Institute of Public Health, Portfolio of Toxicology (TOX). A full, detailed gross necropsy included careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. All gross pathology changes were recorded on CHPPM Form 333 or 23. At necropsy, the ovaries (without oviducts), uterus, testes, epididymides, ventral prostate, dorsolateral prostate, seminal vesicle with coagulating glands and fluid, levator ani plus bulbocavernosus muscles, thyroid (with attached portion of trachea), liver, kidneys, pituitary, and adrenals were removed and weighed. Additionally, the fluid was removed from uterus and seminal vesicle with coagulating glands and the tissues were re-weighed (blotted weight). The Thyroid gland was left in place with the trachea and trimmed and weighed after fixation.

The thyroid from one female (1000 mg/kg-day) was not located during necropsy. Any observed lesions were removed for processing. Sections of the intestines were removed based on observations of pale intestines at necropsy.

The kidneys, ovaries, uterus, and thyroid (with attached trachea) were placed in 10% buffered formalin for at least 24 hours for fixation. The thyroid (with parathyroids) was then dissected from the trachea, blotted and weighed to the nearest 0.01 mg. The left testis and left epididymis from each animal was placed in Bouin's fixative overnight (no longer than 24 hours). After fixation, tissues were rinsed and stored in 70% ethanol until embedded in paraffin.

All tissues were selectively trimmed and placed in cassettes labeled with protocol number, animal identification number and laboratory assigned accession number. Cassettes were placed in labeled, formalin or 70% ethanol filled containers, as specified, and transported to the US Army Institute of Chemical Defense (USAMRICD) for processing. Tissues were routinely processed, paraffin embedded, microtomed at 5*u*m and stained routinely with hematoxylin and eosin. For each testis and epididymis evaluated, two step sections, 5um apart were mounted per slide.
The thyroid gland was paraffin embedded and a minimum of two sections, per lobe, 5um apart, were placed per slide. Thyroid sections were subjectively evaluated for follicular cell height and colloid area using a five point grading scale (1 = shortest/smallest; 5 = tallest/largest) and any abnormalities/lesions noted. A minimum of two sections of each of the two lobes of the thyroid was evaluated, a representative thyroid score was determined per animal based on the evaluation of all serial sections.

Ovarian histology following hematoxylin and eosin (H&E) staining included an evaluation of follicular development (including presence/absence of tertiary/antral follicles, presence/absence of corpora lutea, changes in corpus luteum development, changes in number of both primary and atretic follicles) in addition to any abnormalities/lesions, such as ovarian atrophy. Five random sections were evaluated using the method of Smith, BJ et al. (1991). The ovary was paraffin embedded and 5 sections, 5µm apart were placed on a slide. Five slides per ovary were generated, equating 25 sections of ovary available for evaluation. Ovarian slides were indicated as slides 2-1, 2-2, 2-3, 2-4 and 2-5. Sections were identified from left, the slide label, to right as slide 1: 1-5, slide 2: 6-10, slide 3: 11-15, slide 4: 16-20, slide 5: 21-25. For each slide a random number was generated identifying the ovarian section for evaluation. For example, slide 1, a random number between 1 and 5 was generated, for slide 2, a random number between 6-10 was generated and so on. A black fine point marker was used to make a mark above the ovary selected for evaluation. Random numbers were generated for all slides prior to evaluation and recorded in Table 5. Follicles were counted and recorded per examined ovary section as primordial, growing, antral or atretic per method of Smith, BJ et al. (1991) and as referenced by Pedersen et al. (1968).

Vagina was collected at necropsy. Estrus staging was determined at time of death through evaluation of the vagina, uterus and ovary. Histologic stage was determined for each organ and recorded in Table 7.

The pathologist examined slides for compound-induced histopathologic changes via light microscopy. The prevalence and severity of findings were graded as compared to controls. Control animals were examined for background findings, all findings were recorded. Findings, in all animals and groups, were assigned as none, minimal, mild, moderate or severe. Testis, epididymis, uterus, thyroid, one ovary, and one kidney were evaluated for pathologic abnormalities and potential treatment-related effects for all treated and control animals.

4. RESULTS

4.1 Gross necropsy observations

Pale small intestines was observed grossly in both male and female rats. In females it was observed in 10/15 controls, 15/15, 500 mg/kg rats and 8/15, 1000 mg/kg rats. In males, grossly it was observed in 4/15 controls, 3/15, 250 mg/kg rats and 2/15 500 mg/kg rats. Intestines were only examined microscopically in the females, 8 in the control group, 8 in the 500mg/kg group and 7 in the 100mg/kg group. Although not confirmed microscopically, this observation was likely due to oral corn oil administration. This finding is not compound treatment related.

Congenital hydronephrosis (pelvic dilatation) occurs at a low incidence in the F344 rat. A genetic basis for hydronephrosis has also been shown for other rat strains. Hydronephrosis is more often unilateral, affecting the right side. (Montgomery 1990). In female rats, microscopically, mild to moderate pelvic dilatation was observed in 0/15 control, 3/15 500 mg/kg rats and 1/15 1000 mg/kg rats. In male rats, moderate to severe pelvic dilatation was observed in 0/15 control, 3/15 250 mg/kg rats, and 1/15 500 mg/kg rats. Of the 8 observed, in both males and females, 6/8 were right sided. Since this lesion exhibited itself similarly in control versus treated animals, it was not considered treatment related.

4.2 Microscopic findings

4.2.1 Thyroid

Ultimobranchial cysts were noted in six males (three 250 mg/kg-day and five 500 mg/kg-day) and five females (one control, three 500 mg/kg-day and one 1000 mg/kg-day). Ultimobranchial cysts are considered a common background finding and unrelated to treatmeant. No other histologic findings were appreciated. Generally, the incidence of thyroid codes of control and 250 mg/kg males and control and 500 mg/kg females were equivalent across groups. In females, mean follicle height score was 1.9, 2.3, and 2.1 in control, 500, and 1000 mg/kg-day groups. In males, mean follicle height score was 2.4, 2.3, and 2.3 in control, 250 and 500 mg/kg-day groups. Colloid scores were 3.9, 3.5, and 3.6 and 3.5, 3.7, and 3.9 in females and males, respectively, in the control, low and high dose groups. Neither follicle nor colloid score differed between

treated and control groups for males or females. A follicle score of one and colloid score of five is defined as normal in the scoring scheme (EPA 2009). See Tables 3 and 4 for thyroid coding.

4.2.2 Male reproductive

Predominantly in the 250 mg/kg group, Sertoli cells, spermatogonia, preleptotene, leptotene, zygotene and early pachytene spermatocytes were present and intact. Late pachytene and diplotene spermatocytes were often degenerative. Round spermatids 1-6 were generally present and intact. Elongating spermatids 7-10 were degenerative or necrotic while spermatids 11-19 were absent.

Predominantly, in the 500 mg/kg males with severe tubular atrophy, only Sertoli cells and spermatogonia remained, all other germ cells were absent. In marked tubular atrophy, Sertoli cells and spermatogonia were consistently present with most other first layer germ cells, preleptotene, leptotene and zygotene spermatocytes. Second layer pachytene spermatocytes, stages I-VI, were present but disassociated within lumen and often degenerate or necrotic.

Table 1. Incidence and severity of Testicular lesions

Dose group	Control	250 mg/kg	500 mg/kg
Normal	15/15		
Minimal tubular degeneration/atrophy			
Mild tubular degeneration/atrophy		3/15	
Moderate tubular degeneration/atrophy		12/15	
Marked tubular degeneration/atrophy			7/15
Severe tubular degeneration/atrophy			8/15

The endocytic activity of clear cells is greater than that of any other cell type in the epididymis, and is particularly active in the cauda. These cells are responsible for the uptake of a number of different proteins excreted by the epididymal epithelium, as well as the contents of the cytoplasmic droplet (Joseph A 2011). Clear cell quantity was evaluated and considered consistent across controls and all treated groups. There was no absence of clear cells.

Moderate hypospermia is defined as an absence of mature spermatids in the head and body of the epididymis with mature spermatids evident in the tail section. Mature spermatids were absent in all portions of the epididymis in severe hypospermia/aspermia animals.

Table 2. Incidence and severity of Epididymal lesions			
Dose group	Control	250 mg/kg	500 mg/kg
Normal	15/15		
Mild hypospermia			
Moderate hypospermia			
Severe hypospermia/aspermia		15/15	15/15

4.2.3 Female reproductive

Histology of vagina, uterus and ovary at necropsy were, generally, correlated with the final cytological swab just prior to necropsy. No true uterine hypertrophy or hypotrophy was noted histologically, all instances of increased or decreased luminal diameter correlated with estrus stage. Myometrial, stromal and endometrial gland development appeared normal. All organ reproductive stages at necropsy correlated with the vaginal cytologic stage at necropsy. See ovarian follicle counts, Table 6.

4.2.4 Kidney

All renal findings were considered to be background findings unrelated to treatment.

5. DISCUSSION

Initial responses by follicular cells to TSH are formation of pseudopodia, resulting in increased endocytosis of colloid and the release of preformed hormone stored within the follicular lumen. If the secretion of TSH is sustained for hours or days, thyroid follicular cells become more columnar and follicular lumens become smaller, due to the increased endocytosis of colloid. Conversely, in response to an increase in circulating levels of thyroid hormones, there is a corresponding decrease in circulating pituitary TSH. Thyroid follicles become enlarged and distended with colloid, due to the decreased TSH-mediated endocytosis of colloid. Follicular cells lining the involuted follicles are low cuboidal and have few endocytic vacuoles at the interface with the colloid (Haschek 2010). In the scoring scheme employed in this study, a follicle score of one and colloid score of five are considered normal. With increased TSH; follicular cells would be more columnar, in the F4-F5 range with decreased colloid in the C1-C2 range. The inverse is true with decreased TSH, follicular cells should be shorter, F1-F2 with increased colloid, C4-C5. Rats in this study had slightly increased follicle scores and decreased colloid scores, indicating more columnar follicles and smaller follicular lumens. The treated rats, however, did not differ from the control rats.

Testicular toxicants can target multiple sites within the male reproductive system. Toxicants can act directly on testicular blood supply and cells (leydig cells, sertoli cells, spermatogonia, spermatocytes, spertmatids, spermatozoa) or at extratesticular sites (hypothalamus-pituitary axis and central nervous system) resulting in direct damage to those cells or those cells they physiologically support.

Repetitive and prolonged dosing, regardless of the mechanism of toxicity, will result in germ cell damage and loss. Germ cells are affected because they are dependent on the function and processes of other cell types within the testis; a disruption of the germ cell supporting environment often results in their death (Creasy 1997). Since progressive germ cell loss occurs throughout a repeat dose, long term study, the end result is often seminiferous tubules lined only by Sertoli cells. Even though Sertoli cells are sensitive to alterations in function, they are extremely resistant to cell death (Creasy 2001).

In the 250 mg/kg male group, the initial set of cells with degenerative changes was late pachytene spermatocytes. With progressive maturation there was degeneration and loss of subsequent developmental stages. There was complete absence of elongating spermatids. In the 500 mg/kg group, the higher dosage resulted in more significant damage, with necrosis or loss of all cells developing past first layer spermatogonia, preleptotene, leptotene and zygotene spermatocytes.

If it is necessary to elucidate the target cell, a time course study should be performed in order to identify the earliest stage of pathologic change. Preliminary time-course studies can consist of 1,3,7,14, etc days with a small number of animals, first observation of histologic findings are noted. The most interesting time period then should be chosen for an in-depth analysis (Creasy 1997).

Paraffin tissue blocks and slides will be archived at completion of this report.

6. HISTOPATHOLOGY DATA

6.1 MALES

CONTROL GROUP

12-0178

1. Duodenum; thyroid gland; epididymis, left; testis, left: No significant findings.
2. Kidney, left: Infiltrates, lymphocytic, interstitial, multifocal, minimal.

Thyroid score: F2C3

Gross necropsy findings: Pale small intestines

12-0184

1. Thyroid gland; epididymis; testis; kidney, left: No significant findings.
2. Epididymis, left, tail: Microvacuolated cells, multifocal, moderate.

Thyroid score: F3C3

Gross necropsy findings: Some bedding in stomach.

12-0193

1. Thyroid gland; epididymis, left; testis, left; kidney, left: No significant findings.

Thyroid score: F3C3

Gross necropsy findings: Pale intestines. Bedding in stomach.

12-0213

1. Thyroid gland; epididymis, left; testis, left; kidney, left: No significant findings.

Thyroid score: F2C4

Gross necropsy findings: Pale intestines. Bedding in stomach.

12-0233

1. Thyroid gland; epididymis, left; testis, left: No significant findings.
2. Kidney, left, medulla: Degeneration and apoptosis, interstitium, focal, mild

Thyroid code: F3C3

Gross necropsy findings: No gross lesions noted.

12-0247

1. Thyroid gland: Ultimobranchial cyst, focal.
2. Epididymis, left; testis, left; kidney, left: No significant findings.

Thyroid code: F2C4

Gross necropsy findings: No gross lesions noted.

12-0266

1. Thyroid gland; epididymis, left; testis, left; kidney, left: No significant findings.

Thyroid code F2C3

Gross necropsy findings: Pale intestines. Bedding in stomach.

12-0273

1. Epididymis, left: Infiltrates, lymphocytic, interstitial, focal, minimal.
2. Kidney, left: Infiltrates, lymphocytic, interstitial, multifocal, minimal.

3. Thyroid gland; testis, left: No significant findings.
Thyroid code: F2C4

Gross necropsy findings: Bedding in stomach.

12-0285
1. Kidney, left: Infiltrates, lymphocytic, interstitital, focal, minimal.
2. Thyroid gland; testis, left; epididymis, left: No significant findings.
Thyroid code: F2C4

Gross necropsy findings: Bedding in stomach.

12-0315
1. Kidney: Infiltrates, lymphocytic, interstitial, multifocal, minimal.
2. Thyroid gland; testis; epididymis: No significant findings.
3. Epididymis, body, left: Macrovacuoles, tubular epithelium, multifocal, minimal.
Thyroid code: F2C4

Gross necropsy findings: Bedding in stomach.

12-0320
1. Lung, alveoli: Extravasated red blood cells, multifocal, moderate.
2. Thyroid gland; testis, left; kidney, left: No significant findings.
Thyroid code: F3C3
3. Epididymis, body, left: Macrovacuoles, tubular epithelium, multifocal, minimal.

Gross necropsy findings: Lungs diffusely mottled red. Bedding in stomach.

12-0330
1. Lung, alveoli: Extravasated red blood cells, multifocal, moderate.
2. Thyroid gland; testis left; epididymis, left; kidney, left: No significant findings.
Thyroid code: F2C4

Gross necropsy findings: Red mottled lungs, diffuse.

12-0342
1. Thyroid gland: Ultimobranchial cyst.
3. Kidney: Infiltrates, lymphocytic, interstitial, multifocal, mild with basophilic tubules and cyst.
2. Epididymis, left; testis, left: No significant findings.
Thyroid code: F2C5

Gross necropsy findings: Bedding in stomach. Left kidney 1mm diameter cyst.

12-0352
1. Thyroid gland; epididymis, left; testis, left; kidney, left: No significant findings
Thyroid code: F3C3

Gross necropsy findings: Right side, seminal vesicle small.

12-0362
1. Epididymis, isthmus, left: Infiltrates, monoclonal, interstitial, focal, mild.
2. Thyroid gland; testis, left; kidney, left: No significant finding.
Thyroid code: F3C3

Gross necropsy findings: No gross lesions noted.

250 mg/kg GROUP

12-0232

1. Thyroid gland: Ultimobranchial cyst, focal.
2. Kidney, left: Basophilic tubules, focal, minimal with interstitial mononuclear infiltrates and glomerular amyloidosis.
3. Testis, left: Degeneration/atrophy, tubular, diffuse, moderate with multinucleated giant cells.
4. Epididymis, left: Aspermia, diffuse, severe with intralumenal degenerate germ cells and multinucleated giant cells.
5. Epididymis, head: Dilatation, tubular with interstitial clear space and minimal lymphocytic infiltrates.

Thyroid code: F2C3

Gross necropsy findings: Bedding in stomach

12-0246

1. Epididymis, left: Aspermia, diffuse, severe with intralumenal degenerate germ cells.
2. Epididymis, head, left: Tubular dilatation, diffuse, mild with minimal interstitial clear space.
3. Kidney, left: Infiltrates, mononuclear, interstitial multifocal, mild with minimal basophilic tubules.
4. Testis: Degeneration, tubular, diffuse, moderate with diffuse severe aspermia.
5. Thyroid gland: No significant findings.

Thyroid code: F2C4

Gross necropsy findings: No gross lesions noted.

12-0265

1. Kidney, left: Infiltrates, mononuclear, interstitial, multifocal, minimal.
2. Thyroid gland: No significant findings.
3. Epididymis: Tubular dilatation, diffuse, moderate with diffuse aspermia, severe with intralumenal degenerate germ cells.
4. Epididymis, body-tail junction, left: Cribiform change, diffuse, moderate.
5. Testis: Degeneration, tubular, diffuse, mild.

Thyroid code: F3C3

Gross necropsy findings: No gross lesions noted.

12-0276

1. Kidney, right: Pelvic dilatation, moderate with medullary tubular atrophy.
2. Kidney, left: Infiltrates, mononuclear, interstitial, focal, minimal.
3. Epididymis, left: Tubular dilatation, head and body, moderate with severe intralumenal debris, degenerate cells and diffuse severe aspermia.
4. Testis: Degeneration, diffuse, moderate
5. Thyroid gland: No significant findings.

Thyroid code: F3C3

Gross necropsy findings: Small testes. Pale intestines. Hydronephrosis, right kidney

12-0286

1. Jejunum, goblet cell: Hypertrophy, diffuse, moderate.

2. Epididymis, left: Tubular dilatation, head and body, moderate with intralumenal debris , degenerate cells and diffuse severe aspermia.
3. Testis, left: Degeneration, tubular, diffuse, moderate with multinucleated giant cells.
4. Epididymis, body, left: Cribiform change, diffuse, moderate.
5. Kidney, left and thyroid gland: No significant findings.
Thyroid code: F2C4

Gross necropsy findings: Bedding in stomach.

12-0325
1. Kidney, right: Pelvic dilatation, moderate with medullary tubular atrophy.
2. Epididymis, left: Tubular dilatation, caput and body, moderate with intralumenal debris, degenerate cells and diffuse severe aspermia.
3. Testis, left: Tubular degeneration, diffuse, moderate.
4. Thyroid gland: No significant findings.
Thyroid code: F2C4

Gross necropsy findings: Left thyroid small. Small testes. Right kidney hydronephrosis.

12-0312
1. Kidney, left; thyroid gland: No significant findings.
2. Epididymis, left: Tubular dilatation, diffuse, moderate with intralumenal debris and diffuse severe aspermia.
3. Testis, left: Tubular degeneration/atrophy, diffuse, moderate with multinucleated giant cells.
Thyroid code: F2C4

Gross necropsy findings: Small testes.

12-0334
1. Kidney, right: Pelvic dilatation, moderate with medullary tubular atrophy.
2. Ileum, peyers patches: Hyperplasia, diffuse, mild.
3. Kidney, left; thyroid gland: No significant findings.
4. Epididymis: Tubular dilatation, diffuse, moderate with intralumenal debris/degenerate cells and diffuse severe aspermia.
5. Epididymis, left: Cribiform change, body, minimal.
6. Testis: Tubular degeneration, diffuse, moderate with multinucleated giant cells.
Thyroid code: F2C4

Gross necropsy findings: Small left side of seminal vesicle. Pale intestines. Bedding in stomach. Right kidney hydronephrosis.

12-0344
1. Kidney, left: No significant findings.
2. Thyroid gland: Ultimobranchial cyst, focal.
3. Epididymis, left: Tubular dilatation, diffuse, moderate with intralumenal debris/degenerate cells and diffuse severe aspermia.
4. Epididymis, left: Cribiform change, body, mild.
5. Testis, left: Tubular degeneration, diffuse, moderate with multinucleated giant cells
Thyroid code: F2C4

Gross necropsy findings: No gross lesions noted.

12-0353
1. Kidney, left: Basophilic tubules, focal, minimal.
2. Kidney, left: Infiltrates, mononuclear, interstitial, multifocal, minimal.
3. Epididymis, left: Tubular dilatation, diffuse, moderate with intralumenal debris, degenerate cells and diffuse, severe aspermia.
4. Testis: Tubular degeneration, diffuse, moderate with multinucleated giant cells.
Thyroid code: F3C3

Gross necropsy findings: Small testes.

12-0361
1. Kidney, left: Basophilic tubules, multifocal, minimal
2. Epididymis, left: Tubular dilatation, head and body, moderate with intralumenal debris, eosinophilic amorphous material, degenerate germ cells and diffuse severe aspermia.
3. Testis, left: Tubular degeneration, diffuse, moderate with few multinucleated giant cells.
4. Thyroid gland: No significant findings.
Thyroid code: F4C2

Gross necropsy findings: Small testes.

12-0173
1. Kidney, left: Basophilic tubules, focal, minimal.
2. Thyroid gland: Ultimobranchial cyst.
3. Epididymis, left: Dilatation, diffuse, moderate with moderate intralumenal cellular debris and diffuse, severe aspermia.
4. Testis, left: Degeneration, mild, multifocal.
Thyroid code: F2C3

Gross necropsy findings: Bedding in stomach.

12-0188
1. Kidney, left: Basophilic tubules, focal, minimal.
2. Thyroid gland: No significant findings.
3. Epididymis, left: Dilatation, diffuse, moderate with moderate intralumenal cellular debris and diffuse, severe aspermia.
4. Testis, left: Degeneration, mild, multifocal.
Thyroid code: F2C4

Gross necropsy findings: Bedding in stomach.

12-0195
1. Kidney, left; thyroid gland: No significant findings.
2. Epididymis, left: Dilatation, diffuse, moderate with moderate intralumenal cellular debris and severe aspermia.
3. Testis, left: Tubular degeneration, multifocal, moderate.
Thyroid code: F2C5

Gross necropsy findings: Small testes. Pale intestines.

12-0214
1. Kidney, left: Infiltrates, mononuclear, interstitial, multifocal, minimal with minimal basophilic tubules.

2. Epididymis, left: Dilatation, diffuse, moderate with moderate intralumenal cellular debris and diffuse, severe aspermia.
3. Testis, left: Degeneration, diffuse, moderate with multinucleated giant cells
4. Thyroid gland: No significant findings.

Thyroid code: F1C5

Gross necropsy findings: No gross lesions noted.

500 mg/kg GROUP

12-0175

1. Epididymis, left: Dilatation, diffuse, moderate.
2. Epididymis, left: Aspermia, diffuse, severe.
3. Epididymis, left: Cribiform change, body, mild.
4. Kidney, left: Infiltrates, mononuclear, interstitial, focal, mild with basophilic tubules and fibroplasia.
5. Testis, left: Tubular atrophy, diffuse, severe.
6. Thyroid gland: No significant findings.

Thyroid score: F2C4

Gross necropsy findings: Small testes, small seminal vesicle. Bedding in stomach

12-0183

1. Kidney; thyroid gland: No significant findings.
2. Epididymis, head: Dilatation, diffuse, moderate.
3. Epididymis, body: Cribiform change, mild.
4. Epididymis, left: Aspermia, diffuse, severe.
5. Testis, left: Tubular atrophy, diffuse, severe.

Thyroid score: F2C4

Gross necropsy findings: Small testes.

12-0196

1. Kidney, right: Pelvic dilatation, severe with medullary tubular atrophy.
2. Kidney, left; thyroid gland: No significant findings.
3. Epididymis, left: Tubular dilatation, head and body, moderate, diffuse.
4. Epididymis, left: Aspermia, diffuse, severe.
5. Testis, left: Tubular degeneration/atrophy, diffuse, marked, with increased interstitial clear space and proteinaceous fluid.

Thyroid code: F3C4

Gross necropsy findings: Small testes. Pale intestines. Right kidney hydronephrosis.

12-0216

1. Kidney, left: Basophilic tubules, multifocal, minimal with interstitial mononuclear infiltrates.
2. Epididymis, body, left: Increased clear cells, multifocal, minimal.
3. Epididymis, head and body, left: Tubular dilatation, diffuse, moderate with interstitial clear space and diffuse, severe aspermia.
4. Epididymis, body, left: Cribiform change, diffuse, minimal.
5. Testis, left: Tubular degeneration/atrophy, diffuse, marked with occasional multinucleated giant cells.
6. Thyroid gland: No significant findings.

Thyroid code: F3C4

Gross necropsy findings: Small testes. Bedding in stomach.

12-0237
1. Kidney, left: Basophilic tubules, focal, minimal with fibrosis.
2. Thyroid gland: Ultimobranchial cyst.
3. Epididymis, head and body, left: Tubular dilatation, diffuse, moderate with diffuse severe aspermia.
4. Testis, left: Tubular degeneration/atrophy, diffuse, marked.
5. Epididiymis, body, left: Cribiform change, diffuse, mild.

Thyroid code: F2C4

Gross necropsy findings: Small testes.

12-0242
1. Kidney, left: infiltrates, mononuclear, interstitial, multifocal, mild with rare basophilic tubules.
2. Testis, left: Tubular atrophy, diffuse, severe.
3. Epididymis, left: Tubular dilatation, diffuse, moderate with diffuse severe aspermia and increased interstitial clear space, head and tail.
4. Epididymis, body: Cribiform change, diffuse, mild.
5. Thyroid gland: No significant findings

Thyroid code: F2C3

Gross necropsy findings: Small testes.

12-0264
1. Kidney, left: Basophilic tubules, multifocal, minimal.
2. Thyroid gland: No significant findings.
3. Testis, left: Tubular degeneration, diffuse, marked with occasional multinucleated giant cells.
4. Epididymis, left: Tubular dilatation, focally extensive, moderate with diffuse, severe aspermia.
5. Epididymis, body, left: Cribiform change, diffuse, mild.

Thyroid code: F2C4

Gross necropsy findings: Small testes.

12-0274
1. Kidney, left: Basophilic tubules, multifocal, minimal.
2. Thyroid gland: No significant findings.
3. Testis, left: Tubular degeneration/atrophy, diffuse, marked.
4. Epididymis, head, left: Tubular dilatation, diffuse, moderate with diffuse severe aspermia.
5. Epididymis, body, left: Cribiform change, diffuse, mild.

Thyroid code: F2C4

Gross necropsy findings: Small testes. Bedding in stomach.

12-0282
1. Kidney, left; thyroid gland: No significant findings.
2. Lung: Extravasated red blood cells, multifocal, moderate.
3. Testis: Atrophy, diffuse, severe.
4. Epididymis, head, left: Tubular dilatation, moderate with diffuse severe aspermia.
5. Epididymis, body, left: Cribiform change, diffuse, mild.

Thyroid code: F3C3

Gross necropsy findings: Diffusely red mottled lungs. Small testes.

12-0311
1. Kidney, left: No significant findings.
2. Thyroid gland: Ultimobranchial cyst.
3. Epididymis, head, left: Tubular dilatation, moderate with diffuse severe aspermia.
4. Testis: Tubular degeneration/atrophy, diffuse, marked.
5. Epididymis, body, left: Cribiform change, diffuse, mild.

Thyroid code: F2C5

Gross necropsy findings: Small testes.

12-0321
1. Kidney, left: Basophilic tubules, multifocal, minimal.
2. Small intestine; thyroid gland: No significant findings.
3. Testis: Tubular degeneration/atrophy, diffuse, severe
4. Epididymis, head, left: Tubular dilatation with diffuse, severe aspermia.
5. Epididymis, body, left: Cribiform change, diffuse, mild.

Thyroid code: F3C3

Gross necropsy findings: Small testes. Pale intestines. Bedding in stomach.

12-0331
1. Kidney, left: Infiltrates, mononuclear, interstitial, focal, minimal with basophilic tubules.
2. Thyroid gland: No significant findings.
3. Epididymis: Tubular dilatation, caput, with diffuse severe aspermia.
4. Testis: Tubular atrophy, diffuse, severe.
5. Epididymis, body, left: Cribiform change, diffuse, mild.

Thyroid code: F3C3

Gross necropsy findings: Small testes. Bedding in stomach.

12-0343
1. Kidney, left: Infiltrates, mononuclear, interstitial, multifocal, mild with minimal basophilic tubules.
2. Thyroid gland: No significant findings.
3. Epididymis, head, left: Tubular dilatation, moderate with diffuse severe aspermia.
4. Testis: Tubular degeneration, diffuse, severe.
5. Epididymis, body, left: Cribiform change, diffuse, mild.

Thyroid code: F1C5

Gross necropsy findings: Small testes.

12-0356
1. Kidney, left: Infiltrates, mononuclear, interstitial, multifocal, minimal.
2. Thyroid gland: Ultimobranchial cyst.
3. Epididymis, head, left: Tubular dilatation, moderate with diffuse severe aspermia.
4. Testis: Tubular degeneration, diffuse, marked.
5. Epididymis, body, left: Cribiform change, diffuse, mild.

Thyroid code: F3C4

Gross necropsy findings: Small testes. Bedding in stomach.

12-0364

1. Kidney, left: Infiltrates, mononuclear, interstitial, multifocal, minimal.
2. Thyroid gland: No significant findings.
3. Epididymis, head, left: Tubular dilatation, moderate with diffuse severe aspermia.
4. Testis: Degeneration/atrophy, diffuse, severe.
5. Epididymis, body, left: Cribiform change, diffuse, mild.

Thyroid code: F2C4

Gross necropsy findings: Small testes. Bedding in stomach.

6.2 FEMALES

Control

12-0182

1. Left kidney; jejunum: No significant findings.
2. Thyroid gland: Ultimobranchial cyst, focal.
3. Uterus: Cuboidal to columnar epithelium; mitoses present, no degeneration, some inflammatory cell infiltration. Minimal edema (P)
4. Ovary: Large corpora lutea, some central fibrous tissue. (P)
5. Vagina: stratum granulosum, superficial mucoid layer and stratum corneum. Occasional polymorphonuclear cells. (P)

Thyroid code: F1C4

Gross findings: Pale small intestines

12-0192

1. Thyroid gland; left kidney; jejunum; liver: No significant findings.
2. Uterus: Degenerate endometrial epithelium. Mitoses present (mitoses and degeneration together) (M)
3. Ovary: Corpora lutea with basophilic cell cytoplasm. corpora lutea with central fluid filled cavity; central degeneration; no fibrous tissue (M)
4. Vagina: Virtually complete detachment of cornified layer; desquamation with loss of stratum granulosum and upper germinativum. Leukocyte infiltration (M)

Thyroid code:F1C5

Gross findings: Pale small intestine; pale liver.

12-0202

1. Thyroid gland; left kidney; jejunum; liver: No significant findings.
2. Uterus: Cuboidal to columnar epithelium; mitoses present, no degeneration and some inflammatory cell infiltration. (P)
3. Ovary: Minimal corpora luteal degeneration and central fibrosis. (P)
4. Vagina: Stratum granulosum, superficial mucoid layer and stratum corneum. Polymorphonuclear cells proximal vagina.(P)

Thyroid code: F2C4

Gross findings: Fluid filled uterus; pale small intestines; mildly pale liver.

12-0219

1. Thyroid gland; left kidney; jejunum; liver: No significant findings.
2. Uterus: Columnar epithelium, mitoses present, mild epithelial degeneration. (D)
3. Ovary: Large corpora lutea; finely vacuolated. (D)
4. Vagina: Low epithelium, stratum germanitivum only; variable leukocyte infiltration (D)

Thyroid code: F1C5

Gross findings: Pale intestines

12-0238

1. Thyroid gland; left kidney; jejunum: No significant findings.
2. Liver: Glycogen deposition, multifocal, mild.
3. Uterus: Columnar epithelium; no degeneration, mild inflammatory cell infiltration. Lumen dilatation. (P-Late)

4. Ovary: Large corpora lutea, finely vacuolated. (P)
5. Vagina, proximal: Stratum granulosum, superficial mucoid layer and stratum corneum; shedding of superficial mucoid layer. Occasional polymorphonuclear cells. (P-late)
Thyroid code: F2C3

Gross findings: Fluid filled uterus; pale intestines; mildly pale liver.

12-0248
1. Kidney, left: Fibrosis, interstitial, focal, minimal.
2. Thyroid gland: No significant findings.
3. Uterus: epithelial degeneration/necrosis. leukocyte infiltration. Lumen dilatation. (E)
4. Ovary: Degenerate corpora lutea; small corpora lutea with basophilic cell cytoplasm. Central fluid filled cavity; no fibrous tissue (E)
5. Vagina: Superficial muoid and cornified layer shedding; reduced epithelial height; cell debris; leukocyte infiltration (E)
Thyroid code: F2C4

Gross findings: No gross lesions recognized.

12-0268
1. Kidney, right: Mineral, interstitial, focal, minimal.
2. Thyroid gland; left kidney; jejunum: No significant findings.
3. Uterus: columnar epithelium, mitoses, some degeneration and minimal inflammatory cell infiltration. (D)
4. Ovary: Large corpora lutea; finely vacuolated; central fibrous tissue (D)
5. Vagina: Low epithelium; stratum germanitivum and stratum granulosum present. (D)
Thyroid code:F2C4

Gross findings: Pale kidneys; pale small intestine.

12-0280
1. Kidney, left: Basophilic tubules, multifocal, minimal.
2. Thyroid gland: No significant findings.
3. Uterus: Epithelial degeneration with mitosis; leukocyte infiltration. (M)
4. Ovary: large and corpora lutea with minimal degeneration; central fluid filled cavity; no fibrous tissue (M)
5. Vagina: Complete detachment of cornified layer; desquamation with loss of stratum granulosum and upper germinativum. (M)
Thyroid code: F2C4

Gross findings: Slightly pale kidneys.

12-0288
1. Thyroid gland; left kidney; jejunum: No significant findings.
2. Uterus: Columnar epithelium; few mitoses, no degeneration. (P)
3. Ovary: Corpora lutea often degenerate; cytoplasmic vacuoules ; central fibrous tissue (P)
4. Vagina: Early stratum granulosum, superficial mucoid layer and stratum corneum. (P)
Thyroid code: F2C3

Gross findings: Slightly pale small intestines.

12-0319

1. Thyroid gland; left kidney: No significant findings.
2. Uterus: Small, avascular, slit-like lumen. Columnar epithelium; few mitoses; occasional degenerate cells; minimal stromal edema (D)
3. Ovary: Large corpora lutea; finely vacuolated; central fibrous tissue (D)
4. Vagina: Low epithelium (D)

Thyroid code F2C4

Gross findings: No gross lesions noted.

12-0329

1. Thyroid gland; right and left kidney; jejunum: No significant findings.
2. Uterus: Columnar epithelium; mitoses present; no degeneration, mild polymorphonuclear inflammatory cell infiltration. (P)
3. Ovary: Corpora lutea often degenerate; cyoplasmic vacuoules (P)
4. Vagina: Epithelial mitoses.; stratum granulosum, superficial mucoid layer and stratum corneum present. Occasional polymorphonuclear cells. (P)

Thyroid score: F2C4

Gross findings: Slightly fluid filled uterus. Pale small intestines. Slightly pale kidneys.

12-0337

1. Thyroid gland; left kidney; jejunum: No significant findings.
2. Uterus: Tall columnar, mitoses, no degeneration; mild lumenal dilatation. (P-late)
3. Ovary: Corpora lutea often degenerate; cyoplasmic vacuoules (P)
4. Vagina: Stratum granulosum, mucoid layer and stratum corneum present, shedding beginning (P-late)

Thyroid score: F2C3

Gross findings: Mildly fluid filled uterus. Mildly pale small intestine.

12-0350

1. Thyroid gland; right and left kidney; jejunum: No significant findings.
2. Uterus: Columnar epithelium; few mitoses; occasional degenerate cells;minimal stromal edema (D)
3. Ovary: Varied corpora lutea; finely vacuolated; rare central fibrous tissue(D)
4. Vagina: Moderate numbers of polymorphonuclear cells, minimal desquamation present (M-D transition appearance)

Thyroid score: F2C5

Gross findings: Pale small intestine.

12-0357

1. Thyroid gland; left kidney: No significant findings.
2. Uterus: Notable epithelial degeneration/necrosis. Loss of mitotic activity; mild leukocyte infiltration. (E)
3. Ovary: Degenerate corpora lutea; occasional fluid filled, no fibrous tissue. (E)
4. Vagina: Superficial muoid and cornified layer shedding; mild leukocyte infiltration (E)

Thyroid score: F3C3

Gross findings: No gross lesions noted.

12-0368

1. Thyroid gland; left kidney: No significant findings.

2. Uterus: Endometrial epithelial degeneration with mitoses (M)
3. Ovary: Varied sized corpora lutea; some basophilic; fluid filled. (M)
4. Vagina: Moderate leukocyte infiltrate (PMNs); no cornified layer (M)
Thyroid score: F2C4

Gross findings: No gross lesions noted.

500mg/kg

12-0181

1. Thyroid gland; jejunum: No significant findings.
2. Kidney, left: Pelvic dilatation, diffuse, moderate with medullary atrophy.
3. Kidney, left: Cyst, cortical, focal, mild.
4. Kidney, right: Basophilic tubules, focal, minimal.
5. Lymph node, mesenteric: Sinus dilatation, diffuse, mild.
6. Uterus: Columnar epithelium; mitoses, no degeneration and mild inflammatory cell infiltration. Lumen dilatation (late -P)
7. Ovary: Large corpora lutea, often degenerate; central fibrous tissue (P)
8. Vagina: proximal - granulosum, mucoud and corneum present; distal- cornified, granulosum and germinativum only noted, some cornified shedding. (late -P)

Thyroid score: F3C3

Gross findings: Fluid filled uterus. Pale small intestine. Hydronephrosis, left kidney.

12-0190

1. Thyroid gland; left kidney; jejunum: No significant findings.
2. Lymph node, mesenteric: Sinus histiocytosis, diffuse, mild.
3. Uterus: Degenerate endometrial epithelium. Mitoses present. (M)
4. Ovary: Some corpora lutea degeneration; central fluid filled cavity; no fibrous tissue (M)
5. Vagina: Detachment of cornified layer, not complete; desquamation with loss of stratum granulosum and upper germinativum. Leukocyte infiltration. (M)

Thyroid code: F2C3

Gross findings: Mildly pale small intestine.

12-0197

1. Thyroid gland; left kidney; jejunum: No significant findings.
2. Uterus: Columnar epithelium; mitoses present with no degeneration (P)
3. Ovary: Corpora lutea vacuoulated. (P)
4. Vagina: Formation of stratum granulosum, superficial mucoid layer and stratum corneum. (P-early)

Thyroid code: F3C3

Gross findings: Pale small intestine.

12-0217

1. Thyroid gland; left kidney; jejunum: No significant findings.
2. Uterus: Columnar epithelium; mitoses present, no degeneration. Some endometrial and inflammatory cell infiltration. Some lumen dilatation. (P)
3. Ovary: Corpora lutea with central fibrosis and some degeneration (P)
4. Vagina: Superficial cornified layer shedding. Stratum granulosum, mucoid layer and corneal layer at cervix; proximal vaginal shedding of cornified layer (P-E)

Thyroid code: F2C3

Gross findings: Pale small intestine. Fluid filled uterus.

12-0241

1. Left kidney; jejunum: No significant findings.
2. Thyroid gland: Ultimobranchial cyst, focal.
3. Liver: Glycogen deposition, diffuse, mild.
4. Uterus, myometrium: Epithelial cyst.
5. Uterus: Minimal epithelial degeneration; mid columnar, few mitoses. Slit-like lumen.(D)
6. Ovary: Large corpora lutea; finely vacuolated; central fibrous tissue. (D)
7. Vagina: Moderate leukocyte infiltration.(D)

Thyroid code: F2C4

Gross findings: Pale small intestines. Mildly pale liver.

12-0250

1. Thyroid gland; left kidney; jejunum: No significant findings.
2. Uterus: Columnar epithelium; mitoses with no degeneration. Mild lumen dilatation.(P)
3. Ovary: Corpora lutea degenerate; cyoplasmic vacuoules ; central fibrous tissue (P)
4. Vagina: Epithelial mitoses.; formation of stratum granulosum, superficial mucoid layer and stratum corneum (P)

Thyroid code:F2C4

Gross findings: Pale small intestine.

12-0271

1. Thyroid gland; right and left kidney; jejunum: No significant findings.
2. Uterus: Low epithelium, slit lumen, no degenerative cells noted. (D)
3. Ovary: Large corpora lutea; no fibrous connective tissue noted. (D)
4. Vagina: Low epithelium. (D)

Thyroid code:F2C5

Note: Very small section of vagina to evaluate.

Gross findings: Slightly pale kidneys. Small intestines slightly pale.

12-0277

1. Thyroid gland; jejunum: No significant findings.
2. Kidney, left: Basophilic tubules, multifocal, minimal.
3. Uterus: Tall columnar epithelium, mitoses with no degeneration(P).
4. Ovary: Large corpora lutea, finely vacuolated, with some fibrous connective tissue (P).
5. Vagina: Stratum granulosum and mucoid layer (P).

Thyroid code: F2C3

Gross findings: Slightly dilated uterus. Pale small intestine.

12-0290

1. Thyroid gland: Ultimobranchial cyst, focal.
2. Right and left kidney; small intestine: No significant findings.
3. Uterus: Tall columnar epithelium, mitoses, some edema. No degeneration. Mildly dilated lumen. (P)
4. Ovary: Large corpora lutea with some degeneration (P).
5. Vagina: Stratum mucificatioin present (P).

Thyroid code: F2C3

Gross findings: Slightly pale kidneys. Slightly pale small intestine.

12-0318

1. Thyroid gland; jejunum: No significant findings.
2. Kidney, left: Fibrosis, interstitial, focal, mild with basophilic tubules.
3. Kidney, left: Tubular dilatation, focal, moderate.
4. Uterus: Tall columnar epithelium, rare mitoses. Rare to no degenerative cells. Small lumen. (M)
5. Ovary: Large corpora lutea with fine vacuolation. Central fibrous connective tissue. (M-D)
6. Vagina: Numerous neutrophils, minimal sloughed cells in lumen. (M)

Thyroid code: F2C4

Gross findings: Pale small intestine.

12-0326

1. Thyroid gland: Ultimobranchial cyst.
2. Thyroid gland: Follicular dilatation, focal, mild with intraluminal foamy macrophages and neutrophils.
3. Kidney, right: Pelvic dilatation, diffuse, moderate with medullary atrophy.
4. Kidney, medulla, right: Tubular dilatation, focal, with peritubular fibrosis.
5. Kidney, cortex, left: Basophilic tubules, multifocal, with lymphocytic infiltrates.
6. Small intestine: No significant findings.
7. Uterus: Tall columnar epithelium, moderate epithelial degeneration, rare mitoses. (E)
8. Ovary: Medium sized, darker copora lutea, some degeneration and fluid. (E)
9. Vagina: Shedding of cornified layer. (E)

Thyroid code: F2C3

Gross findings: Slightly pale kidneys. Slightly pale small intestine.

12-0338

1. Thyroid gland; jejunum: No significant findings.
2. Kidney, left: Pelvic dilatation, diffuse, mild.
3. Uterus: Small lumen, tall columnar epithelium, mild epithelial degeneration. Some mitoses. (D)
4. Ovary: Moderate sized corpora lutea with fibrous centers. Mild vacuolation and degeneration. (D)
5. Vagina: Thin stratum corneum, moderate number of leukocytes. Some cornified cells in lumen. (D)

Thyroid code: F2C4

Gross findings: Pale small intestine.

12-0349

1. Kidney, left: Cystic tubular dilataion, focal, mild with rare basophilic tubules.
2. Ovary: Small to moderate sized corpora lutea, cystic centers with degeneration and vacuolation. (E)
3. Uterus: Mildly dilated lumen. Tall columnar epithelium with moderate degeneration; leukocyte infiltration. (E)
4. Vagina: Shedding of cornified layer. (E)
5. Thyroid gland; jejunum: No significant findings.

Thyroid code: F3C4

Gross findings: Pale small intestine.

12-0358

1. Thyroid gland; small intestine: No significant findings.
2. Kidney, left: Basophilic tubules, multifocal, minimal.

3. Uterus: Tall columnar epithelium, no degeneration and few mitoses. (M).
4. Ovary: Small corpora lutea. (M)
5. Vagina: Leukocyte infiltration, some squamous cells and leukocytes in lumen. (M).

Thyroid code: F3C3

Gross findings: Pale small intestine.

12-0370

1. Ovary: Medium to large corpora lutea with mild degeneration and vacuolation. (D)
2. Uterus: Slit lumen. Few mitoses, no degeneration and tall columnar epithelium. (D)
3. Vagina: Mild leukocytes. (late-D)
4. Thyroid gland; kidney; jejunum: No significant findings.

Thyroid code: F2C4

Gross findings: Slightly pale intestines.

1000mg/kg GROUP

12-0180

1. Thyroid gland; left kidney; jejunum: No significant findings.
2. Uterus: Mild degeneration, some mitoses. (M)
3. Ovary: Large corpora lutea with central hemorrhagic fluid filled cavity. (M)
4. Vagina: Some leukocytes, no stratum corneum. (M)

Thyroid code:F2C4

Gross findings: No gross lesions noted.

12-0191

1. Thyroid gland: No significant findings.
2. Kidney, medulla, left: Cystic tubular dilatation, focal, mild.
3. Uterus: Tall columnar epithelium, dilatation of lumen. Moderate degeneration, no mitoses noted. (E)
4. Ovary: Large corpora lutea with degeneration. Basophilic corpora lutea noted. (E)
5. Vagina: Stratum corneum present. No mucification noted. (E)

Thyroid code:F3C3

Gross findings: Pale liver

12-0199

1. Thyroid gland; left kidney; small intestine: No significant findings.
2. Liver: Glycogen deposition, diffuse, mild.
3. Uterus: Slit lumen, no degeneration, few mitoses. (D)
4. Ovary: Mild degeneration and vacuolation of corpora lutea. (D)
5. Vagina: Epithelial thickening. (D)

Thyroid code: F2C3

Gross findings: Mildly pale liver. Mildly pale intestine.

12-0218

1. Thyroid gland; left kidney; small intestine: No significant findings.
2. Uterus: Mild degeneration, mild lumen dilatation. (E)
3. Ovary: Fluid filled cavities in corpora lutea. (E)
4. Vagina: Stratum corneum sloughing. (E)

Thyroid code: F3C3

Gross findings: Pale intestines. Bedding throughout intestines.

12-0239

1. Thyroid gland; left kidney; jejunum: No significant findings.
2. Uterus: Mildly dilated lumen, epithelial degeneration, few mitoses. (Late M)
3. Ovary: some vacuolation and central cavitation of corpora lutea. (late M)
4. Vagina: Low epithelium. (late M)

Thyroid code: F1C5

Gross necropsy findings: Bedding in colon. Slightly pale small intestines.

12-0251

1. Thyroid gland; left kidney: No significant findings.
2. Uterus: Tall columnar epithelium with degeneration. (E)
3. Ovary: Degenerate corpora lutea; few basophilic CLs. (E)
4. Vagina: Stratum corneum present, no mucoid layer. (E)

Thyroid code: F2C4

Gross findings: Small amount of bedding in colon.

12-0270

1. Thyroid gland; urinary bladder: No significant findings.
2. Kidney, left: Basophilic tubules, multifocal, mild with lymphocytic infiltrates.
3. Uterus: Mildly dilated lumen, mid-columnar epithelium, minimal mitoses. No degeneration. (P)
4. Ovary: Small to medium corpora lutea. (P).
5. Vagina: Mucin layer shedding. Some stratum corneum shedding. (P).

Thyroid code: F2C4

Gross findings: No gross lesions noted.

12-0281

1. Thyroid gland: No significant findings.
2. Kidney, right: Pelvic dilatation, diffuse, moderate with medullary atrophy.
3. Kidney, right and left: Basophilic tubules, focal, minimal.
4. Uterus: Tall columnar epithelium. Dilated lumen, no degeneration, few mitoses. (P)
5. Ovary: Mild degeneration of corpora lutea. (P)
6. Vagina: Some loss of stratum corneum. No mucin layer. (P)

Thyroid code: F2C3

Gross findings: Dilated uterus. Mildly pale kidneys.

12-0287

1. Thyroid gland; jejunum; heart: No significant findings.
2. Kidney, left: Fibrosis, interstitial, focal, mild with basophilic tubules and mononuclear infiltrates.
3. Lung: Extravascated red blood cells, multifocal, mild.
4. Uterus: Moderate degeneration. Some mitoses. (M)
5. Ovary: Vacuolated corpora lutea varied in size. (M)
6. Vagina: Leukocyte infiltrates. (M)

Thyroid code: F2C4

Gross findings: Pale intestines. Diffusely red mottled lungs.

12-0316
1. Thyroid gland: No significant findings.
2. Kidney, left: Cyst, multifocal with lymphocytic infiltrates.
3. Uterus: Mid to tall columnar epithelium. Moderate mitoses. Minimal degeneration. (D)
4. Ovary: Corpora lutea mildly vacuolated. Central clearing with fibrosis. (D)
5. Vagina: Low epithelium. Moderate leukocyte infiltrates. (D)

Thyroid code: F2C4

Gross findings: Yellow staining around mouth.

12-0328
1. Thyroid gland; jejunum: No significant findings.
2. Kidney, left: Basophilic tubules, focal, minimal with lymphocytic infiltrates.
3. Lymph node, mesenteric: Sinus dilatation, diffuse, mild.
4. Uterus: Slit lumen, few mitoses. Rare epithelial degeneration. (D)
5. Ovary: Large corpora lutea with central fibrous tissue. (D)
6. Vagina: Mild to moderate leukocyte infiltrates. Thick epithelium. (D)

Thyroid code: F2C4

Gross findings: Pale small intestine.

12-0335
1. Thyroid gland; jejunum: No significant findings.
2. Kidney, left: Cystic tubule, medullary, focal, moderate with fibrosis.
3. Uterus: Tall columnar epithelium. Mildly dilated lumen. Mitoses. No degenerative cells. (P)
4. Ovary: Corpora lutea vacuolated, degenerative. (P)
5. Vagina: Not evaluated.

Note: Only vulva on slide, vagina not in sections examined.

Thyroid code:F2C3

Gross findings: Pale small intestine.

12-0347
1. Thyroid gland; left kidney; jejunum: No significant findings.
2. Uterus: Tall columnar epithelium. Degenerative epithelium. (E)
3. Ovary: Corpora lutea degenerative. Basophilic CLs. (E)
4. Vagina: Some cornified cells remain. Cornified cells in lumen. (E)

Thyroid code: F2C3

Gross findings: Pale small intestines.

12-0359
1. Thyroid gland: Ultimobranchial cyst.
2. Kidney, left: Basophilic tubules, multifocal, mild with mononuclear infiltrates.
3. Lymph node, mesenteric: Sinus dilatation, diffuse, mild.
4. Jejunum: No significant findings.
5. Uterus: Tall columnar epithelium. Mild epithelial degeneration. Mild mitoses. (M)
6. Ovary: Mildly vacuolated and degenerative corpora lutea. (M)
7. Vagina: Low epithelium. Mild leukocyte infiltrates. (M)

Thyroid code: F3C3

Gross findings: Pale small intestines.

12-0366

1. Right and left kidney; heart: No significant findings.
2. Lung: Extravasated red blood cells, multifocal, mild.
3. Uterus: Tall columnar epithelium. Mild degeneration, few mitoses. (D)
4. Ovary: Mildly vacuolated corpora lutea with fibrous connective tissue. (D)
5. Vagina: Mild leukocyte infiltrates. (D)

NOTE: No thyroid gland.

Gross findings: Mottled right and left lung. Mildly pale kidneys.

6.3 Table 3. Female thyroid codes

Table 3. Thyroid grading incidence: Females			
Thyroid code	Control	500 mg/kg	1000 mg/kg
F1C1			
F1C2			
F1C3			
F1C4	1/15		
F1C5	2/15		1/14
F2C1			
F2C2			
F2C3	3/15	5/15	4/14
F2C4	7/15	5/15	6/14
F2C5	1/15	1/15	
F3C1			
F3C2			
F3C3	1/15	3/15	3/14
F3C4		1/15	
F3C5			
F4C1			
F4C2			
F4C3			
F4C4			
F4C5			
F5C1			
F5C2			
F5C3			
F5C4			
F5C5			

6.4 Table 4. Male thyroid codes

Table 4. Thyroid grading incidence: Males			
Thyroid code	Control	250 mg/kg	500 mg/kg
F1C1			
F1C2			
F1C3			
F1C4			
F1C5		1/15	1/15
F2C1			
F2C2			
F2C3	2/15	2/15	1/15
F2C4	6/15	7/15	6/15
F2C5	1/15	1/15	1/15
F3C1			
F3C2			
F3C3	6/15	3/15	3/15
F3C4			3/15
F3C5			
F4C1			
F4C2		1/15	
F4C3			
F4C4			
F4C5			
F5C1			
F5C2			
F5C3			
F5C4			
F5C5			

6.5 Table 5. Cytology based reproductive staging

0FP3-95-12-02-01 Consolidated vaginal cytology data

Animal #	4/14/2012	4/15/2012	4/16/2012	4/17/2012	4/18/2012	4/19/2012	4/20/2012	4/21/2012	4/22/2012	4/23/2012	4/24/2012	4/25/2012
180				PE	M	D	D	DP	P	E	D	
181		P	E	M	D	P	E	D	D	P	E	
182				D	D	D	P	E	D	D	D	
190			D	D	P	E	D	D	P	E	D	
191		D	D	E	D	P	E	D	D	P	E	
192		M	MD	D	P	E	M	D	P	E	D	
197			E	M	D	D	P	E	D	D	P	
199					D	D	D	P	E	D	D	
202			D	DP	E	D	D	PE	D	D	PE	
217		P	M	D	D	P	E	D	D	D	P	
218					P	E	D	D	D	P	E	
219					E	M	D	D	P	E	D	
238			M	D	D	P	E	D	D	D	P	
239			E	M	D	D	D	DP	P	E	D	
241				E	E	D	D	P	E	D	D	
248			MD	D	D	P	E	M	D	D	E	
250					P	PE	undetermined	E	M	D	P	
251		E	M	D	D	P	E	D	D	P	E	
268				E	D	D	D	D	P	E	D	
270				D	P	E	D	D	D	D	P	
271					undetermined	D	D	DP	P	undetermined	D	D
277					M	D	D	P	E	D	D	D
280		M	D	D	P	E	D	D	P	E	D	
281		PE	D	D	D	P	E	D	D	P	P	
287						E	D	D	P	P	E	M
288				E	M	D	D	P	E	D	D	P
290					M	D	D	P	E	D	D	D
316					E	M	D	D	P	E	D	D
318				E	E	D	D	D	P	E	D	D
319					E	D	D	D	P	E	M	D
326				E	M	D	DP	E	M	D	P	E
328					D	D	D	P	E	E	M	D
329	M	D	D	DP	E	D	D	P	E	D	D	P
335								E	E-M	M	D	D
337			E	M	D	D	P	E	D	P	E	DP
338					E	E	M	D	P	E	M	D
347		E	M	D	DP	P	E	D	D	DP	E	E
349				E	D	D	DP	E	D	D	P	E
350		E	D	D	DP	E	M	D	P	E	D	D

6.6 Table 6. Ovarian follicle counts

OFP3-95-12-02-01
Pubertal Development and Thyroid Function in Intact Juvenile Rats Exposed to NTO

Animal number	slide 2-1						slide 2-2						slide 2-3						slide 2-4						slide 2-5						slide 2-6					
	Primordial	Growing	Antral	Atretic	CL		Primordial	Growing	Antral	Atretic	CL		Primordial	Growing	Antral	Atretic	CL		Primordial	Growing	Antral	Atretic	CL		Primordial	Growing	Antral	Atretic	CL		Primordial	Growing	Antral	Atretic	CL	
12-0182	21	5	4	2	3		16	5	4	2	3		9	6	7	4	3		17	7	6	4	3		23	4	10	11	4		19	2	14	20	4	
12-0192	1	1	1	0	4		0	1	1	0	5		0	1	2	0	6		7	3	7	3	7		0	4	8	5	8		4	6	14	6	9	
12-0202	1	1	5	3	5		0	3	5	4	5		2	0	6	5	4		4	1	5	3	4		3	0	5	4	5		2	2	7	5	7	
12-0219	16	1	8	3	4		9	2	9	3	4		8	5	14	5	4		8	4	18	7	5		11	9	13	9	8		12	6	18	5	7	
12-0238	17	8	16	4	4		22	4	17	1	5		17	6	18	5	6		15	8	15	7	6		18	7	20	8	6		9	8	17	8	7	
12-0248	6	2	1	2	9		2	1	2	5	9		5	0	3	4	8		7	1	3	2	7		3	2	3	1	7		1	2	3	3	6	
12-0268	13	1	5	3	7		11	3	7	6	7		12	4	9	6	7		22	3	9	6	7		17	2	9	3	7		14	1	14	5	7	
12-0280	13	2	13	2	6		9	3	14	2	6		20	6	14	2	7		16	5	14	3	6		24	4	19	4	7		17	7	20	8	7	
12-0288	4	1	8	2	4		9	0	9	1	4		5	1	12	1	4		6	3	17	1	5		10	3	14	1	5		9	3	15	5	4	
12-0319	40	2	28	6	5		25	3	31	6	7		28	11	30	12	8		34	8	34	19	9		49	9	28	14	9		39	8	20	28	9	
12-0329	7	4	15	5	8		9	2	16	9	8		13	6	17	12	7		9	3	20	14	7		10	3	18	12	7		7	7	22	8	8	
12-0337	7	2	13	6	5		11	4	12	13	7		10	5	12	15	7		14	3	18	15	10		8	6	18	21	10		14	4	14	15	10	
12-0350	8	7	19	14	10		11	5	18	20	12		1	7	16	17	14		9	7	18	15	13		10	7	21	14	13		10	11	13	16	12	
12-0357	0	3	7	2	5		4	2	8	3	5		10	2	9	7	7		6	6	11	1	7		13	8	11	1	7		5	3	15	5	7	
12-0368	33	13	32	3	9		38	9	36	9	12		51	10	33	6	12		48	9	31	10	12		21	6	26	5	12		12	2	26	9	10	
12-0181	13	5	19	3	3		9	2	20	7	4		16	2	19	6	4		5	3	17	11	5		8	3	14	7	6		11	3	15	6	6	
12-0190	6	1	6	0	5		11	1	6	0	5		6	1	8	0	8		14	2	10	1	9		10	1	10	2	9		11	1	11	2	8	
12-0197	14	6	20	7	5		14	3	24	10	5		18	4	17	11	5		15	10	30	13	6		16	8	28	16	6		12	10	27	13	6	
12-0217	3	1	16	8	2		17	1	14	8	2		18	2	18	4	2		6	2	16	12	2		1	2	14	14	2		6	3	18	12	3	
12-0241	2	1	6	0	5		0	0	7	0	5		2	0	6	0	8		3	0	6	0	8		4	1	7	0	7		11	1	8	1	6	
12-0250	17	2	16	8	5		21	4	14	7	5		19	2	4	4	5		28	1	9	5	5		31	2	8	6	5		17	2	11	8	6	
12-0271	3	5	16	4	8		14	4	16	9	9		28	2	24	10	10		25	4	22	9	9		22	4	18	8	9		13	1	10	8	11	
12-0277	42	3	27	2	6		35	5	27	7	6		26	8	22	8	6		45	6	22	10	6		32	8	23	8	6		15	4	17	8	6	
12-0290	5	4	11	2	3		4	3	9	2	3		8	3	8	1	3		6	3	7	2	3		7	2	9	1	3		11	1	8	3	3	
12-0318	7	5	7	7	5		10	3	5	12	5		11	8	9	9	6		17	7	8	7	6		17	3	152	12	6		92	5	8	4	7	
12-0326	1	4	8	6	8		2	2	13	5	8		2	1	14	9	7		3	6	18	12	6		4	4	14	12	7		0	8	14	8	7	
12-0338	15	0	15	4	8		10	8	11	8	9		21	3	16	14	9		26	3	12	13	9		4	5	16	10	10		5	8	14	5	10	
12-0349	5	4	12	2	4		3	10	18	2	8		3	7	18	6	8		8	7	15	9	10		9	4	15	14	10		1	8	10	18	11	
12-0358	5	1	4	0	5		10	1	5	0	5		12	2	5	1	5		10	4	6	2	7		18	3	10	4	7		30	3	11	4	7	
12-0370	9	4	21	8	6		7	4	16	18	6		21	10	5	19	5		13	9	21	10	5		6	15	25	8	8		65	9	25	4	8	
12-0180	2	2	8	0	4		6	3	9	1	4		8	4	11	1	4		6	3	10	0	4		11	11	12	2	5		6	12	10	2	5	
12-0191	3	1	9	8	7		4	3	8	8	9		3	2	9	4	9		3	3	8	3	9		2	5	11	4	9		1	6	10	4	8	
12-0199	7	1	7	6	7		8	4	5	4	7		19	5	9	5	8		20	9	9	6	8		8	5	11	14	9		25	7	15	12	9	
12-0218	7	2	2	4	5		1	1	2	1	5		0	2	2	3	6		11	12	15	7	7		11	11	12	13	7		4	13	14	17	8	
12-0239	18	8	10	5	6		14	7	11	5	6		12	6	13	7	7		30	9	13	2	6		28	3	18	8	6		30	3	13	7	6	
12-0251	16	4	7	1	5		11	3	9	4	4		11	5	9	4	6		6	6	9	3	6		11	6	9	2	6		12	6	6	3	6	
12-0270	32	7	11	7	3		15	6	12	10	5		23	5	11	10	5		24	4	12	14	5		19	5	11	16	5		25	4	7	9	5	
12-0281	13	5	15	5	4		19	5	17	3	4		22	9	16	9	4		15	8	16	12	5		11	10	17	6	5		20	3	20	4	6	
12-0287	3	5	8	4	5		9	4	8	6	5		11	5	10	8	5		10	5	12	6	7		3	9	12	6	8		10	11	12	6	7	
12-0316	7	4	8	4	5		10	3	8	4	5		10	3	8	6	5		15	3	8	3	5		11	5	11	8	5		14	8	9	4	6	

6.7 Table 7. Reproductive stage at necropsy

Animal #	Uterus	Ovary	Vagina
12-0182	P	P	P
12-0192	M	M	M
12-0202	P	P	P
12-0219	D	D	D
12-0238	P-late	P	P-late
12-0248	E	E	E
12-0268	D	D	D
12-0280	M	M	M
12-0288	P	P	P
12-0319	D	D	D
12-0329	P	P	P
12-0337	P-late	P	P-late
12-0350	D	D	M-D transition
12-0357	E	E	E
12-0368	M	M	M
12-0181	P-late	P	P-late
12-0190	M	M	M
12-0197	P	P	P
12-0217	P	P	P-E
12-0241	D	D	D
12-0250	P	P	P
12-0271	D	D	D
12-0277	P	P	P
12-0290	P	P	P
12-0318	M	M-D	M
12-0326	E	E	E
12-0338	D	D	D
12-0349	E	E	E
12-0358	M	M	M
12-0370	D	D-late	D
12-0180	M	M	M
12-0191	E	E	E
12-0199	D	D	D
12-0218	E	E	E
12-0239	M-late	M-late	M-late
12-0251	E	E	E
12-0270	P	P	P
12-0281	P	P	P
12-0287	M	M	M
12-0316	D	D	D
12-0328	D	D	D
12-0335	P	P	P
12-0347	E	E	E
12-0359	M	M	M
12-0366	D	D	D

6.8 Table 8. Random number generator

Animal number	slide 2-1	slide 2-2	slide 2-3	slide 2-4	slide 2-5	slide 2-6		TYPE TO RESET
12-0182	1	9	15	18	23	30		fgh
12-0192	2	9	11	20	23	26		
12-0202	3	7	15	17	23	28		
12-0219	4	8	11	19	21	30		
12-0238	5	10	13	16	23	27		
12-0248	1	10	14	20	22	26		
12-0268	1	10	14	16	23	28		
12-0280	1	7	11	17	24	27		
12-0288	4	7	11	20	23	30		
12-0319	1	7	13	20	24	29		
12-0329	2	9	12	20	21	26		
12-0337	5	10	14	17	25	30		
12-0350	1	10	11	16	23	29		
12-0357	5	9	14	17	22	27		
12-0368	2	10	13	18	23	28		
12-0181	3	9	15	19	21	27		
12-0190	1	9	13	18	21	28		
12-0197	1	6	11	16	21	27		
12-0217	2	7	15	20	22	28		
12-0241	2	10	15	16	22	26		
12-0250	5	7	11	19	21	27		
12-0271	3	10	11	17	22	26		
12-0277	3	7	11	16	22	28		
12-0290	5	7	14	19	25	29		
12-0318	4	9	15	18	21	29		
12-0326	4	10	11	16	25	30		
12-0338	3	9	11	16	23	27		
12-0349	4	8	15	18	24	27		
12-0358	5	9	13	19	24	29		
12-0370	2	9	13	18	22	29		
12-0180	1	9	14	17	24	28		
12-0191	3	8	14	20	21	29		
12-0199	2	6	15	16	22	27		
12-0218	4	6	15	19	24	27		
12-0239	3	9	15	18	23	30		
12-0251	5	6	14	17	22	26		
12-0270	4	8	13	16	22	29		
12-0281	2	6	13	17	22	27		
12-0287	4	7	12	17	22	27		
12-0316	5	6	12	18	24	28		
12-0328	2	10	13	17	24	28		
12-0335	5	6	12	18	21	30		
12-0347	2	9	14	18	23	30		
12-0359	3	10	11	17	24	30		
12-0336	2	10	13	19	25	28		
OFP3-95-12-02-12 Pubertal Development and Thyroid Function in Intact Juvenile Rats								
Exposed to NTO		Generated 8 August 2012						

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8. GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The portion of the study described in this contributing scientist report, including the tissue processing piece conducted at the United States Army Medical Research Institute of Chemical Defense's Comparative Pathology Branch, was conducted in compliance with Title 40, Code of Federal Regulations (CFR) Part 792, Good Laboratory Practice Standards.

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23 July 2013
Date

Appendix M

Summary of Clinical Chemistry Data

Table M-1
Protocol No. 0FP3-95-12-02-01
Pubertal Deveopment and Thyroid Function in Intact Juvenile Rats Exposed to NTO

Summary of Clinical Chemistry Data in Female Rats

NTO	Effect	Tranform or nonparam	Pairwise test	p-value	Normal range^	Vehicle Control				500 mg/kg-day					1000 mg/kg-day				
						Mean	SD	CV	N	Mean	SD	CV	N	p-value	Mean	SD	CV	N	p-value
ALKP (U/L)				0.313	133-219	416.53	72.25	17.34	15	397.87	72.95	18.33	15		379.67	47.67	12.55	15	
ALT (U/L)				0.763	23-28	79.47	8.45	10.63	15	83.33	17.09	20.51	15		83.60	22.85	27.33	15	
AST (U/L)				0.188	78-109	164.87	39.32	23.85	15	200.40	63.32	31.59	15		196.33	65.02	33.12	15	
BUN (mg/dL)				0.708	10-13	12.13	1.85	15.22	15	11.13	3.74	33.59	15		11.67	3.87	33.14	15	
CHOL (mg/dL)				0.291	69-92	90.40	13.64	15.09	15	92.47	12.17	13.16	15		97.33	10.72	11.01	15	
CREA (mg/dL)				0.949	0.5-0.6	0.28	0.07	24.14	15	0.27	0.06	21.76	15		0.28	0.07	24.14	15	
GLU (mg/dL)				0.145	100-179	176.87	17.28	9.77	15	171.33	12.95	7.56	15		165.93	14.08	8.48	15	
TBIL (mg/dL)				0.050	0.10-1.00	0.20	0.08	42.25	15	0.24	0.09	37.92	15		0.16	0.08	51.75	15	
TP (g/dL)				0.970	5.7-6.4	5.37	0.13	2.51	15	5.35	0.16	2.99	15		5.35	0.21	3.92	15	
Na (mmol/L)				0.609	140-147	146.27	1.16	0.80	15	145.87	0.92	0.63	15		145.93	1.39	0.95	15	
K (mmol/L)				0.562	4.3-6.0	5.94	0.53	8.95	15	5.94	0.57	9.62	15		5.75	0.59	10.29	15	
Cl (mmol/L)	*		Tukey	0.018	100-108	105.73	1.39	1.31	15	106.93	1.44	1.34	15		107.33	1.76	1.64	15	0.018

^Giknis, M.L.A and Clifford, C.B. 2006. Clinical Laboratory Parameters for Crl:CD(SD) Rats.
http://www.criver.com/SiteCollectionDocuments/rm_rm_r_clinical_parameters_cd_rat_06.pdf

Table M-2
Protocol No. 0FP3-95-12-02-01
Pubertal Deveopment and Thyroid Function in Intact Juvenile Rats Exposed to NTO

Summary of Clinical Chemistry Data in Male Rats

NTO	Effect	Tranform or nonparam	Pairwise test	p-value	Normal range^	Vehicle Control				250 mg/kg-day					500 mg/kg-day				
						Mean	SD	CV	N	Mean	SD	CV	N	p-value	Mean	SD	CV	N	p-value
ALKP (U/L)				0.123	136-188	497.87	95.61	19.20	15	439.27	70.49	16.05	15		446.60	82.02	18.36	15	
ALT (U/L)				0.082	28-40	79.53	8.62	10.84	15	71.67	8.29	11.56	15		77.60	11.92	15.35	15	
AST (U/L)	*		Tukey	0.015	87-114	136.40	17.05	12.50	15	137.53	24.46	17.78	15		158.07	23.26	14.71	15	0.025
BUN (mg/dL)	*		Tukey	0.012	13-16	14.13	3.07	21.71	15	11.93	2.76	23.17	15		11.07	2.43	21.99	15	0.011
CHOL (mg/dL)				0.972	54-74	98.40	10.08	10.24	15	97.93	14.48	14.79	15		99.00	12.04	12.16	15	
CREA (mg/dL)				0.945	0.5-0.6	0.26	0.06	24.31	15	0.26	0.06	24.31	15		0.27	0.06	23.11	15	
GLU (mg/dL)				0.917	112-176	171.80	9.12	5.31	15	172.53	12.30	7.13	15		173.40	10.01	5.77	15	
TBIL (mg/dL)				0.383	0.10-1.00	0.20	0.09	46.30	15	0.25	0.07	30.08	15		0.23	0.11	47.77	15	
TP (g/dL)				0.938	5.9-6.6	5.83	0.26	4.52	15	5.81	0.20	3.43	15		5.80	0.13	2.16	15	
Na (mmol/L)				0.634	141-150	150.13	1.51	1.00	15	150.20	1.47	0.98	15		149.73	1.34	0.89	15	
K (mmol/L)				0.936	4.7-6.2	7.62	0.53	6.93	15	7.66	0.60	7.83	15		7.71	0.92	11.94	15	
Cl (mmol/L)				0.924	102-105	102.00	1.87	1.83	15	102.20	2.21	2.16	15		102.27	1.91	1.86	15	

^Giknis, M.L.A and Clifford, C.B. 2006. Clinical Laboratory Parameters for Crl:CD(SD) Rats.
http://www.criver.com/SiteCollectionDocuments/rm_rm_r_clinical_parameters_cd_rat_06.pdf

Appendix N

Summary of Hormone Data

Table N-1
Protocol No. 0FP3-95-12-02-01
Pubertal Deveopment and Thyroid Function in Intact Juvenile Rats Exposed to NTO

Summary of Serum Hormone Data in Female Rats

NTO	Effect	Tranform or nonparam	Pairwise test	p-value	Vehicle Control				500 mg/kg-day					1000 mg/kg-day				
					Mean	SD	CV	N	Mean	SD	CV	N	p-value	Mean	SD	CV	N	p-value
Serum T4, total (ug/dl)				0.247	3.313	0.549	16.57	15	2.933	0.606	20.64	15		2.967	0.843	28.42	15	
Serum TSH (ng/ml)				0.798	1.866	1.552	83.21	15	1.765	1.283	72.69	15		1.560	0.865	55.42	15	

Table N-2
Protocol No. 0FP3-95-12-02-01
Pubertal Deveopment and Thyroid Function in Intact Juvenile Rats Exposed to NTO

Summary of Serum Hormone Data in Male Rats

NTO	Effect	Tranform or nonparam	Pairwise test	p-value	Vehicle Control				250 mg/kg-day					500 mg/kg-day				
					Mean	SD	CV	N	Mean	SD	CV	N	p-value	Mean	SD	CV	N	p-value
Serum T4, total (ug/dl)				0.348	3.907	0.480	12.29	15	4.020	0.492	12.23	15		4.153	0.402	9.67	15	
Serum TSH (ng/ml)				0.624	2.969	1.092	36.79	15	3.141	2.025	64.47	15		3.628	2.395	66.01	15	
Serum testosterone, total (ng/ml)				0.460	2.221	1.238	55.71	15	2.538	1.636	64.46	15		1.947	0.878	45.07	15	

Appendix O

Study Protocol with Modifications

MCHB-IP-AU 7 February 2012

MEMORANDUM FOR Dr. Emily Lent

SUBJECT: Protocol Approval

1. USAPHC's Institutional Animal Care and Use Committee (IACUC) has approved your protocol entitled, "Pubertal Development and Thyroid Function in Intact Juvenile Rats Exposed to NTO".

2. Your protocol has been assigned an IACUC approval number: **12 - 02 - 01**. Please refer to this number in all further correspondence regarding this protocol. The complete protocol study number is 0FP3-95-12-02-01.

3. Please report the total number of animals actually used and pain categories to the IACUC Administrator when your protocol is completed or at the end of the fiscal year, whichever is sooner. If you have not finished your protocol by the end of the fiscal year, report the animal numbers used through 30 September and their corresponding pain categories for the USDA report on the protocol annual review form. You will receive this annual form in October of each year the protocol is open. The annual review form requests additional information that is required for the DoD Annual Report to Congress on Animal Use.

4. The protocol should be followed exactly as you have described it to the IACUC. If you wish to make procedural changes, or require more animals than you anticipated, you must submit a protocol modification using CHPPM Form 28-R-E, being careful to include a justification for the change(s) to your original protocol. Refer to the most current versions of the USAPHC IACUC SOPs for more information concerning modifications.

5. As the Principal Investigator/Study Director of this protocol, you are responsible for coordinating a pre-protocol planning meeting with the Veterinary Staff, in advance of ordering animals.

KRISTIN T. NEWKIRK
IACUC Chair

ANIMAL USE PROTOCOL
TOXICOLOGY PORTFOLIO
ARMY INSTITUTE OF PUBLIC HEALTH
U.S. ARMY PUBLIC HEALTH COMMAND
ABERDEEN PROVING GROUND, MD 21010-5403

PROTOCOL TITLE: Pubertal Development and Thyroid Function in Intact Juvenile Rats Exposed to NTO

PROTOCOL NUMBER: ØFP3-95-12-Ø2-Ø1

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I. NON-TECHNICAL SYNOPSIS

The endocrine disrupting potential of 3-nitro-1,2,4-triazol-5-one (NTO), an insensitive, energetic material used in explosive formulations, will be assessed by conducting two Environmental Protection Agency (EPA) Tier I Endocrine Disruptor Screening Program (EDSP) assays. Groups of 15 rats per sex will be orally exposed to NTO from post natal day (PND) 22/23 through a period approximating normal pubertal development (females: PND 42-43; males: PND 53-54). All rats will be monitored throughout the study for body weight changes and clinical signs of toxicity and effects on reproductive development. Females will be examined daily (starting on PND22) for vaginal opening and, beginning at vaginal opening, vaginal fluid will be collected to monitor estrous cyclicity. Males will be examined daily, starting on PND30, for preputial separation. At the end of the study, rats will be euthanized, blood samples collected for clinical chemistry and hormone assays, necropsy conducted and selected tissues weighed and processed for histopathology. The endocrine disrupting potential of NTO on peripubertal rats will be based on the weight of evidence of effects observed for body weight, age at puberty, organ weights, clinical chemistry, reproductive tissue histopathology, and reproductive and thyroid hormones.

II. BACKGROUND

II.1. Background: 3-Nitro-1,2,4-triazol-5-one (NTO) is being investigated as a less sensitive direct replacement for traditional explosives such as TNT and RDX. NTO is a crystalline powder that is one of the components used in the formulation of an insensitive explosive referred to as IMX101. NTO was first reported in 1905, but was not used as an explosive until the early 1980's when it was revealed that the French were developing a "new insensitive explosive", which was later reported to be NTO. Renewed interest in the energetic properties of NTO has been fueled by the need to develop munitions that are less prone to inadvertent initiation during transport and routine handling. The reduced sensitivity to environmental stimuli and nearly equal performance during testing make NTO-based formulations desirable replacements for currently fielded munitions (references 1 and 2). As a potential component of new munitions formulations, NTO must not only meet certain performance criteria, but must also be acceptable from the perspective of human health and the environment. To ensure its safe use by military personnel and production employees handling the material on a daily basis, the toxicity of NTO must be investigated. Toxicological testing will be conducted by the Army Institute of Public Health (AIPH), Portfolio of Toxicology (TOX).

II.2. Literature Search for Duplication

II.2.1. Literature Source(s) Searched: BRD (Biomedical Research Database), DOAC (DTIC Online Access Controlled)* Technical Reports, DOAC Research in Progress, FEDRIP, PubMed, Web of Science

II.2.2. Date of Search: 9 Dec 2011

II.2.3. Period of Search: 1900-2011

II.2.4. Key Words of Search: ((3-nitro-1,2,4-triazol-5-one or 3 nitro 1,2,4 triazol 5 one or triazole* or nitro compound*) and ((endocrine near disrupt*) or (reproduc* or thyroid near hormone*) or (sex* near develop*)) and (rat or rats))

II.2.5. Results of Search: A total of 11 references resulted from the literature search that was performed for NTO. However, no reproductive/developmental toxicity studies, pubertal development, thyroid function, or endocrine disruption tests for NTO were found that would suggest that this study would be a duplicate effort. As such, the present study is not a duplication of the information available in the literature.

III. OBJECTIVE/HYPOTHESIS

The primary objective of this study is to assess the endocrine disrupting potential of NTO through the use of two screening assays. The purpose of these assays is to determine whether NTO has the potential to interact with the endocrine system *in vivo* by identifying effects on pubertal development and thyroid function in the intact juvenile rat. These assays can detect anti-thyroid, estrogenic, anti-estrogenic, androgenic, and anti-androgenic activity as well as agents acting via gonadotropins, prolactin or hypothalamic function.

IV. MILITARY RELEVANCE

As a result of an initiative by the Department of Defense (DOD) to improve munitions safety, the US Army is developing insensitive munitions (IM) for incorporation into its inventory of conventional military munitions systems. The Army's IM Program is dedicated to developing munitions that reliably perform as they are intended but are less prone to inadvertent initiation from external stimuli such as bullet/fragment impact, heat from fire, and shock from neighboring explosions (reference 3). The production of insensitive munitions requires the use of intrinsically less sensitive explosives. Despite the slightly lower performance of NTO compared to TNT, there has been a renewed interest in NTO use in explosive formulations based on its lower sensitivity as a melt-cast medium observed during testing and the less stringent shipping requirements. This has led to the development of a range of melt-castable explosives at Picatinny Arsenal, collectively known as "PAX" explosives (reference 4). To support possible fielding of these PAX explosives, a Toxicity Clearance would have to be granted and occupational exposure guidelines developed. Consequently, toxicity data in a mammalian system need to be generated to assess occupational health hazards associated with the use and production of this material.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures: This study consists of two Environmental Protection Agency (EPA) Tier I Endocrine Disruptor Screening Program (EDSP) assays: "Pubertal Development and Thyroid Function in Intact Juvenile/Peripubertal Male Rats" (OPPTS 890.1500) and "Pubertal Development and Thyroid Function in Intact Juvenile/Peripubertal Female Rats" (OPPTS 890.1450)

(references 5 and 6). These assays will be conducted on pups derived from timed pregnant dams. Groups of 15 rats per sex will be exposed to NTO via oral gavage from post natal day (PND) 22/23 through a period approximating normal pubertal development (females: PND 42-43; males: PND 53-54). All rats will be monitored throughout the study for body weight, clinical signs of toxicity, and effects on reproductive development. Females will be examined daily (starting on PND22) for vaginal opening and, beginning at vaginal opening, vaginal fluid will be collected to monitor estrous cyclicity. Males will be examined daily, starting on PND30, for preputial separation. Rats will be euthanized, blood samples collected for clinical chemistry and hormone assays, necropsy conducted and selected tissues weighed and processed for histopathological evaluation.

V.1.1. Pubertal Development and Thyroid Function in Intact Juvenile Rats

This study will be conducted following the methods outlined in EDSP Test Guideline OPPTS 890.1450 and 890.1500. This protocol uses a randomized complete block design (time-separated necropsy is the blocking factor) with 15 female and 15 male juvenile rats in each treatment group. The treatment groups are: (1) vehicle control; (2) NTO at or just below the Maximum Tolerated Dose (MTD); and (3) NTO at ½ the MTD. The route of administration will be by oral gavage with pure NTO suspended/dissolved in corn oil or polyethylene glycol (PEG). Corn oil used on study will be clear and free of sediment and will have a bland odor that is free from rancid, musty, metallic, putrid or any other undesirable odor.

Juvenile rats will be derived from 20 timed pregnant females obtained from an external supplier such that all dams are pregnant for the first time and timed to deliver on the same day. Dams will be on gestation day (GD) 7, 8, 9, or 10 at the time of arrival. Any litters with fewer than 8 total pups/litter and any litters not delivered by GD 23 will be excluded from the study. To maximize uniformity in growth rates, the litters will be standardized to the same number of pups per litter (8, 9, or 10) between PND 3 and 5. Cross-fostering of pups is not allowed. Body weights will be monitored weekly and any unthrifty litters or runted pups excluded from the study.

On PND21 the pups will be weaned, identified as described in V.4.4.5 and weighed individually to the nearest 0.1 g. Males and females, separately, will then be ranked by body weight and an equal number of pups will be eliminated from the heavy end and the light end of the distribution, leaving the number of animals needed for the study in the middle of the distribution. Pups will then be assigned to treatment groups such that the mean body weights and variances for all groups are similar. Per the EDSP Guidelines, placing of littermates in the same treatment group will be avoided and will not be done unless an insufficient number of pups are available. After assignment to treatment groups, pups will be pair housed according to sex and treatment group (it will be necessary to add a 3rd rat to one cage to maintain social housing for all juvenile rats).

NTO or vehicle will be administered daily by oral gavage beginning on PND 22/23. Females will be dosed through PND 42-43 and males through PND 53-54. Each animal will be weighed daily prior to treatment and the NTO or vehicle will be administered on a

mg/kg body weight basis using the current day's weight, and volume of the dose administered recorded each day. Clinical observations will also be recorded daily.

Beginning on PND 22, females will be examined for vaginal opening (VO) at approximately the same time each day. Starting on the day of vaginal opening through and including the day of necropsy, vaginal fluid will be obtained and evaluated at approximately the same time each day to determine estrous cyclicity. Beginning on PND 30, males will be examined at approximately the same time each day for preputial separation (PPS). Observations of vaginal opening, estrous cyclicity and preputial separation will be performed after daily dosing.

Following the dosing period, animals will be euthanized, blood will be collected, and a necropsy performed with selected tissues collected, weighed, and submitted for histopathological evaluation. Due to necropsy being conducted over two days, females will be euthanized on PND 42 and 43 with animals in each treatment group being approximately equally dispersed between the two necropsy days. Similarly, males will be euthanized on PND 53 and 54. Animals euthanized on the second day of necropsy will be dosed on the day of their necropsy and data collected in the same manner as animals euthanized on the first day of necropsy. All animals will be dosed between 0700 and 0900 hours and euthanized beginning 2 hours following dosing. It is critical that euthanasia be completed by 1300 hours due to normal diurnal fluctuation in thyroid hormone levels. Anesthesia, euthanasia, blood sampling, and necropsy procedures are described in Sections V.4.1.2.1, V.4.6, V.1.9, and V.1.116, respectively.

Experiment 1. Pubertal Development and Thyroid Function in Intact Juvenile Rats

Group	No. of Male Rats	No. of Female Rats
DAMS/PUPS		
Timed-Pregnant Females		20
Total Pups*	100	100
	TOTAL = 100	**TOTAL = 120**
ANIMALS USED ON STUDY		
Experimental Groups		
Vehicle Control	15	15
MTD mg/kg-day NTO	15	15
½ MTD mg/kg-day NTO	15	15
	TOTAL = 45	**TOTAL = 45**
	GRAND TOTAL = 100	**GRAND TOTAL = 120**

(*) The estimation of "Total Pups" was made with the assumptions that each timed-pregnant female would produce 10 pups and the sex ratio of the offspring would be 1:1. The "Total Pups" includes the 45 male and 45 female pups allotted into the treatment groups.

V.1.2. Test Substance: This study will be conducted with 3-nitro-1,2,4-triazol-5-one (NTO). The test material will be supplied by BAE SYSTEMS, Ordnance Systems, Kingsport, TN. All dosing solutions/suspensions will be prepared using NTO from the same batch and lot number. To facilitate the oral gavage procedure, NTO will be mixed with corn oil or PEG. Samples of each batch of the resulting solutions/suspensions will be submitted to the AIPH Laboratory Sciences (LS) portfolio for concentration verification. In addition, appropriate samples will be submitted to verify the stability and homogeneity of NTO in corn oil or PEG, if not previously done, IAW LS SOP 801 (reference 7).

Test Substance Chemical/Physical Properties

Name	3-nitro-1,2,4-triazol-5-one
Synonym	NTO
CAS#	932-64-9
Physical State	White to pale yellow crystalline powder
Molecular Formula	$C_2H_2N_4O_3$
Molecular Weight	130
Density	1.93 g/cm^3
Solubility	Soluble in water (16 g/L)
Purity (by HPLC)	99.6%

V.1.3. Administration of Test Substance by Oral Gavage Dose: Per the EDSP Guidelines, treatments will be administered in 2.5 to 5.0 ml vehicle/kg body weight (same volume for all experimental groups) at 0700-0900 daily using an 18 gauge gavage needle (1 to 1½ inch length, 2.25 mm ball). Needle size may be optimized to animal size but must be constructed of metal to avoid the potential for absorption by or leaching of substances from rubber or plastic tubing.

V.1.4. Dose Selection for Oral Gavage: Per the EDSP Guidelines, the highest dose level will be set at or just below the Maximum Tolerated Dose (MTD) level, but will not exceed the limit dose of 1 g/kg-day. The EDSP Guidelines use a definition of MTD such that a dose level is at or just below the Maximum Tolerated Dose level if it causes a statistically significant reduction in final body weight in treated animals relative to controls and the reduction is no greater than approximately 10%, and no clinical signs of toxicity are observed. Abnormal blood chemistry and histopathology may indicate that a dose exceeded the MTD, even in the absence of a reduction in terminal body weight compared to controls.

Due to the duration of dosing for this study (21/22 and 31/32 days), dose selection is based on the findings from both the 14- and 90-day oral studies that were performed previously by this Institute. A statistically significant reduction in body weight relative to the control was only observed in males in the 14-day study at the 2000 mg/kg-day NTO dose. As this is beyond the limit dose for the current study, body weight will not be a useful marker for determination of the MTD and for dose selection. Limited abnormal clinical chemistry was observed in the 14-day study at doses at or above 1000 mg/kg-day. Testes weights and weight ratios were significantly reduced compared to controls in male rats administered 500 mg/kg-day NTO and above in the 14-day study. Histopathology was not performed on any tissues from the 14-day study. No effects on body weight were observed in the 90-day study and effects on blood parameters were limited to slight anemia and reduced albumin and protein in males at or above 1000 mg/kg-day. The 90-day study on NTO revealed significant reductions in testes and epididymides weights and sperm counts at dosages of 315 mg/kg-day and above. Histopathology performed on the 90-day tissues revealed significant incidences of testicular hypoplasia at dosages of 315 mg/kg-day and above as well as insignificant, less severe, testicular hypoplasia at dosages of 100 mg/kg-day and below (reference 8). The only additional abnormal histopathology, and the only effect noted for females, was hepatocellular hypertrophy at doses at or above 1000 mg/kg-day. Based on these results, it was determined that the MTD differed between males and females and, thus,

the doses selected for this study differ between the sexes. A MTD of 1000 mg/kg-day was selected for females based on the very limited effects at this dose and the lack of effects observed below this dose in either the 14- or 90-day study. The low dose for females is calculated as ½ the MTD, or 500 mg/kg-day. A MTD of 500 mg/kg-day was selected for the males based on the presence of abnormal clinical chemistry/hematology and liver histopathology at 1000 mg/kg-day. The pattern of changes in reproductive tissue weights and histopathology was used in selecting the dose; however, the MTD was not selected to be below doses causing reproductive effects (despite the definition of MTD) as doing so would likely ensure a negative outcome for the assay. The low dose for males is calculated as ½ the MTD, or 250 mg/kg-day.

Due to the restricted dose volume (2.5 - 5 ml/kg), the relatively high MTD selected for females results in a concentration of dosing solution (200 mg/ml) that may clog the small gavage tube (18 gauge). If particle size cannot be sufficiently reduced to ensure delivery of the full dose, the top dose selected for females will be the maximum dose that can be successfully administered.

V.1.5. Observations: Pregnant females will be checked by study personnel each morning for new births and the duration of gestation will be calculated from day 0 of pregnancy as indicated by records from the supplier. Live pups will be counted and sexed on PND 0/1. In addition to the observation of parental animals, any abnormal behavior of the offspring will also be recorded.

A thorough physical examination of each rat being administered the test compound will be performed by study personnel at least once per day. The examination process will consist of each rat being removed from its home cage, individually handled, and carefully observed. Observations will include, but not be limited to, evaluation of skin and fur, eyes and mucous membranes, respiratory and circulatory effects, autonomic effects such as salivation, central nervous system effects, including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypies or bizarre behavior (e.g., self mutilation, walking backwards). All data related to the observation of rats will be documented in the study records by study personnel.

V.1.6. Body Weight and Food Consumption: Pups/litters will be weighed on PND 0/1 and weekly thereafter until PND 21. Additionally, pups will be weighed between PND 3 and 5 when litters are standardized. Male and female juvenile rats will be weighed daily prior to dosing. Food consumption will be monitored weekly by weighing the food hopper.

V.1.7. Vaginal Opening and Estrous Cyclicity: Beginning on PND 22, females will be examined after daily dosing for vaginal opening. Females will be examined for the appearance of a small "pin hole," a vaginal thread, or complete vaginal opening. Each observation will be recorded on the day (PND) it is observed. Beginning on the day of complete vaginal opening through the day of necropsy, vaginal smears will be obtained daily to evaluate estrous cyclicity. Vaginal smears will be collected via vaginal lavage (references 9-10). A small amount (approximately 0.2 ml) of sterile saline will be drawn

up into a disposable pipette tip. The rat will be restrained by grasping around the thorax with one hand, scruffing, or placing in dorsal recumbency and placing one hand over the thorax and applying gentle pressure. The tip of the pipette will be pushed gently into the entrance of the vagina to a depth of 2-5 mm and the fluid flushed into the vagina and back up into the pipette two or three times by gently squeezing and releasing the bulb of the pipette. If the fluid is 'cloudy' after the first flushing, no subsequent flushings will be done. The cell suspension will then be expelled onto a labeled glass slide and may be covered with a cover slip to provide a uniform field of depth and prevent movement of samples during transport. Slides will be evaluated shortly after collection to obviate fixing and staining; slides will be discarded after evaluation. Slides will be examined under low-power (10-40X) using a light microscope for the presence of leukocytes, nucleated epithelial cells, or cornified epithelial cells (references 6, 9-10). The vaginal smears will be classified as diestrus (predominance of leukocytes mixed with some cornified epithelial cells), proestrus (predominance of clumps of round, nucleated epithelial cells), or estrus (predominance of cornified epithelial cells). The estrous stage will be determined daily after vaginal opening and the age at first vaginal estrus noted. The vaginal opening and estrous cyclicity observations will be collected at approximately the same time each day.

V.1.8. Preputial Separation: Beginning on PND 30, males will be examined after daily dosing for preputial separation (PPS). Preputial separation will be determined by attempting to manually retract the prepuce using gentle pressure (reference 11). The appearance of partial and complete preputial separation, or a persistent thread of tissue between the glans and prepuce, will be recorded on the days they are observed. The PPS observations will be collected at approximately the same time each day.

V.1.9. Hormonal Assays: Euthanasia procedures are described in V.4.6. Blood from the trunk of the animal will be collected immediately following euthanasia by decapitation by inverting the trunk over a funnel and collecting the blood in serum separation tubes (*i.e.*, without EDTA or heparin). After collection, blood will be allowed to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 g. Serum will be removed and assayed immediately or aliquotted into microcentrifuge tubes and stored at -20 °C or colder for subsequent hormone and/or blood chemistry analyses. Hormonal measurements will be conducted using enzyme-linked immunosorbent assay (ELISA) and/or time-resolved immunofluorescent procedures. Details concerning use of the TOSOH Automated Enzyme Immunoassay System for measurement of thyroid and reproductive hormones are outlined in TOX SOP 145 (reference 12). Analysis of thyroid-stimulating hormone (TSH) will be conducted using a rat TSH ELISA kit per the manufacturer's (ALPCO Immunoassays or similar) instructions (reference 13). Briefly, 25µl of standard, blank, or sample will be added to the appropriate wells, 200µl of enzyme-labeled anti-rat TSH-antibody added to all wells, plate covered with the adhesive strip, and incubated for 18-20 hours at 4±2°C. Liquid will then be aspirated from each well and the plated washed 4 times (Wash: Each well filled with diluted wash solution (300µl) and let it stand for 2 minutes, then liquid removed by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel). The 3,3',5,5'-Tetra-Methyl-Benzidine (TMB) Substrate Solution (200µl) will be added to each well and the plate incubated in the dark for 10-30 minutes (timing based on color development), keeping the plate away from drafts and

other temperature fluctuations. Stop Solution (50µl) will be added to each well when the first four wells containing the highest concentration of standards develop obvious blue color. The optical density of each well will be determined within 30 minutes, using a microplate reader set to 450 nm. Test samples and quality control (QC) samples will be run in duplicate, with QC samples dispersed among the test samples. The hormone tests and kit(s) will be validated (i.e. kit standards perform as expected and hormone measures fall within assay performance criteria for controls) using blood and tissues collected from rats used on another protocol prior to use in the full study.

V.1.10. Clinical Chemistry: Euthanasia procedures are described in V.4.6. Blood from the trunk of the animal will be collected immediately following euthanasia by decapitation by inverting the trunk over a funnel and collecting the blood in serum separation tubes (*i.e.*, without EDTA or heparin). After collection, blood will be allowed to clot (minimum 30 minutes at room temperature), centrifuged, and serum separated for analysis. The serum may be aliquotted into microcentrifuge tubes and stored at -20 °C or colder for subsequent hormone and/or blood chemistry measurements if not completed on the day of collection. Serum will be evaluated for blood urea nitrogen (BUN) and creatinine (CREA). Additional chemistries may be analyzed if sufficient blood is available including, alkaline phosphatase (ALK P), aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose (GLU), total protein (TP), albumin (ALB), globulin (GLOB), cholesterol (CHOL), lactate dehydrogenase (LDH), total bilirubin (TBIL), calcium (CA), inorganic phosphate (PHOS), and electrolytes (sodium, potassium, chloride). Details concerning clinical chemistry parameters are outlined in TOX SOP 034 (reference 14). Animals will not be fasted prior to blood collection due to possible effects on hormone levels.

V.1.11. Gross Necropsy: Euthanasia procedures are described in section V.4.6. Necropsy will occur over two days for both females (PND 42-43) and males (PND 53-54), with an approximately equal number of animals from each treatment group being euthanized on each necropsy day. The order of necropsy will be randomized or evenly distributed across treatment groups each day. All animals are dosed the day of necropsy (between 0700 and 0900) and euthanasia will begin 2 hours following dosing. Due to diurnal fluctuations in thyroid hormone levels, all euthanasia will be completed by 1300 hours each day.

A full, detailed gross necropsy which includes careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents will be performed on all experimental animals at the termination of the study. Special attention will be paid to the organs of the reproductive system. Endpoint measures (organ weights, hormone levels, histology etc.) will not be collected from animals found dead or euthanized as moribund. All gross pathology changes will be recorded on CHPPM Form 333 or 23. At necropsy, the ovaries (without oviducts), uterus, testes, epididymides, ventral prostate, dorsolateral prostate, seminal vesicle with coagulating glands and fluid, levator ani plus bulbocavernosus muscles, thyroid (with attached portion of trachea), liver, kidneys, pituitary, and adrenals will be removed and weighed (except the thyroid/trachea which will be preserved prior to weighing). Weights will be recorded to one decimal place for organs reported in milligrams and to two decimal places for organs reported in grams. Kidneys, adrenals and ovaries will be

weighed as pairs. The left and right testes and the left and right epididymides will be weighed individually. Care will be taken to remove mesenteric fat with small surgical scissors from these tissues such that the fluid in the sex accessory glands is retained. After weighing the seminal vesicle with coagulating glands and fluid, the fluid will be removed and the combined weight of the seminal vesicle weight and the coagulating glands minus the fluid will be recorded. Care must also be taken to remove mesenteric fat from the uterine horns. The uterus and cervix will be separated from the vagina. The uterus will be placed on filter paper, slit to allow the fluid contents to leak out, gently blotted dry and weighed. Small tissues such as the adrenals and pituitary, as well as tissues that contain fluid, will be weighed immediately to prevent tissues from drying out prior to weighing. If such organs cannot be weighed immediately, they may be placed in a weigh-boat and a moist paper towel used to cover the weigh-boat, but the paper towel must not come into contact with the organs at any time. Additional tissues may be collected based on gross observations at the time of necropsy and at the discretion of the study director.

The kidneys, ovaries, uterus, thyroid (with attached trachea) will be placed in 10% buffered formalin for at least 24 hours for fixation. The thyroid (with parathyroids) will then be dissected from the trachea, blotted and weighed to the nearest 0.01 mg. A single testis and epididymis from each animal (either left or right, but the same side from all animals) will be placed in Davidson's fixative overnight (no longer than 24 hours) or 10% buffered formalin for at least 24 hours; however, fixing in Davidson's solution for less than 24 hours is preferred. After fixation, all tissues will be rinsed and stored in 70% ethanol until embedded in paraffin. Tissues will then be stained with hematoxylin and eosin (H&E) for subsequent histological evaluations.

V.1.12. Histopathology: Testis, epididymis, uterus, thyroid, one ovary, and one kidney will be evaluated for pathologic abnormalities and potential treatment-related effects for all treated and control animals.

Thyroid sections will be subjectively evaluated for follicular cell height and colloid area using a five point grading scale (1 = shortest/smallest; 5 = tallest/largest) and any abnormalities/lesions noted. A minimum of two sections of each of the two lobes of the thyroid will be evaluated.

Ovarian histology following H&E staining will include an evaluation of follicular development (including presence/absence of tertiary/antral follicles, presence/absence of corpora lutea, changes in corpus luteum development, changes in number of both primary and atretic follicles) in addition to any abnormalities/lesions, such as ovarian atrophy. Five random sections will be evaluated using the method of Smith BJ et al. (reference 15).

Uterine histology will document cases of uterine hyper- or hypotrophy as characterized by changes in uterine horn diameter and myometrial, stromal, or endometrial gland development. The final histological assessment will take into account the stage of the estrous cycle of the female at the time of necropsy as ovarian and uterine cellular changes are dependent upon endocrine status.

The testis will be examined for lesions including atrophy and tumors as well as treatment related effects including retained spermatids, missing germ cell layers or types, multinucleated giant cells, or sloughing of spermatogenic cells into the lumen (references 5 and 16). A longitudinal section of the intact epididymis, to include the caput, corpus, and cauda, will be examined in order to identify such lesions as sperm granulomas, leukocytic infiltration (inflammation), aberrant cell types within the lumen, or the absence of clear cells in the cauda epididymal epithelium.

V.1.13. Study Conduct: This study will be conducted in a manner consistent with the principles of 40 CFR (Code of Federal Regulations) Part 792 "Toxic Substance Control Act" (TSCA) Good Laboratory Practice (GLP) Regulation (reference 17). All study records will be made available to oversight organizations such as the EPA as needed. The investigators and technicians will adhere to The Guide for Care and Use of Laboratory Animals, 2011 (reference 18).

Records will be kept in standard USAPHC laboratory notebooks and/or three ring binders. Daily records will be kept on survival and clinical signs collected on the animals during the study. Procedures for preparation of any euthanasia solution, drug administration, animal blood collection, observation logs, morbidity/mortality logs, etc., will be stored with the study records. These records will be made available to oversight organizations such as the US EPA, Quality Systems Office, and the IACUC. The protocol, protocol amendments, raw data, statistical analysis, tabular calculations, and graphic analysis of the data will be saved with the study records. Additionally, memoranda to the study file, study logs, signature logs, final reports, and final report amendments will be archived at USAPHC. Some ancillary records such as maintenance and calibration logs, environmental monitoring logs, animal room log books, all veterinarian staff duties logbooks, training files, etc. may be stored in the archives but not stored with the study files.

V.1.14. Study Time Frame: Estimated initiation date for the study is February 2012. Estimated completion date for the study is May 2012.

V.2. Data Analysis: The day of complete preputial separation (PPS) and vaginal opening (VO) are the endpoints used in the analysis for the age at PPS and VO; however, if any animal within any treatment group shows incomplete PPS or VO for greater than three days, a separate analysis will be conducted using the ages at which partial separation or incomplete opening was first observed.

At the end of the study, the overall pattern of each female will be characterized as regularly cycling (having recurring 4- to 5-day cycles), irregularly cycling (having cycles with a period of diestrus longer than 3 days or a period of cornification longer than 2 days), or not cycling (having prolonged periods of either vaginal cornification or leukocytic smears). If there are too few days between vaginal opening and the end of the study to observe more than one cycle, the default assumption will be that animals are cycling regularly if the partial data fit the definition, and are irregularly cycling if the study ends without being able to distinguish between irregular cycling and not-cycling. Cycle length will be calculated consistently as either the number of days from one proestrus to the next proestrus, or from one diestrus to the next diestrus. Incomplete

cycles will not be counted in calculating mean cycle length. Mean cycle length for each animal will be calculated first, and the mean of these means will then be calculated to represent the group.

In cases where preputial separation or vaginal opening had not occurred prior to necropsy, the last day of observation +1 will be used as the age at PPS or VO when determining the mean for each group.

All data, except histology and estrous cyclicity evaluations, will be analyzed using a two-way Analysis of Variance (ANOVA) with Block (necropsy day) and Experimental Group as main effects. Age and body weight at vaginal opening, and all organ weights will also be analyzed by Analysis of Covariance (ANCOVA) using the body weight at PND 21 as the covariate. When statistically significant effects are observed ($p \leq 0.05$), post hoc tests will be used to compare pairs of dose groups and dose groups to the control group; Tukey's multiple, comparison test if the variance of the groups is similar and Dunnett's T3 test if the variances are unequal. Variance equality will be determined by Levene's test. If the data are not normally distributed, the data may be transformed appropriately prior to ANOVA/ANCOVA, or analyzed using a nonparametric Kruskal-Wallis test. Non-parametric analysis will be the method of last resort since it does not allow analysis of covariation. The unadjusted and adjusted values will also be analyzed for linear trend with dose level.

Chi-square analysis will be used to determine significant differences between the cycling status (cycling vs. not cycling) and percent of animals cycling between treated and control groups. When possible, appropriate statistical analysis, such as Chi-square analysis, will be applied to the histology results.

SPSS 16.0 will be used to perform the analyses and statistical significance will be defined as $p \leq 0.05$ for all tests.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered: The objective(s) addressed by this study are adverse health effects observed in rats administered the test substance by oral administration. The data from this study will aid in the assessment and evaluation of the toxic characteristics of the test substance. There are no appropriate animal substitutes (e.g., computer models, tissue/cell cultures) for the data that will be produced in this study. No non-animal alternative would provide the necessary toxicological information provided by this study. Therefore, it is necessary to perform this study in an animal model.

V.3.2. Animal Model and Species Justification: The test guidelines for the EPA state that the rat is the preferred species (references 5-6). Sprague Dawley or Wistar strains of rat are preferred for the assay. Sprague Dawley rats have been historically used for oral toxicity studies by USAPHC TOX and are the recommended species due to an historical and extensive database.

V.3.3. Laboratory animals

V.3.3.1. Genus and Species: *Rattus norvegicus*

V.3.3.2. Strain/Stock: Sprague Dawley

V.3.3.3. Source vendor: Charles River Laboratories, Wilmington, MA (USDA 14-R-0144) or other USAPHC approved vendor (for the timed-pregnant females)

V.3.3.4. Age (at exposure): PND 22-23

V.3.3.5. Weight (at exposure): Age appropriate

V.3.3.6. Sex: Male and female

V.3.3.7. Special Considerations: Timed-pregnant females must be pregnant for the first time and timed to deliver on the same day. All dams should be on GD 7, 8, 9 or 10 (all the same day) at the time of arrival in the facility.

V.3.4. Number of Animals Required (By Species): N=220

Minimum of 20 timed-pregnant female rats. Minimum of 90 juvenile rats obtained from the timed-pregnant females and selected for study treatment groups

Group	No. of Male Rats	No. of Female Rats
DAMS/PUPS		
Timed-Pregnant Females		20
Total Pups*	100	100
	TOTAL = 100	**TOTAL = 120**
ANIMALS USED ON STUDY		
Treatment Groups		
Vehicle Control	15	15
MTD mg/kg-day NTO	15	15
½ MTD mg/kg-day NTO	15	15
	TOTAL = 45	**TOTAL = 45**
	GRAND TOTAL = 100	**GRAND TOTAL = 120**

(*) The estimation of "Total Pups" was made with the assumptions that each timed-pregnant female would produce 10 pups and the sex ratio of the offspring would be 1:1. The "Total Pups" includes the 45 male and 45 female pups allotted into the treatment groups.

EPA EDSP Test Guidelines state that enough litters should be available to assure that a sufficient number of juveniles are available for 15 pups per treatment group. Enough litters also need to be available to obviate the need for placing littermates in the same experimental group, necessitating a minimum of 15 litters. Additionally, any dam that does not give birth by GD 23 and any litter with fewer than 8 total pups must be excluded from the study. Unthrifty litters and runted pups will also be excluded. To reduce variance in body weight, the heaviest and lightest pups of each sex will not be selected for study. Although a sex ratio of 1:1 is assumed for planning purposes, ratios other than 1:1 may require drawing pups from additional litters. Therefore, 20 timed-pregnant females are required to provide sufficient litters.

After animals have been assigned to experimental groups, females and extra pups will either be euthanized or transferred to another IACUC-approved protocol.

V.3.5. Refinement, Reduction, Replacement

V.3.5.1. Refinement: Standard rat enrichment will be implemented in accordance with TOX SOP 122 (reference 19) with the exception that no plastic materials will be allowed and all food must be certified to genestein+daidzein content <300 µg/g. Timed-pregnant females will be single-housed. After weaning and upon assignment to treatment groups (PND 22), juvenile rats will be pair housed (or 3 per cage for one group per treatment) with rats of the same sex and same treatment group housed together. All animals on this study will be handled on a frequent basis and provided a form of environmental enrichment throughout the study period. Due to the endocrine disrupting potential of plastics, nylabone and rodent retreats will not be used as environmental enrichment. Pair-housing and nesting material (nestlets, Alpha-dri Plus, Alpha Twist, or similar) will serve as alternate forms of enrichment. Another refinement is that moribund animals will be euthanized.

V.3.5.2. Reduction: This study combines two Guideline tests (male and female) into one study in order to maximize the use of the litters produced by the time-pregnant females and reduce the overall number of animals used when compared to running the studies separately. Additionally, data from previous studies was used to determine the MTD dose to eliminate the need for a range finding study. As indicated above, an effort is made to use the fewest number of dams as possible that will produce the quantity of offspring required for the study design. Tissue sharing may be allowed, however, only if doing so will not affect the validity of the study. The extra pups and mothers will be made available to transfer to other animal protocols.

V.3.5.3. Replacement: No non-animal alternatives are known to exist that will provide the required data. At this time, there are no non-animal alternatives that can fully replicate the complex processes that occur within an intact mammalian organism.

V.4. Technical Methods

V.4.1. Pain/Distress Assessment:

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals
NOTE: Estimates listed in Columns B-E below are based on a maximum number of animals.

V.4.1.1.1.1. Column B: 130 rats (20 timed-pregnant females, 110 pups born in-house but not placed in experimental groups)

V.4.1.1.1.2. Column C: 90 rats (15 rats per sex per experimental group; no pain or distress is anticipated from the administration of the test substance based on previous studies)

V.4.1.1.1.3. Column D: None

V.4.1.1.1.4. Column E: None

V.4.1.2. Pain Relief/Prevention

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization: For the female rats, anesthesia will be administered prior to euthanasia via decapitation in order to facilitate handling by study personnel. Anesthesia will be provided using ketamine (70-80mg/kg) in combination with xylazine (7-10mg/kg) given either intramuscularly or intraperitoneally in the same syringe using a 23-25 gauge needle. Males will not be anesthetized prior to decapitation due to the potential for effects of anesthetics such as ketamine on testosterone levels (references 5, 20-21).

V.4.1.2.2. Pre- and Post-procedural Provisions: A careful clinical examination will be made at least once each day during the pregnancy, lactation, and dosing phases. Appropriate actions will be taken to minimize loss of animals to the study or associated relevant data. Observations will be detailed and carefully recorded in the study records. Observations will include, but not be limited to, evaluation of skin and fur, eyes and mucous membranes, respiratory and circulatory effects, autonomic effects such as salivation, central nervous system effects, including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypes or bizarre behavior (e.g., self mutilation, walking backwards). Observation and body weight frequency are described in detail in sections V.1.5 and V.1.6.

V.4.1.2.3. Paralytics: None

V.4.2. Prolonged Restraint: N/A

V.4.3. Surgery: None

V.4.3.1. Pre-Surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-Surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures: None

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations

V.4.4.1. Injections: Anesthetics (ketamine and xylazine) will be administered in the same syringe either intraperitoneally (IP) or intramuscularly (IM) using a 23-25 gauge needle.

V.4.4.2. Biosamples: Blood will be collected from all experimental animals following euthanasia. All blood will be collected by placing the trunk of the animal over a funnel following decapitation as outlined in Sections V.1.9 and V.1.10.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: Animals will be identified by cage cards according to TOX SOP 003 (reference 22). An identification number (e.g., the last 3 digits of the animal number) will also be marked on the tail of each rat with a water-insoluble marker in order to ensure proper identification of rats when removed from their cages or when group-housed. Between PND 3 and 5, pups will be individually identified by markings on the tail or head with water-insoluble marker (due to the size of the pups coded markings may be used instead of numbers). On or about PND 21, individual animal numbers will be marked on the tails of juvenile rats as described above.

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures: The method of test substance administration will be oral gavage. Starting on PND 22/23, each experimental rat will be gently restrained either by placing the index and middle finger on either side of the animal's neck with the remainder of the hand used to support the body or by scruffing. Just prior to dosing, the index and middle finger can be used to tilt the animal's head back and the gavage needle inserted into either the side or the top of the mouth. The gavage needle will then be gently slid down the animal's esophagus until the hub of the gavage needle is at the opening of the animal's mouth. The 18 gauge gavage needle (1 to 2 inch length, 2.25 mm ball) is the proper size for juvenile rats; however, needle size may be optimized to animal size. If any resistance is felt during the gavage procedure, the gavage needle will be removed and the animal briefly released before the procedure is attempted again. Once the material has been dispensed, the animal will be briefly observed for any signs of aspiration.

V.4.4.8. Tissue Sharing: Tissue sharing may be allowed upon request provided there is no affect on the validity of the study.

V.4.5. Study Endpoint: The study endpoint is euthanasia. The scheduled euthanasia for female rats will be PND 42-43 while male rats will be euthanized on PND 53-54. Intervention euthanasia will be conducted on moribund animals, but animals are not expected to become ill on this study. Animals will be assessed for moribundity based on a weight of evidence of the following signs: impaired ambulation which prevents

animals from reaching food/water; excessive weight loss or emaciation (≥ 20% body weight loss compared to controls); lack of physical or mental alertness; prolonged labored breathing (e.g. lasting longer than 8 hours and accompanied by extreme lethargy); unabated seizure activity (e.g. lasting longer than 1 hour); inability to urinate or defecate for greater than 24 hours; or a prolonged inability to remain upright (e.g. lasting more than 2 hours). Animals considered to be moribund will be immediately euthanized as described in section V.4.6. The Attending Veterinarian will be consulted to evaluate potentially moribund animals, unless the PI/SD plans to immediately euthanize the animal. At the time of weaning, all dams and juvenile rats not placed in experimental groups or selected for use in validation assays, will either be transferred to another approved protocol or euthanized by CO_2.

V.4.6. Euthanasia: Euthanasia will be performed by study staff. Euthanasia of juvenile rats placed in treatment groups will be accomplished through decapitation due to effects of CO2 and anesthetics on hormone levels (references 5, 20-21). Euthanasia of moribund animals, dams, juvenile rats not placed in treatment groups, and pups culled to standardized litter size will be accomplished by asphyxiation from CO_2 exposure according to TOX SOP 066 (reference 23). Death of all rats euthanized by CO_2 will be ensured by thoracotomy or by decapitation. Thoracotomy will be accomplished by inserting a sharp blade into the chest cavity behind a rib and moving the blade the length of the rib. Decapitation of young pups will be accomplished using sharp scissors. Decapitation of juvenile rats will be accomplished using a guillotine. Female rats will first be anesthetized as described in V.4.1.2.1. All rats will be placed in a decapicone then positioned in the guillotine. When the animal is properly positioned, the guillotine will be used to decapitate the animals.

V.5. Veterinary Care

V.5.1. Husbandry Considerations: Animal rooms will be maintained according to the conditions specified in TOX SOP 004 (reference 24) with the exceptions: the light:dark cycle will be 14:10 with lights on at 0500 and off at 1900; animals will be provided **distilled water** (tap water is not acceptable per the EDSP Guidelines); and only certified rodent feed that contains **< 300 µg/g genistein + daidzein** will be used. The animals will be housed in plastic, solid-bottom shoebox cages and provided food and water *ad libitum*. Timed-pregnant females will be single-housed. After weaning and upon assignment to treatment groups (PND 21), juvenile rats will be pair housed (or 3 per cage for one group per treatment) with rats of the same sex and same treatment group housed together. Timed-pregnant female rats will be allowed to acclimate for 24 hours prior to handling by study personnel. After a 24 hour acclimation period, study personnel will conduct observations as described in V.4.1.2.2.

V.5.1.1. Study Room: Studies will be conducted at the AIPH Toxicology Portfolio animal facility, Bldg E-2100 or Bldg E-2101, study room as assigned. The animal facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

V.5.1.2. Special Husbandry Provisions: Animals on study will be provided **distilled water** only (tap water is not acceptable). Distilled water will be provided in glass water

bottles fitted with silicone stoppers and metal sipper tubes. Polycarbonate will not be used for drinking water storage or supply at any time. All feed provided to animals on study will be certified to contain **<300 µg/g genistein + daidzein** and all control and treatment groups will be provided feed from the same lot. Due to the endocrine disrupting potential of plastics, nylabone and rodent retreats will not be used as environmental enrichment. Pair-housing and nesting material (nestlets, Alpha-dri Plus, Alpha Twist, or similar) will serve as alternate forms of enrichment. Plastic materials will be avoided in all situations. The PI/SD will be consulted before using plastic in the study room for any purpose involving study animals, their feed, water, or housing. The light cycle in the room will be set to 14:10 with lights on at 0500 and off at 1900. General husbandry procedures performed by the animal care staff (e.g. cage changes) will need to be performed following morning dosing/observations, and collection of PPS, VO and cyclicity data.

V.5.1.3. Exceptions: None

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care: All animals will be observed at least twice daily by assigned Veterinary Medicine personnel (once daily on weekends and holidays) for husbandry conditions, humane care, and general health. Appropriate actions will be taken to minimize loss of animal data during the study (e.g., necropsy or refrigeration of those animals found dead).

V.5.2.2. Emergency Veterinary Medical Care: Veterinary care is available 24 hours a day, 7 days a week. In the case of an emergency health problem, if the PI or co-PI is unavailable or if the investigator staff and veterinary staff cannot reach consensus on treatment, the veterinarian has the authority to treat the animal, remove it from the experiment, institute appropriate measures to relieve severe pain or distress, or perform euthanasia if necessary. A veterinarian will conduct a physical exam of the animal if the veterinarian orders treatment or euthanasia and the PI/SD does not concur. To facilitate communication, the vet med staff will maintain an emergency contact roster in the vet tech office. In an emergency, the veterinary staff will phone the numbers (office, home, and mobile) listed for the PI, primary co-PI, or on-call designee. If the PI, primary co-PI, or on-call designee cannot be reached by phone within 15 minutes, then they are considered unavailable.

V.5.3. Environmental Enrichment

V.5.3.1 Enrichment Strategy: All enrichment will be provided in accordance with TOX SOP 122 (reference 19) with the exception that no plastic materials will be allowed and all food will be certified to genestein+daidzein content <300 µg/g. Pair-housing and nesting material (nestlets, Alpha-dri Plus, Alpha Twist, or similar) will serve as alternate forms of enrichment. Animals will be handled by study staff on a frequent basis and provided a form of environmental enrichment (e.g., nesting material) throughout the study.

V.5.3.2. Enrichment Restriction: Due to the endocrine disrupting potential of plastics, nylabone and rodent retreats will not be used as environmental enrichment. Pair-housing and nesting material (nestlets, Alpha-dri Plus, Alpha Twist, or similar) will serve as alternate forms of enrichment. Timed-pregnant females will be single-housed. After weaning and upon assignment to treatment groups (PND 22), juvenile rats will be pair housed (or 3 per cage for one group per treatment) with rats of the same sex and same treatment group housed together.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

Staff Member	Procedure	Training	Experience	Qualifications
Emily Lent	Oral gavage, observations, handling, blood collection, vaginal lavage, anesthesia, CO_2 euthanasia, decapitation (scissors) Necropsy	Rat handling, gavage, injections, blood collection, euthanasia (July 2007); Rat bleeding techniques & tissue collection (Apr 2008); necropsy (Jul/Oct 2007, Apr 2008, Nov 2010); Rat oral gavage (March 2008); Oral gavage in rats (May 2009); Rat euthanasia via CO_2 (Nov 2010); Vaginal lavage (To be provided (TBP)) Anesthesia (TBP) Decapitation (TBP)	13+ Yrs Animal Research	M.S., Wildlife Biology; Ph.D., Natural Resources and Environmental Studies
Mike Quinn	Oral gavage, observations, handling, blood collection, vaginal lavage CO_2 euthanasia, anesthesia, decapitation (guillotine and scissors) necropsy	Necropsy (May 2005, Oct 2007, Dec 2009); Oral gavage (March 2008); Rodent Handling Workshop (June 2005) Vaginal lavage (TBP) Anesthesia (TBP) Decapitation (TBP)	13+ Yrs Animal Research	Ph.D., Animal Science
Desmond Bannon	Oral gavage, observations, handling, blood collection, vaginal lavage CO_2 euthanasia, anesthesia, decapitation (guillotine and scissors) necropsy	Rodent & small animal handling workshop (MRICD, 1/2005) Vaginal lavage (TBP) Anesthesia (TBP) Decapitation (TBP)	14 years animal research 12 years clinical toxicology	Ph.D., D.A.B.T.
Lee Crouse	Oral gavage, observations, handling, blood collection, vaginal lavage	Humane Care & Use of Lab Animals (May 2000); Rodent Handling Techniques, WRAIR (includes oral gavage in	16+ Yrs Animal Research	M.S., Environmental Science

	CO_2 euthanasia, anesthesia, decapitation (guillotine and scissors) necropsy	rats; Nov 1996); Rat handling, gavage, injections, blood collection (July 2007); Rat oral gavage (March/May 2008); Necropsy procedures (bleeding, euthanasia, trimming weighing, April 2000); Rat Isoflurane anesthesia cardiac blood draw and CO_2 euthanasia (May 2009); necropsy (Oct/Dec 2007) Vaginal lavage (TBP) Anesthesia (TBP) Decapitation (TBP)		
Terry Hanna	Observations, handling, vaginal lavage, CO_2 euthanasia, decapitation (scissors) necropsy,	Rodent Handling & Techniques (1992); Rodent & Small Animal Handling Workshop (2004, 2005, 2006); Rat handling and gavage (2007), rat euthanasia via CO_2 with thoracotomy (3/2009); rat isoflurane anesthesia, cardiac blood draw, & CO_2 euthanasia (2009); necropsy (2009, 2010); Vaginal lavage (TBP) Anesthesia (TBP) Decapitation (TBP)	15+ Yrs Animal Research	ALAT
Alicia Shiflett	Observations, handling, vaginal lavage, CO_2 euthanasia, decapitation (scissors) necropsy	Rodent handling & techniques training; observations, handling/restraint, weighing, basic bleeding (Nov 2008); rat CO_2 euthanasia with thoracotomy (Mar 2009); rat necropsy & tissue collection (Mar 2008, Jan 2010)	2+ Yrs Animal Research	Associates Degree, Histology/Science
SPC Versteegh	Observations, handling, vaginal lavage, CO_2 euthanasia, decapitation (guillotine and scissors) necropsy	TBP	6 months clinical veterinary technician	Academy of Health Sciences Diploma, Animal Care Specialist

VII. BIOHAZARD/SAFETY:

In accordance with PHC Reg. 385-1, CHPPM Reg. 385-5, and TOX SOP 083, standard laboratory protection (e.g., glasses, gloves, labcoat) shall be used when handling the neat test substance. The test substance shall be stored in a sealed container at room temperature when not in use. Although the precise toxicity of the test substance may

not be known, information regarding its chemical family will be provided to study staff so that a reasonable assessment of its safety can be made (references 25, 26, and 27). While in animal rooms or while handling animals in a laboratory, personnel will wear appropriate PPE (e.g., lab coat, gloves, mask).

VIII. ENCLOSURES:

A. References

IX. ASSURANCES:

IX.1. As the Study Director/ Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard/Safety: I have taken into consideration, and I have made the proper coordinations regarding all applicable rules and regulations regarding radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training: I verify that the personnel performing the animal procedures/manipulations/ observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures/manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical, and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedures: I am conducting biomedical experiments which may potentially cause more than momentary ~~or slight~~ pain or distress to animals. This potential pain and/or distress WILL or (WILL NOT) be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

Emily May Lent — 20120125

Emily May Lent, Study Director (PI) — Date (YYYYMMDD)

IX.2. As the Primary Co-Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Authority: I understand that, as the Primary Co-Investigator, I am authorized and responsible for performing all procedures and manipulations as assigned to the SD/PI in the SD/PI's absence. This includes euthanasia of distressed animals.

C. Training: I verify that I am technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures/manipulations.

D. Responsibility: I acknowledge the inherent moral, ethical, and administrative obligations associated with the performance of this animal use protocol, and I assure that I will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

E. Painful Procedures: I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress WILL or (WILL NOT) be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

Michael J. Quinn – Primary Co-Investigator

20120130
Date (YYYYMMDD)

IX.3. ASSURANCES: As a Co-Investigator on this protocol, I provide the following assurances:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Authority: I understand that, as a Co-Investigator, I am authorized, responsible for, and willing to perform all procedures and manipulations as assigned to me by the SD/PI.

C. Training: I verify that I am technically competent and have been or will be properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the assigned procedures/manipulations performed by me.

D. Responsibility: I acknowledge the inherent moral and administrative obligations associated with the performance of this animal use protocol, and I assure that I will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to participate in this study in the spirit of the fourth "R", namely "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible, and conducting humane and lawful research.

E. Painful Procedures: I am participating in biomedical experiments, which may potentially cause more than momentary or slight pain or distress to animals. I will follow the direction of the SD/PI relative to potential pain and/or distress and relief by the use of anesthetics, analgesics and/or tranquilizers.

DESMOND BANNON — 1/30/2012
(PRINT) (Signature) (Date)
First name, MI, Last name of Co-Investigator

Lee CB Crouse — 1/30/2012
(PRINT) (Signature) (Date)
First name, MI, Last name of Co-Investigator

(PRINT) (Signature) (Date)
First name, MI, Last name of Co-Investigator

(PRINT) (Signature) (Date)
First name, MI, Last name of Co-Investigator

APPENDIX A

REFERENCES

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PROTOCOL REVIEW, SUPPORT, APPROVAL SHEET

PROTOCOL NUMBER: 0FP3 - 95 - 12-02-01 (SUB-JONO / TEST TYPE / IACUC NUMBER)	TITLE: Pubertal Development and Thyroid Function in Intact Juvenile Rats Exposed to NTO		
1. SCIENTIFIC MERIT (PEER REVIEW)			
1a. Printed Name (First, MI, Last) Wilfred McCain	1b. Title Toxicologist	1c. Signature MCCAIN.WILFRED.CARL.1229781845	1d. Date (yyyy/mm/dd) 20111215
2. DIRECTOR			
2a. Printed Name (First, MI, Last) COL Chris E. Hanson	2b. Title Portfolio Director, Toxicology	2c. Signature HANSON.CHRIS.E.1149169069	2d. Date (yyyy/mm/dd) 20111220
3. PROGRAM MANAGER			
3a. Printed Name (First, MI, Last) Glenn J. Leach	3b. Title Program Manager, TEP	3c. Signature	3d. Date (yyyy/mm/dd) 20111215
4. ATTENDING VETERINARIAN			
4a. Printed Name (First, MI, Last) MAJ Dawn Fitzhugh	4b. Title Attending Veterinarian	4c. Signature FITZHUGH.DAWN.CATHERINE.1036926127	4d. Date (yyyy/mm/dd) 20111222
5. ANALYTICAL CHEMISTRY (If Applicable)			
5a. Printed Name (First, MI, Last) David Morrow	5b. Title Chief, Laboratory Consultants Division	5c. Signature	5d. Date (yyyy/mm/dd) 20111228
6. SAFETY MANAGER			
6a. Printed Name (First, MI, Last) Roy Valiant	6b. Title Safety Manager	6c. Signature VALIANT.ROY.A.1081780591	6d. Date (yyyy/mm/dd) 20111215
7. STATISTICIAN (If Applicable)			
7a. Printed Name (First, MI, Last) Karen Deaver	7b. Title Statistician	7c. Signature DEAVER.KAREN.DEVILBISS.1400519672	7d. Date (yyyy/mm/dd) 20111228

PROTOCOL NUMBER: 0FP3 - 95 - 12-02-01 (SUB-JONO / TEST TYPE / IACUC NUMBER)	TITLE: Pubertal Development and Thyroid Function in Intact Juvenile Rats Exposed to NTO		
8. SIO-QAT (GLP COMPLIANCE AND QA SUPPORT)			
8a. Printed Name (First, MI, Last) Michael P. Kefauver	8b. Title Quality Assurance Specialist, USAPHC Quality Systems Office	8c. Signature KEFAUVER.MICHAEL.P.1229209678	8d. Date (yyyy/mm/dd) 20111228
9. CHAIRMAN, IACUC			
9a. Printed Name (First, MI, Last) Kristin Newkirk	9b. Title Chairman, IACUC	9c. Signature NEWKIRK.KRISTIN.TORELL.1014786895	9d. Date (yyyy/mm/dd) 20120201
10. INSTITUTIONAL OFFICIAL			
10a. Printed Name (First, MI, Last) John Resta	10b. Title Director, IPH	10c. Signature RESTA.JOHN.J.1229129303	10d. Date (yyyy/mm/dd) 20120206
11. STUDY DIRECTOR/PRINCIPAL INVESTIGATOR			
11a. Printed Name (First, MI, Last) Emily May Lent	11b. Title Toxicologist	11c. Signature LENT.EMILY.MAY.1296114371	11d. Date (yyyy/mm/dd) 20120207
12. OTHER ORGANIZATION(S) PROVIDING SUPPORT (AS NEEDED):			
12a. Printed Name (First, MI, Last)	12b. Title	12c. Signature	12d. Date (yyyy/mm/dd)
13. STUDY SPONSOR:			
13a. Printed Name (First, MI, Last) Kimberly Watts	13b. Title Logistics & Environmental Deputy Program Director	13c. Signature for	13d. Date (yyyy/mm/dd) 2013/10/23

PROTOCOL NUMBER: 0FP3 - 95 - 12-02-01 (SUB-JONO / TEST TYPE / IACUC NUMBER)	TITLE: Pubertal Development and Thyroid Function in Intact Juvenile Rats Exposed to NTO		
8. SIO-QAT (GLP COMPLIANCE AND QA SUPPORT)			
8a. Printed Name (First, MI, Last) Michael P. Kefauver	8b. Title Quality Assurance Specialist, USAPHC Quality Systems Office	8c. Signature KEFAUVER.MICHAEL.P.1229209678	8d. Date (yyyy/mm/dd) 20111228
9. CHAIRMAN, IACUC			
9a. Printed Name (First, MI, Last) Kristin Newkirk	9b. Title Chairman, IACUC	9c. Signature NEWKIRK.KRISTIN.TORELL.1014786893	9d. Date (yyyy/mm/dd) 20120201
10. INSTITUTIONAL OFFICIAL			
10a. Printed Name (First, MI, Last) John Resta	10b. Title Director, IPH	10c. Signature RESTA.JOHN.J.1229129303	10d. Date (yyyy/mm/dd) 20120206
11. STUDY DIRECTOR/PRINCIPAL INVESTIGATOR			
11a. Printed Name (First, MI, Last) Emily May Lent	11b. Title Toxicologist	11c. Signature LENT.EMILY.MAY.1296114371	11d. Date (yyyy/mm/dd) 20120207
12. OTHER ORGANIZATION(S) PROVIDING SUPPORT (AS NEEDED):			
12a. Printed Name (First, MI, Last)	12b. Title	12c. Signature	12d. Date (yyyy/mm/dd)
13. STUDY SPONSOR:			
13a. Printed Name (First, MI, Last) Kimberly Watts	13b. Title Logistics & Environmental Deputy Program Director	13c. Signature	13d. Date (yyyy/mm/dd)

USACHPPM PROTOCOL MODIFICATION

For use of this form, see DTOX SOP 085

1. DATE: (YYYY/MM/DD) 2012/02/29	2. PROTOCOL NUMBER: 0FP3-95-12-02-01	3. MODIFICATION#: 1

4. PROTOCOL TITLE: Pubertal Develpment and Thyroid Function in Intact Juvenile Rats Exposed to NTO

5. STUDY DIRECTOR/PRINCIPAL INVESTIGATOR:	6. WORK PHONE:	7. OFFICE SYMBOL:
Emily May Lent	436-7749	MCHB-IP-TEP

SECTION I. PREVIOUSLY APPROVED AND CURRENTLY IN USE PROTOCOL MODIFICATIONS:

1. MODIFICATION NUMBER	2. SHORT DESCRIPTION OF PRIOR APPROVED MODIFICATION(S)	3. NO. & SPECIES OF ANIMAL REQUESTED	4. APPROVED DATE (XX XXX XXXX

SECTION II. CHANGE IN TOTAL # OF ANIMALS USED AND/OR CHANGE IN USDA PAIN CATEGORY

1a. CHANGE: INCREASE TOTAL APPROVED ANIMALS BY: 22 — 1b. N/A ☐

2. ORIGINAL PROTOCOL TOTAL: 220 — 3. PROTOCOL TOTAL AFTER MODIFICATION: 242

2a. USDA pain cat:	B: 130	C: 90	D: 0	E. 0	3a. USDA pain cat:	B: 152	C: 90	D: 0	E: 0

4. Yes	No	
☐	☒	Modification requires specific changes or additions to the experimental design of the protocol. (Section V.I. of the template.)
☒	☐	Modification requires changes to the technical methods, i.e., procedures, routes of administration, biosample collection, etc. (Section V.4. of the protocol template.) Indicate training of personnel for new methods, procedures being used.
☐	☒	Modification requires additions or changes in personnel performing procedures. (Section VI of the protocol template.) Include training and qualification information and tasks that each individual will be performing. If changing the Study Director/PI, a signed Assurance Statement needs to be submitted with the modifications.

SECTION III. MODIFICATION/JUSTIFICATION

Explain the modification indicated above in the area below. Indicate any changes to the 3R's (Refinement, Reduction, Replacement) resulting from changes in number of animals

PROTOCOL Page, paragraph, section	
Page 13 V.3.4. Number of Animals Required	1. MODIFICATION: Increase the number of animals required from 220 to 242 (2 timed-pregnant females plus an estimated 10 pups per dam = 22 additional animals).

1a. JUSTIFICATION/REASON:

Two additional timed-pregnant females were sent by Charles River beyond the 20 approved and ordered. Although the protocol indicates that 20 dams will provide sufficient litters and pups, weight differences among the dams upon arrival suggest that some of the dams may not be pregnant, may be carrying very small litters, or may be at an earlier gestational day than indicated by the vendor and thus may not provide the required number of litters/pups within the required timeframe. (Body weights on day of receipt ranged from 195 to 289 gms.) Adding the additional dams to the protocol will reduce the likelihood of having insufficient litters/pups and will reduce the chances of needing to place litter mates in the same treatment group. It is currently the intention that the pups/juvenile rats not placed in experimental groups in this protocol will be transferred to another approved protocol that requires 60 male and 40 female rats. Adding these two dams to this protocol may also ensure that a sufficient number of pups of the proper sexes are available for transfer (must provide all 100 rats as mixed sources are not allowed) and may obviate the need to euthanize the unused pups from this protocol or order additional rats for the subsequent protocol.

PROTOCOL Page, paragraph, section	*Explain the modification indicated above in the area below. Indicate any changes to the 3R's (Refinement, Reduction, Replacement) resulting from changes in number of animals used.*
Page 17 V.4.6. Euthanasia	2. MODIFICATION: Replace the sentences: "All rats will be placed in a decapicone then positioned in the guillotine. When the animal is properly positioned the guillotine will be used to decapitate the animals." With: "Rats may be placed in a decapicone then positioned in the guillotine. When the animal is properly positioned, the guillotine will be used to decapitate the animals."
	2a. JUSTIFICATION/REASON: Instructor providing guillotine decapitation training indicated that the use of decapicones can impair the blood collection procedure and can hinder the movement of the guillotine blade.
	3. MODIFICATION:
	3a. JUSTIFICATION/REASON:
	4. MODIFICATION:
	4a. JUSTIFICATION/REASON:

Continued on next page YES ☐ NO ☒

SECTION IV. SIGNATURES AND DATES

1. STUDY DIRECTOR: (Printed Name) Emily May Lent	Signature Emily May Lent	DATE: (yyyy/mm/dd) 2012/03/02
2. PROGRAM MANAGER:: (Printed Name) Shannon M. WALLACE, LTC	Signature	DATE: (yyyy/mm/dd) 2012/03/02
3. ATTENDING VETERINARIAN: (Printed Name) Patty C Fitzhugh VMD	Signature	DATE: (yyyy/mm/dd) 2012/03/02
4. CHPPM SAFETY OFFICER/OSC HEALTH REP: (IF APPLICABLE) N/A	Signature	DATE: (yyyy/mm/dd)
5. CHAIR, IACUC OR QA (If no animal related changes): (Printed Name) KRISTIN T. NEWKIRK	APPROVED / REVIEWED YES ☒ NO ☐ Signature	DATE: (yyyy/mm/dd) 2012/03/02

USACHPPM PROTOCOL MODIFICATION

For use of this form, see DTOX SOP 085

1. DATE: (YYYY/MM/DD) 2012/04/04	2. PROTOCOL NUMBER: 0FP3-95-12-02-01	3. MODIFICATION#: 2

4. PROTOCOL TITLE: Pubertal Development and Thyroid Function in Intact Juvenile Rats Exposed to NTO

5. STUDY DIRECTOR/PRINCIPAL INVESTIGATOR:	6. WORK PHONE:	7. OFFICE SYMBOL:
Emily Lent	436-7749	MCHB-IP-TTE

SECTION I. PREVIOUSLY APPROVED AND CURRENTLY IN USE PROTOCOL MODIFICATIONS:

1. MODIFICATION NUMBER	2. SHORT DESCRIPTION OF PRIOR APPROVED MODIFICATION(S)	3. NO. & SPECIES OF ANIMAL REQUESTED	4. APPROVED DATE (XX XXX XXXX
1	Increased number of animals by 22 to 242 animals due to extra TP females received from vendor. Added option of using decapicones with the guillotine.	22	2 Mar 2012

SECTION II. CHANGE IN TOTAL # OF ANIMALS USED AND/OR CHANGE IN USDA PAIN CATEGORY

1a. CHANGE: INCREASE TOTAL APPROVED ANIMALS BY: 0	1b. N/A ☒

2. ORIGINAL PROTOCOL TOTAL: 242					3. PROTOCOL TOTAL AFTER MODIFICATION: 242				
2a. USDA pain cat:	B:	C:	D:	E.	3a. USDA pain cat:	B:	C:	D:	E:

4. Yes	No	
☐	☒	Modification requires specific changes or additions to the experimental design of the protocol. (Section V.I. of the template.)
☒	☐	Modification requires changes to the technical methods, i.e., procedures, routes of administration, biosample collection, etc. (Section V.4. of the protocol template.) Indicate training of personnel for new methods, procedures being used.
☐	☒	Modification requires additions or changes in personnel performing procedures. (Section VI of the protocol template.) Include training and qualification information and tasks that each individual will be performing. If changing the Study Director/PI, a signed Assurance Statement needs to be submitted with the modifications.

PROTOCOL Page, paragraph, section	SECTION III. MODIFICATION/JUSTIFICATION *Explain the modification indicated above in the area below. Indicate any changes to the 3R's (Refinement, Reduction, Replacement) resulting from changes in number of animals*
Page 17, V.4.5. Study Endpoint	1. MODIFICATION: Change the last sentence of the section beginning "At the time of weaning..." to: "All dams and juveniles that are not placed in experimental groups or selected for use in validation assays, will either be transferred to another approved protocol or euthanized by CO_2. The exact date of transfer or euthanasia will be determined by the SD/PI, but will not exceed 10 days past PND21."
	1a. JUSTIFICATION/REASON: The extra pups that are not needed on this study may be transferred for use on other studies. Adding a holding timeframe of up to 10 days past PND21 will allow for sufficient time for a protocol receiving the animals to be modified as necessary (via an IACUC approved modification).

PROTOCOL Page, paragraph, section	*Explain the modification indicated above in the area below. Indicate any changes to the 3R's (Refinement, Reduction, Replacement) resulting from changes in number of animals used.*
Pg 17, V.5.1. Husbandry K. Newkirk 5 April 2012	2. MODIFICATION: See attached dated 5 April 2012. K. Newkirk 5 April 2012 Emily May Lent 5 April 2012
	2a. JUSTIFICATION/REASON: See attached dated 5 April 2012. K. Newkirk 5 April 2012 Emily May Lent 5 April 2012
	3. MODIFICATION:
	3a. JUSTIFICATION/REASON:
	4. MODIFICATION:
	4a. JUSTIFICATION/REASON:

Continued on next page YES ☐ NO ☒

SECTION IV. SIGNATURES AND DATES

1. STUDY DIRECTOR: (Printed Name) Emily Lent, PhD	Signature: Emily May Lent	DATE: (yyyy/mm/dd) 2012/04/04
2. PROGRAM MANAGER:: (Printed Name)	Signature	DATE: (yyyy/mm/dd)
3. ATTENDING VETERINARIAN: (Printed Name)	Signature: STUTLER.SHANNON.AMES.1094317378 Digitally signed by STUTLER.SHANNON.AMES.1094317378 DN: c=US, o=U.S. Government, ou=DoD, ou=PKI, ou=USA, cn=STUTLER.SHANNON.AMES.1094317378 Date: 2012.04.05 16:33:10 -04'00'	DATE: (yyyy/mm/dd)
4. CHPPM SAFETY OFFICER/OCC HEALTH REP: (IF APPLICABLE)	Signature	DATE: (yyyy/mm/dd)
5. CHAIR, IACUC OR QA (If no animal related changes): (Printed Name) Kristin Newkirk	APPROVED / REVIEWED YES ☐ NO ☐ Signature: Kristin D Newkirk	DATE: (yyyy/mm/dd) 2012/04/04

Newkirk, Kristin T Ms CIV USA MEDCOM PHC

From: Newkirk, Kristin T Ms CIV USA MEDCOM PHC
Sent: Thursday, April 05, 2012 7:39 AM
To: Lent, Emily M Dr CIV USA MEDCOM PHC
Subject: FW: Mod to animal use protocol (UNCLASSIFIED)

Importance: High

Classification: UNCLASSIFIED
Caveats: NONE

Emily,

I have not heard from LTC Stutler on her taking any dams yet....

This is the additional text that I propose to add to the modification based on LTC Stutler's comments yesterday evening:

Page 17, V.5.1. Husbandry considerations:
Modification: Add to the end of the paragraph, "Juveniles that are not placed in experimental groups or selected for use in the validation assays will be weaned at PND21 +/- 3 days. They will be group or pair-housed. Dams may be pair housed after juveniles are weaned."

Justification: The experiment and assays on the study must adhere to the recommendations in the test guidelines of weaning at age PND21. Flexibility for the weaning day is acceptable for the extra animals that will not be used in the experimental groups or the assays. These extra animals will be available for use on another protocol (will be transferred) or will be euthanized.

Let me know if you want anything changed, otherwise I will write it in and bring it over.

Thanks,
Kristin

-----Original Message-----
From: Stutler, Shannon A LTC MIL USA MEDCOM USAMRICD
Sent: Wednesday, April 04, 2012 6:40 PM
To: Newkirk, Kristin T Ms CIV USA MEDCOM PHC
Subject: RE: Mod to animal use protocol (UNCLASSIFIED)

Classification: UNCLASSIFIED
Caveats: NONE

PS- let me check with the techs re: needing the dams. Hate to waste something we could potentially use...

-----Original Message-----
From: Newkirk, Kristin T Ms CIV USA MEDCOM PHC
Sent: Wednesday, April 04, 2012 6:26 PM
To: Stutler, Shannon A LTC MIL USA MEDCOM USAMRICD
Cc: Lent, Emily M Dr CIV USA MEDCOM PHC
Subject: Re: Mod to animal use protocol (UNCLASSIFIED)

Thanks Shannon. I will make the pen and ink changes and give it to Emily tomorrow to initial. I don't think there will be any issues with removing the dams- hopefully they can just fit that into their busy schedule dosing for this next phase. Lee will not be needing the dams at all, and Dawn didn't say anything about keeping any for training- do you want any? If not they will be euthanized. Is weaning by COB Friday okay, or can they go through the weekend? The "generally not exceeding..." phrasing can be added too, but I don't anticipate a need for this again on this protocol; TP females were only to be ordered once to produce the juveniles needed for the main part of the study.
Thanks again,
Kristin

----- Original Message -----
From: Stutler, Shannon A LTC MIL USA MEDCOM USAMRICD
Sent: Wednesday, April 04, 2012 05:57 PM
To: Newkirk, Kristin T Ms CIV USA MEDCOM PHC
Cc: Fitzhugh, Dawn C MAJ MIL USA MEDCOM PHC
Subject: RE: Mod to animal use protocol (UNCLASSIFIED)

Classification: UNCLASSIFIED
Caveats: NONE

Hey, Kristin,

Either one is fine, I support the mod described below (bless your little blackberry fingers!) except I would like one thing added and recommend another thing be added: suggest you do NOT limit the holding time to 10 days past the PND 21. You could say something like "generally not to exceed 10 days past..." That gives you some flexibility in the case of unforeseen circumstances. I would also like to see it clearly stated that the pups will be weaned at PND 21 (or, give a range of a couple days +/-). This would actually modify the husbandry section of the protocol.

Please make the weaning change, and if you feel compelled (I always recommend as much flexibility as possible) make the 10 day limit change and consider it approved as of today (4-4-12).

Thanks,
Shannon

-----Original Message-----
From: Newkirk, Kristin T Ms CIV USA MEDCOM PHC
Sent: Wednesday, April 04, 2012 5:17 PM
To: Stutler, Shannon A LTC MIL USA MEDCOM USAMRICD
Subject: Mod to animal use protocol

Shannon,

Normally I wouldn't bug you but a need for a modification to a protocol came up today that I wanted to inform you of since you are our alternate vet on the committee. I had to leave work already so I can't send you the mod form; I'll summarize below (typing away on my BB). The mod is to hold dams and their 21 day old pups on a protocol past the day the protocol stated they would be euthanized or transferred to another protocol. Currently it reads this is done at time of weaning at PND21. The pups are extras that were born but ultimately not needed on the next part of the study (timed-pregnant females received from CRL). Emily Lent is the PI (her protocol is posted on the IACUC project SP site if you want to look it up). Lee Crouse is going to take all the pups for his new protocol but has to modify his protocol to do that (I sent the mod for this to the IACUC yesterday).

I helped Emily write the modification, changing the last sentence of section V.4.5. Study Endpoint, to read "All dams and juveniles that are not placed in experimental groups or selected for use in validation assays, will either be transferred to another approved protocol or euthanized by CO2. The exact date of transfer or euthanasia will be determined by the SD/PI, but will not exceed 10 days past PND21." The justification for this is: "The extra pups that are not needed on this study may be transferred for use on other studies. Adding a holding timeframe of up to 10 days past PND21 will allow for sufficient time for a protocol receiving the animals to be modified as necessary (via an IACUC approved modification)." There was only one section in the protocol that needed to be changed. The protocol already addressed animal sharing in the Reduction section.
Today and yesterday was PND21 for the pups, which is why we needed this in place. We missed this timing loop-hole in the protocol. Our current practice for minor mods is for the SD, AV, and Chair to sign the mod form. I have already signed it, but I wanted to make you aware of it as well since you are MAJ Fitzhugh's alternate on the committee. I can scan the mod first thing tomorrow morning to sign and you can scan and email back, or you can reply via email and I will keep the response with the mod.
Let me know which is easier.

Thanks,
Kristin
Classification: UNCLASSIFIED
Caveats: NONE

Classification: UNCLASSIFIED
Caveats: NONE

Classification: UNCLASSIFIED
Caveats: NONE

USACHPPM PROTOCOL MODIFICATION

For use of this form, see DTOX SOP 085

1. DATE: (YYYY/MM/DD) 2012/04/27	2. PROTOCOL NUMBER: 0FP3-95-12-02-01	3. MODIFICATION#: 3

4. PROTOCOL TITLE: Pubertal Development and Thyroid Function in Intact Juvenile Rats Exposed to NTO

5. STUDY DIRECTOR/PRINCIPAL INVESTIGATOR:	6. WORK PHONE:	7. OFFICE SYMBOL:
Emily Lent	436-7749	MCHB-IP-TEP

SECTION I. PREVIOUSLY APPROVED AND CURRENTLY IN USE PROTOCOL MODIFICATIONS

1. MODIFICATION NUMBER	2. SHORT DESCRIPTION OF PRIOR APPROVED MODIFICATION(S)	3. NO. & SPECIES OF ANIMAL REQUESTED	4. APPROVED DATE (XX XXX XXXX
1	Increased number of animals by 22 to 242 animals due to extra TP females received from vendor. Added option of using decapicones with the guillotine.	22	2 Mar 2012
2	Study endpoint modified for rats not placed in experimentals groups to allow holding up to 10 days past PND21 to facilitate transfer to another protocol.		4 Apr 2012

SECTION II. CHANGE IN TOTAL # OF ANIMALS USED AND/OR CHANGE IN USDA PAIN CATEGORY

1a. CHANGE: INCREASE TOTAL APPROVED ANIMALS BY: 0 — 1b. N/A ☒

2. ORIGINAL PROTOCOL TOTAL: 242 — 3. PROTOCOL TOTAL AFTER MODIFICATION: 242

2a. USDA pain cat:	B:	C:	D:	E.	3a. USDA pain cat:	B:	C:	D:	E:

4. Yes	No	
☐	☒	Modification requires specific changes or additions to the experimental design of the protocol. (Section V.I. of the template.)
☒	☐	Modification requires changes to the technical methods, i.e., procedures, routes of administration, biosample collection, etc. (Section V.4. of the protocol template.) Indicate training of personnel for new methods, procedures being used.
☐	☒	Modification requires additions or changes in personnel performing procedures. (Section VI of the protocol template.) Include training and qualification information and tasks that each individual will be performing. If changing the Study Director/PI, a signed Assurance Statement needs to be submitted with the modifications.

SECTION III. MODIFICATION/JUSTIFICATION

Explain the modification indicated above in the area below. Indicate any changes to the 3Rs (Refinement, Reduction, Replacement) resulting from changes in number of animals.

Protocol Page, paragraph section	
Page 15, V.4.1.2.1. Anesthesia/ Analgesia/ Tranquilization	1. MODIFICATION: Replace the sentence: "Males will not be anesthetized prior to decapitation due to the potential for effects of anesthetics such as ketamine on testosterone levels." With: "For male rats, CO2 may be administered prior to euthanasia via decapitation, for a period of no more 60 seconds, to facilitate handling by study personnel. Males may be placed in a bell-jar chamber pre-charged with CO2, exposure will be limited to 60 seconds, after which decapitation will be performed even if the animal has not been fully anesthetized. Alternatively, anesthesia using ketamine (70-80 mg/kg) in combination with xylazine (7-10 mg/kg) may be given, either itramuscularly or intraperitoneally in the same syringe using a 23-25 gauge needle, prior to euthanasia via decapitation. If injectable anesthetics are used, decapitation will be performed within 2 minutes of administration regardless of the level of anesthesia achieved." 1a. JUSTIFICATION/REASON: Necropsy of the female rats revealed that precise positioning of rats in the guillotine is necessary to avoid damage to the thyroid, a crucial tissue in this study. Such positioning of alert or un-anesthetized rats will not be possible. Providing CO2 or injectable anesthesia will facilitate handling and proper positioning in the guillotine to reduce loss of thyroid tissue. Limiting the exposure to very short periods of time will minimize the potential effects on testosterone levels.

PROTOCOL Page, paragraph, section	Explain the modification indicated above in the area below. Indicate any changes to the 3R's (Refinement, Reduction, Replacement) resulting from changes in number of animals used.
	2. MODIFICATION:
	2a. JUSTIFICATION/REASON:
	3. MODIFICATION:
	3a. JUSTIFICATION/REASON:
	4. MODIFICATION:
	4a. JUSTIFICATION/REASON:

Continued on next page YES ☐ NO ☒

SECTION IV - SIGNATURES AND DATES

1. STUDY DIRECTOR: (Printed Name) Emily Lent, PhD	Signature Emily May Lent	DATE: (yyyy/mm/dd) 2012/05/02
2. PROGRAM MANAGER:: (Printed Name) Shannon Wallace	Signature	DATE: (yyyy/mm/dd) 2012/05/02
3. ATTENDING VETERINARIAN: (Printed Name) Dawn Fitzhugh	Signature	DATE: (yyyy/mm/dd) 2012/05/02
4. CHPPM SAFETY OFFICER/OCC HEALTH REP: (IF APPLICABLE)	Signature	DATE: (yyyy/mm/dd)
5. CHAIR, IACUC OR QA (If no animal related changes): (Printed Name) Kristin Newkirk	APPROVED / REVIEWED YES ☒ NO ☐ Signature	DATE: (yyyy/mm/dd) 2012/05/03