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Toxicological Study No. S.0008062-15, March 2016
Toxicology Directorate
Division of Toxicologic Pathology

Pathology Report for One-Generation Study of the Effects of 3-nitro-1,2,4-triazol-5-one (NTO) to the Northern Leopard Frog (Lithobates pipiens) Under Static Renewal Test Conditions, December 28, 2015

Protocol No. RP4OK.468.001
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14. ABSTRACT
Larval (tadpole) northern leopard frogs were exposed to 3-nitro-1,2,4-triazol-5-one (NTO) or to water control until they developed into froglets (up to 90 days) at Gosner Stage 46 (Gosner, 1960). Animals were humanely euthanized and kidney and liver tissues examined microscopically to detect indications of toxicity. No evidence of toxicity or developmental delay was observed, suggesting NTO may not affect anuran renal or hepatic development. Tissues did have inflammatory changes normally associated with infection by mycobacteria, fungi or foreign materials.

15. SUBJECT TERMS
Anuran; histopathology; kidney; Lithobates pipiens; liver; Northern Leopard frogs; 3-nitro-1,2,4-triazol-5-one (NTO); toxicity

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This pathology investigation was conducted in a manner consistent with the principles of the United States Environmental Protection Agency (USEPA) Good Laboratory Practice regulations of the Toxic Substances Control Act (TSCA), as detailed in 40 CFR Part 792, plus amendments.

CARROLL.ERICA.E.1027432413

28 December 2015
Date

Erica E. Carroll, DVM, PhD, Diplomate ACVP
LTC, VC
Study Pathologist
Toxicology Portfolio
Army Public Health Center (Provisional)

Table of Contents

Toxicological Study No. S.0008062-15
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1 Summary

1.1 Purpose

Larval (tadpole) anuran *Lithobates pipiens (*northern leopard frog, formerly recognized as *Rana pipiens)* were exposed to 3-nitro-1,2,4-triazol-5-one (NTO) or to NTO-free dilution water control. Organisms were exposed in water until the majority of control organisms emerged from the water as air-breathing froglets (up to 90 days), which is equivalent to Gosner Stage 46 (Gosner, 1960). All subjects were sacrificed on the day they reached Gosner Stage 46; that varied from ~45 days post-hatch (PH) in the controls to 60+ days PH in the high NTO concentration. Froglets were humanely euthanized using MS-222 and liver and kidney tissues dissected, fixed in formalin and shipped overnight to the Division of Toxicologic Pathology, Army Public Health Center (Provisional) (APHC (Prov)) for histologic processing and evaluation.

1.2 Authority

Funding for this work was provided under Military Interdepartmental Purchase Request No. W74RDV53092356. This Toxicology Study addresses, in part, the environment, safety and occupational health (ESOH) requirements outlined in DoD Directive 4715.1E, “Environment, Safety, and Occupational Health,” 2005, and was performed as SERDP Project ER-2223, DEVELOPMENT OF ENVIRONMENTAL HEALTH CRITERIA FOR INSENSITIVE MUNITIONS (IMX-101-104). The Sponsor is the Strategic Environmental Research and Development Program (SERDP), U.S. Department of Defense.

2 References

Goff G.L., F.L. Frye, and E.R. Jacobson, eds. 1984. *Disease of Amphibians and Reptiles*. New York: Plenum Press. p.627

Gosner, K.L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*. 16(3):183-190.

Norris D.A. and J.A. Carr, eds. 2013. *Vertebrate Endocrinology*, *5th Edition*. London, UK: Elsevier. p.302.

3 Methods

Due to their small size, samples were placed whole into labelled cassettes with protective sponges, processed per standard operating procedures, embedded in paraffin, sectioned to 4 micrometers (um) thickness onto microscope slides, stained with hematoxylin and eosin, and cover-slipped.

4 Individual Animal Descriptions

- 00A1 Kidney (Control): Disrupting approximately 20% of the renal parenchyma (tubules and hematopoietic tissue) are multiple aggregates of flattened macrophages encircling necrotic cellular debris and granulocytes (granulomas). There is moderate amount of black pigment at the periphery and multifocally scattered within the section. About 10% of the section is autolyzed. There is ureter tissue with a few mitotic figures.

- 00A1 Kidney (Control): Granulomas, multifocal, mild. Liver (Control): Approximately 25% of the tissue is autolyzed. There is extracellular and intracellular pigment, unassociated with red blood cells; not considered to be “formalin pigment” (acid hematin).

- 00B1 Kidney (Control): Abundant subcapsular hematopoiesis. Ovary with germ cells (primary oocytes) is present. There is a small amount of peripheral autolysis. Pigment is moderate, superficial, around the ovary and vessels. Ureteral tissue is present. Few small granulomas are present.

- 00B1 Kidney (Control): Granulomas, multifocal, minimal.
  Liver (Control): About 5% autolyzed. No observed lesions.

- 00B2 Kidney (Control): Pigment only at periphery, at capsule. Ureter is present.
  Liver (Control): 10% autolysis. No lesions observed.

- 00C1 Kidney (Control): Granulomas, multifocal, minimal.
  Liver (Control): No observed lesions.

- 00C2 Kidney (Control): Granulomas, multifocal, minimal.
  Liver (Control): No lesions observed.

- 20A1 Kidney (High): Granulomas, multiple, mild. Ovary with primary oocytes.
  Liver (High): No lesions observed.

- 20B1 Kidney (High): Granulomas, multiple, mild.
  Liver (High): No lesions observed.

- 20B2 Kidney (High): Granulomas, multifocal, moderate.
  Liver (High): Approximately 30% autolyzed. No lesions observed.

- 20C1 Kidney (High): Granulomas, multiple, mild.

Liver (High): No lesions observed.

- 20C2 Kidney (High): Granulomas, multiple, mild. Ovarian tissue with oocytes.
  Liver (High): 10% autolyzed. No lesions observed.

5 Results

Histopathologic changes are itemized in Table 1. Pathology was absent in liver sections. Intranuclear vacuoles were present in liver sections in two control frogs, which are believed to be artifact. The only recognized pathology was in renal tissue, was present in five of five examined high-exposure frogs and four of five control frog tissues.

Table 1. Histopathologic Findings in NTO-Exposed Anurans

identity #'s-->	00A1	00B1	00B2	00C1	00C2	20A1	20B1	20B2	20C1	20C2
Treatment-->	CTRL	CTRL	CTRL	CTRL	CTRL	HIGH	HIGH	HIGH	HIGH	HIGH
Histopathologic Findings:										
KIDNEY										
Granulomas	2	1	0	1	1	2	2	3	2	2
LIVER										
Intranuclear vacuoles (seen at 10X)*	0	1	0	0	2	0	0	0	0	0

* 20C1-A few nuclei are refractile, like precursors to the change seen in 00C2. Suspect artifact.

Scoring Criteria: 0 means present in less than 1% of affected cell type. '1' (minimal) up to 5%; '2' (mild) 6-20%. '3' (moderate) 21-40% of cell type affected. '4' (marked) >40% of cell type affected.

6 Discussion

Granulomas were identified in all five high-exposure frogs and in four of five control frog kidneys. They were characterized by flattened macrophages or giant cells at the periphery of necrotic cellular debris, a few granulocytes with eosinophilic cytoplasmic granules. Granulomas or granulomatous inflammation is generally associated with acid-fast bacteria (such as mycobacteria) or fungal pathogens. Ziehl-Neelsen (an acid-fast stain) is used to detect mycobacteria; Gomori methenamine silver or periodic acid Schiff stains (PAS) are used to detect fungal pathogens, if present. PAS, however, only works on living fungal organisms. Granulomas can also represent a response to foreign material, such as aluminum-based adjuvants or inoculated keratin.

One high-exposure frog (20C1) exhibited an amorphous eosinophilic (amyloid-like) material in the renal hematopoietic area adjacent to granulomas. Spawning salmon brains, presumably stressed, have been reported to have beta-amyloid deposits (Norris and Carr, 2013) detectable immunologically. Severe splenic amyloidosis has also been reported in an adult male captive spiny-tailed iguana, *Ctenosaura acanthura* (Goff et al., 1984). It is reasonable to hypothesize that frogs stressed by whatever elicited the robust granulomatous inflammatory response observed in these animals may also elicit deposition of amyloid-like substance at that site.

The severity (extent and number) of granulomas in high-dose frogs may have been slightly greater than that seen in control frogs, although the small sample size prohibits definitive statements. Biologically, the high-dose animals may have exhibited renal impairment due to disruption of tubule or hematopoietic function, which may have been less likely in control frogs. This suggests greater susceptibility by high-exposure animals than control frogs to the cause of the granulomatous response. Clinical data was not available for correlation with this study. A larger study including more animals may be useful to evaluate effect of NTO exposure on the innate or adaptive immune response of *L pipiens.* Efforts to eliminate the source of the granulomatous response may help eliminate confounding pathology in this toxicity study.

No evidence of toxicity or developmental delay was observed in the examined tissues, suggesting NTO may not affect anuran renal or hepatic development. NTO, however, has been associated with adversely effects on rat spermatogenesis (the author's personal observation, 2015), and some substances that do not affect mammals are more injurious to animals of other phyla; therefore, it may be informative to submit anuran reproductive or central nervous tissues for histologic examination.

7 Photomicrographs

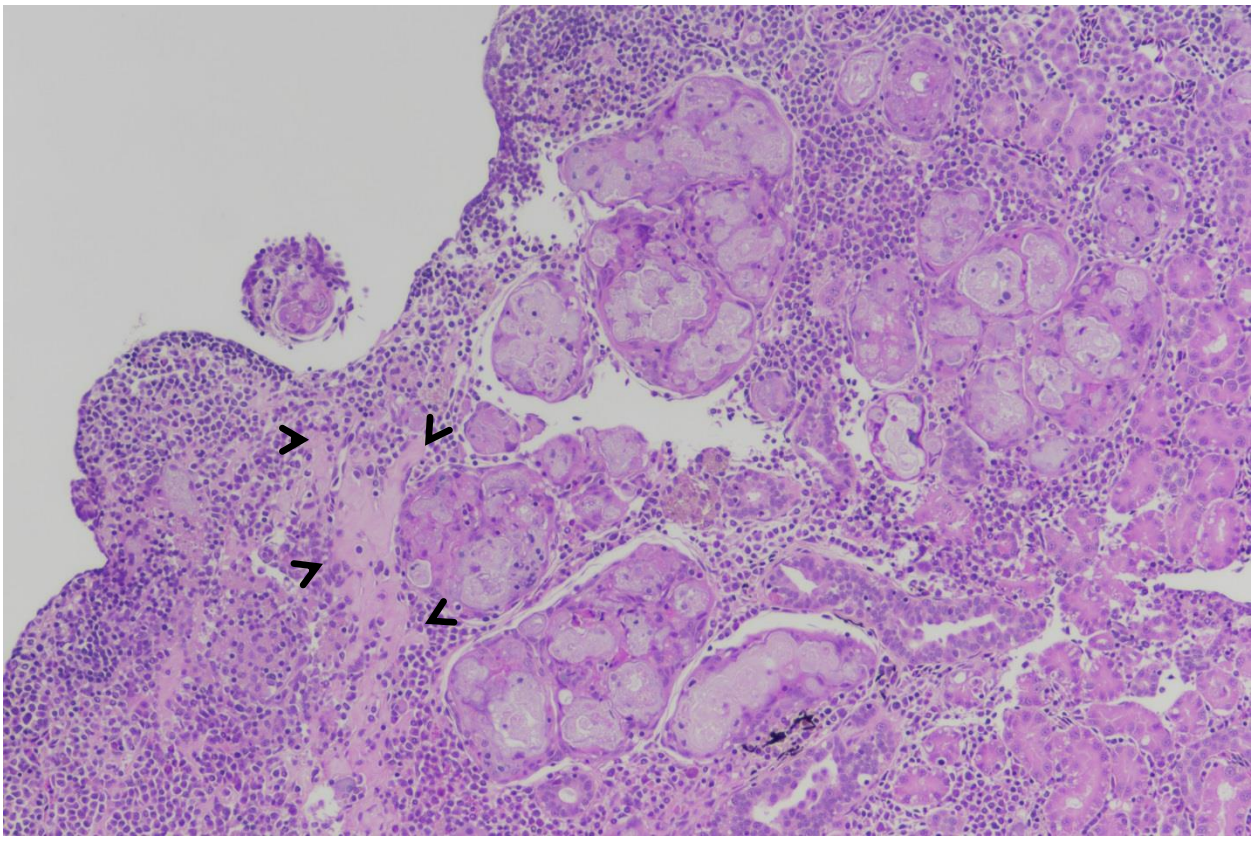

Figure 1. 20C1 High-dose Frog, Kidney

The subcapsular hematopoietic tissue is disrupted by numerous aggregates of macrophages encircling necrotic areas (granulomas). Pale eosinophilic amorphous material is also present (arrowheads) 10X

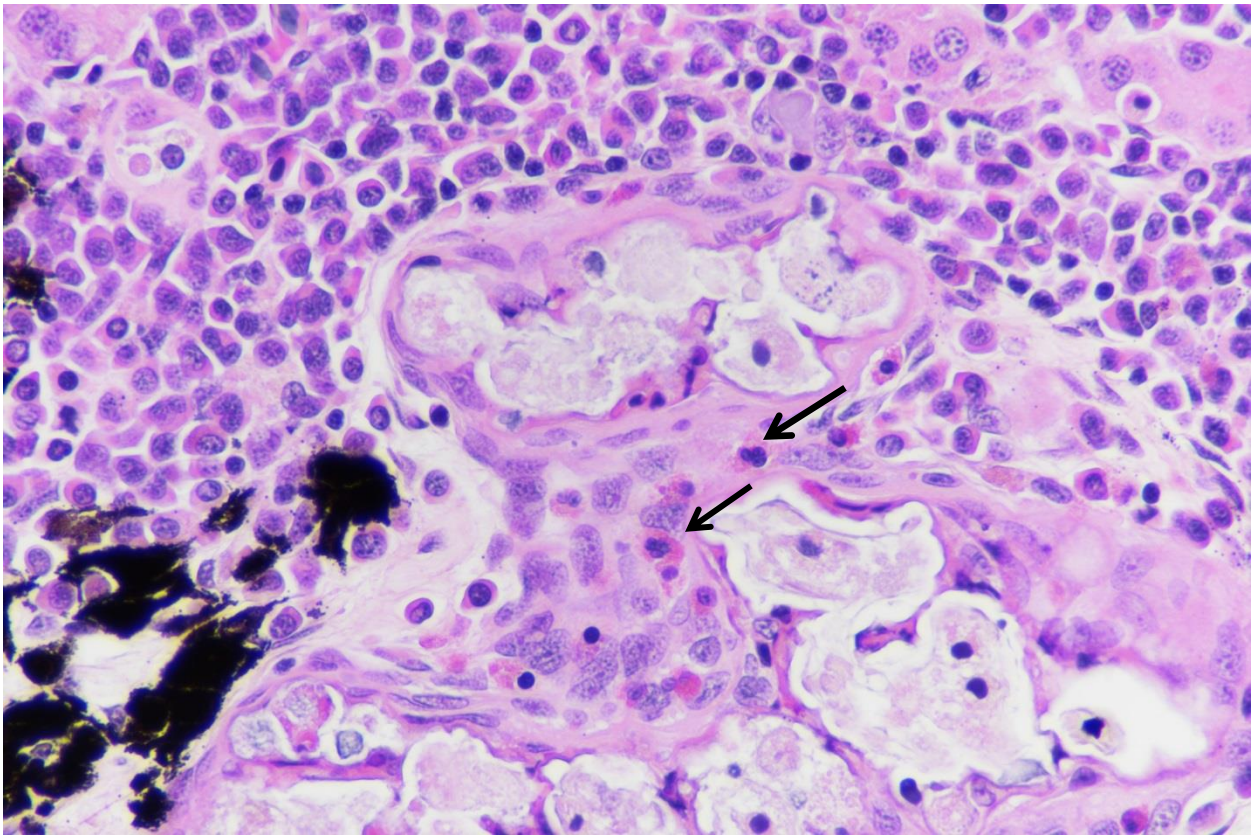

Figure 2. 00A1 Control, Kidney

Disrupting hematopoietic tissue in the kidney are multiple granulomas consisting of macrophages, some flattened, admixed with granulocytes with eosinophilic cytoplasmic granules (arrows) encircling necrotic cellular debris with pyknotic nuclei. 40X

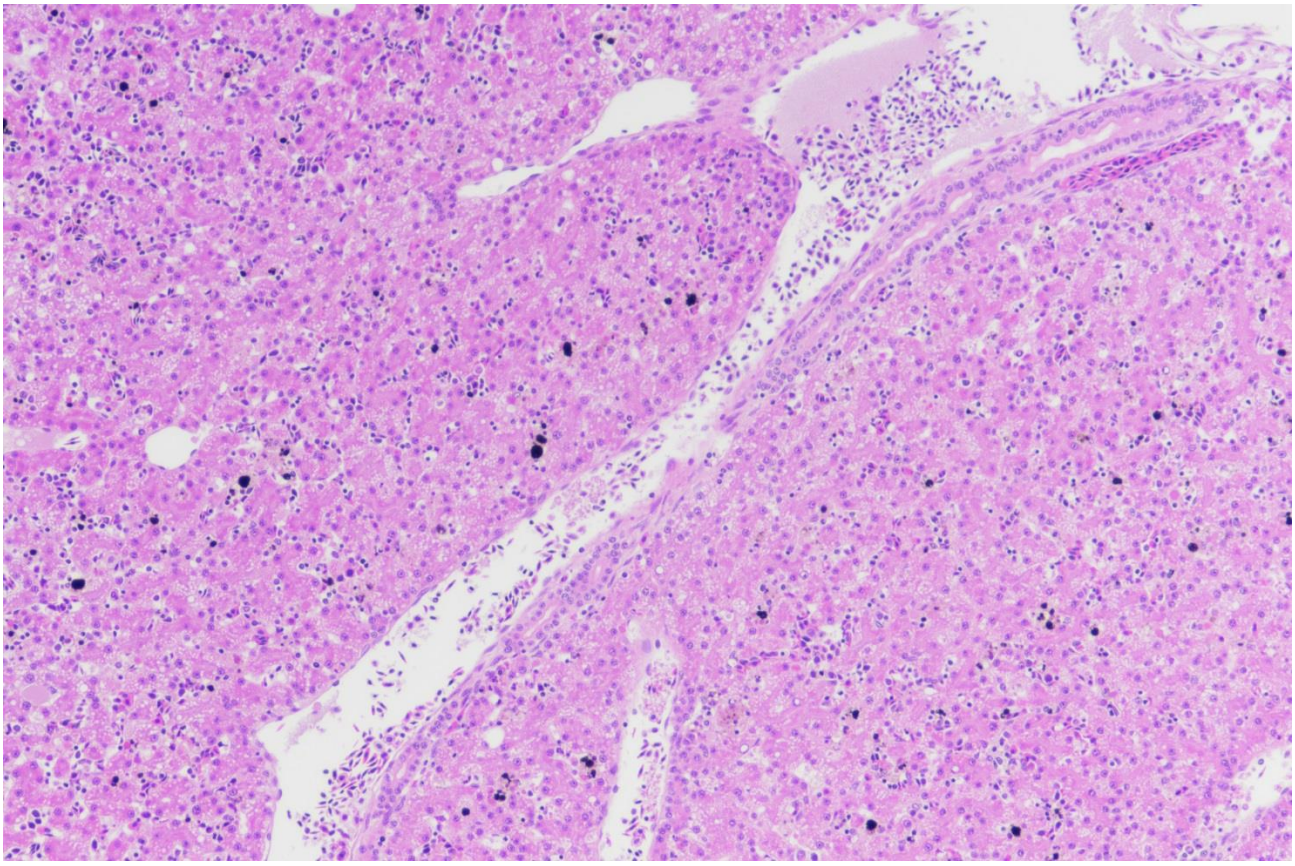

Figure 3. 20C1 High NTO-exposure Frog, Liver

Hepatocellular cords, sinuses central veins and bile ducts appear within normal limits. Pigment is commonly present in normal and NTO-exposed frogs. 10X

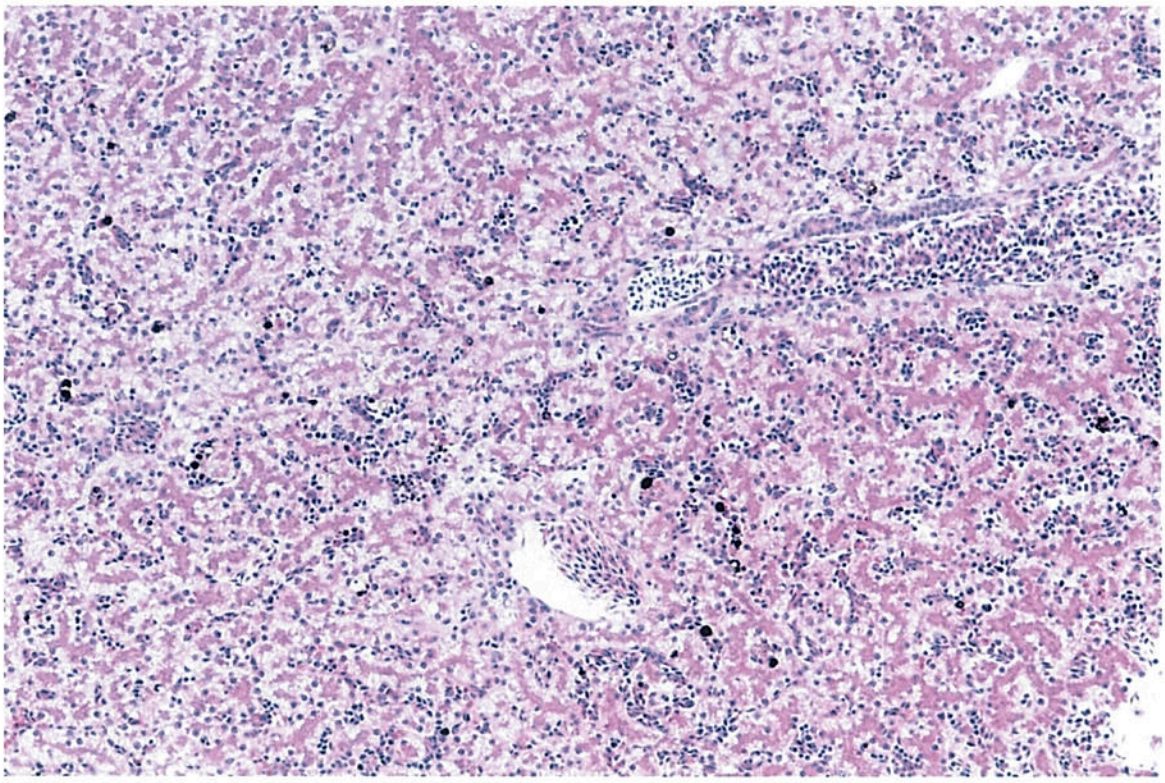

Figure 4. 00A1 Control Frog, Liver

Hepatocellular cords, sinuses central veins and bile ducts appear within normal limits. Pigment is commonly present in normal and NTO-exposed frogs. 10X

8 Point of Contact

The APHC (Prov) point of contact for this assessment is LTC Erica E. Carroll. She may be contacted at DSN 584-3980, commercial 410-436-3980, or via email at usarmy.apg.medcom-aphc.mbx.tox-info@mail.mil.

Erica Eggers Carroll

ERICA E. CARROLL, DVM, PhD, DACVP
LTC, VC
Chief, Division of Toxicologic Pathology

Approved:

MARK S. JOHNSON, PhD
Director, Toxicology

Appendix A

Quality Assurance Statement

The following critical phases were audited by the APHC (Prov) Quality Systems and Regulatory Compliance Office (QSARC), Laboratory and Toxicology Accreditation and Compliance Office (LTACO):

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/ SD
Pathology Contributing Scientist Inspection - Quality Assurance Audit of Excel Entered Data	12/23/2015	12/24/2015
Pathology Contributing Scientist Inspection - Interim Pathology Report GLP Standard Regulation Review	12/23/2015	12/24/2015
Pathology Contributing Scientist Inspection - Final Pathology Report GLP Standard Regulation Review	12/24/2015	12/24/2015

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 inspections not specifically related to this study are done monthly or annually in accordance with QSARC, LTACO Standing Operating Procedures.

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

Michael P. Kefauver
Quality Assurance Specialist, QSARC

24 December 2015
Date

Appendix B

Archives

B-1 Archives

All trim sheets, equipment logs, master data file (when present), and a copy of the final pathology report generated, as a result of providing pathology support, will be archived in the storage facilities of the Directorate of Toxicology, APHC (Prov), for a minimum of (10) years following submission of the final pathology report to the Sponsor.

A signed final pathology report is, for the purposes of Good Laboratory Practices, considered the raw data. The investigator will be provided a copy and the Army Public Health Center (Provisional) (APHC (Prov))-Toxicology (TOX) Portfolio will keep an electronic copy and one scanned copy to a computer disk. Scanned study files will be stored electronically in building E-2100, room 3010, APHC (Prov), Aberdeen Proving Ground, MD 21010. Any remaining wet tissue, paraffin blocks, and histology slides will be stored in building E-5158, unless the investigator requests them.

Pathology standing operating procedures are maintained by the Quality Assurance Unit in the Master Control database. Instrument maintenance logs are stored in room 1026 upon completion.

The archivist is Martha L. Thompson.

Appendix C

Addendum to Pathology Report

ADDENDUM TO

PATHOLOGY REPORT

For

One-Generation Study of the Effects of 3-nitro-1,2,4-triazol-5-one (NTO) to the Northern Leopard Frog (*Lithobates pipiens*) Under Static Renewal Test Conditions

TRE Project No.: 15001-468
Protocol No.: RP4OK.468.001

Study Director:
David Pillard, PhD

Prepared by:

LTC Erica Eggers Carroll, DVM, PhD, Diplomate, ACVP

Acknowledgement

Dr. Jeffrey C. Wolf, DVM, Diplomate ACVP, very kindly provided expertise and mentorship on anuran histopathology, for which this pathologist is very grateful.

Addendum to the Pathology Report for One-Generation Study of the Effects of 3-nitro-1,2,4-triazol-5-one (NTO) to the Northern Leopard Frog (*Lithobates pipiens*) Under Static Renewal Test Conditions

1 Background

Approximately 60 days after completion of the first phase of this project, a second shipment was received by Army Public Health Center (Provisional) (APHC (Prov)) Toxicology Directorate, Division of Toxicologic Pathology. Formalin-fixed testes from eight Northern Leopard frogs (*Lithobates pipiens)* were collected when the animals completed metamorphosis (Gosner Stage 46), and were humanely euthanized. Upon receipt, due to the small size of specimens, they were "sandwiched" between histology cassette sponges and processed using a biopsy setting on the tissue processor. Tissues were embedded, sectioned (2 micrometers (um) to maximize resolution for this study) and stained (with periodic acid Schiff [PAS] in the event spermatozoa were developing). In spite of extraordinary care in processing, of the eight animal tissues submitted, tissues from only five frogs (two controls and three "high concentration") were successfully processed onto microscope slides.

2 References

Papoulias D.M., M.S. Schwarz, and L. Mena. 2013. Gonadal abnormalities in frogs (Lithobates spp.) collected from managed wetlands in an agricultural region of Nebraska, USA. *Environ Pollut*. 172:1-8.

Haczkiewicz K. and M. Ogielska. 2013. Gonadal sex differentiation in frogs: how testes become shorter than ovaries. *Zoolog Sci*. 30(2):125-134.

Table 1. Summary of Testicular Samples Collected from Male *Lithobates pipiens*

Sample ID	Organ/ Tissue	Treatment	~NTO Concn. (mg/L)	Replicate	Specimen #	Comment – Histological Evaluation
00C2-TES	Testes	Control	0	C	2	2 testes collected
00D1-TES	Testes	Control	0	D	1	1 testis collected – n/a
00D2-TES	Testes	Control	0	D	2	1 testis collected – n/a
00D4-TES	Testes	Control	0	D	4	2 testes collected
20B2-TES	Testes	High Concn.	3300	B	2	Likely male; 1 testis collected
20B3-TES	Testes	High Concn.	3300	B	3	Likely male, 1 testis collected - n/a
20D1-TES	Testes	High Concn.	3300	D	1	2 testes collected
20D2-TES	Testes	High Concn.	3300	D	2	2 testes collected

Note:
n/a = not available for evaluation; tissue lost during processing

3 Results

- 00C2 Control testis: There are some distinct seminiferous tubules, numerous spermatogonia, with 1-2 prominent nucleoli, often indented or kidney bean-shaped nuclei. Mitotic figures or binucleate cells average 1–2/40x field. Vacuoles commonly disrupt the spermatogonia cytoplasm. Scant black pigment is just beneath the tissue surface (Figure 1).

- 00D4 Control testis: Seminiferous tubules are not evident. Spermatogonia are numerous with prominent chromatin. Some nuclei have 1–2 distinct nucleoli. Nuclei are occasionally offset by 1–2 cytoplasmic vacuoles. There is scant subsurface black pigment (Figure 2).

- 20B2 High-concentration NTO testis: There are distinct seminiferous tubules with possibly fewer spermatogonia (3–4 per tubule instead of 5 or more per tubule as seen in the two controls. Chromatin is less prominent than in 00D4 but there are 1–2 distinct nucleoli. There is scant superficial pigment (Figure 3).

- 20D1 High-concentration NTO testis: Distinct seminiferous tubules are at the margins of the section with densely packed smaller, darker cells in the center (fibroblasts and early Leydig cells). Spermatogonia chromatin is less distinct than in 00D4 (control). Binucleate, indented and kidney-bean shaped nuclei are common (Figure 4).

- 2D02 High-concentration NTO testis: Seminiferous tubules appear smaller than in controls, contain fewer spermatogonia which have indistinct chromatin. Binucleate spermatogonia are common, each nucleus with one distinct nucleolus (Figure 5).

4 Discussion

Figures 1 and 2 demonstrate normal inter-individual variation in testicular development. Although collected at Gosner stage 46, upon completion of metamorphosis, formation of seminiferous tubules varied among control frogs with some exhibiting apparently randomly scattered spermatogonia and precursors to Sertoli cells, interpreted as less mature (00D4). Other specimens exhibited clearly formed tubules with Sertoli cells arranged in support of Spermatogonia, some with mitotic figures (00C2). A similar range of maturity was present in the NTO-exposed anurans (Figures 3–5). Black pigment is normal in several frog tissues. The effect of NTO exposure is unclear and one cannot make definitive statements upon evaluating five animals but some sections have fewer, less robust spermatogonia than others; it remains to be determined if this occurs more often in NTO-exposed animals compared to control frogs. As tissues were collected prior to the development of mature spermatozoa, spermatogenesis could not be evaluated.

Tissues from three frogs were lost during processing of eight submitted tissues. At this stage of development *L. pipiens* testes are so small (often < 3 millimeters (mm) in any dimension) as to be extremely challenging to collect and process with optimal results. We suggest, henceforth, for structures of interest measuring less than 4 mm in any dimension, that transverse sections of the entire animal be submitted, no thicker than 4 mm thick. This method will help preserve tissue architecture.

5 Photomicrographs

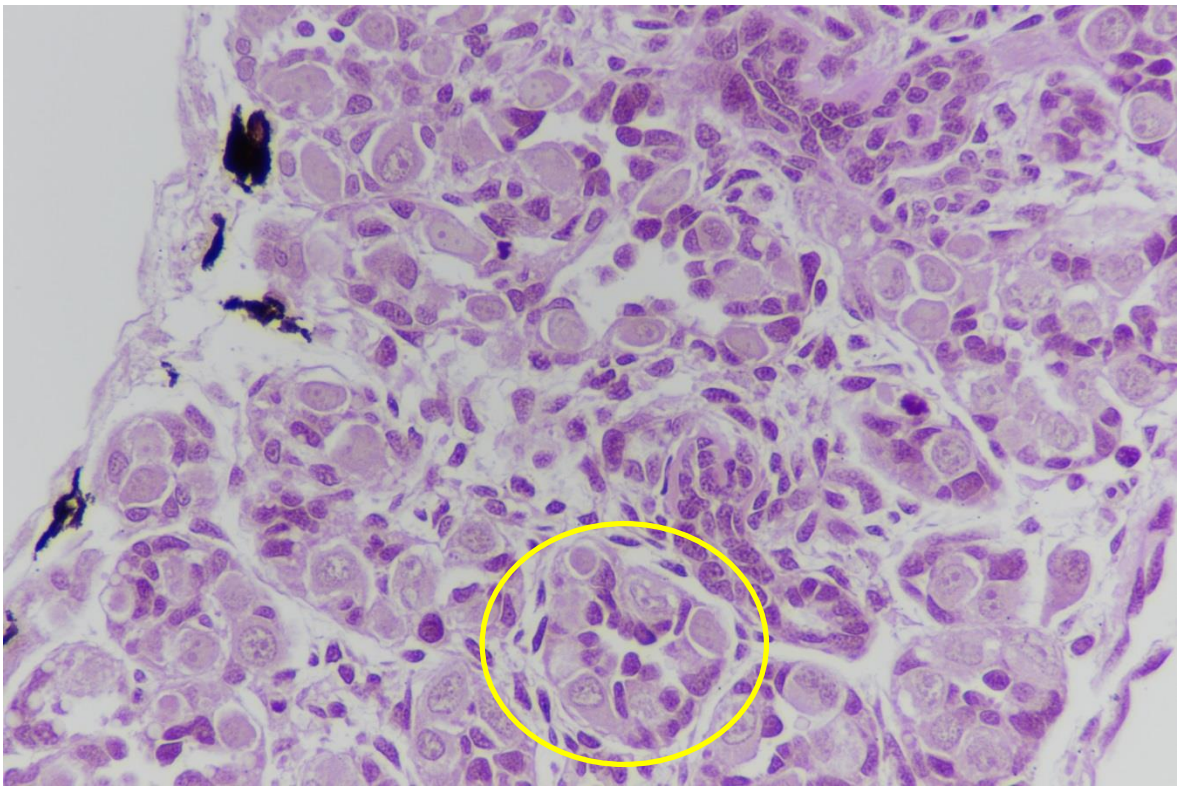

Figure 1. Control Frog (00C2) Testis

In this section seminiferous tubules are more mature as spermatogonia and early Sertoli cells form recognizable seminiferous tubules (outlined). 40X PAS

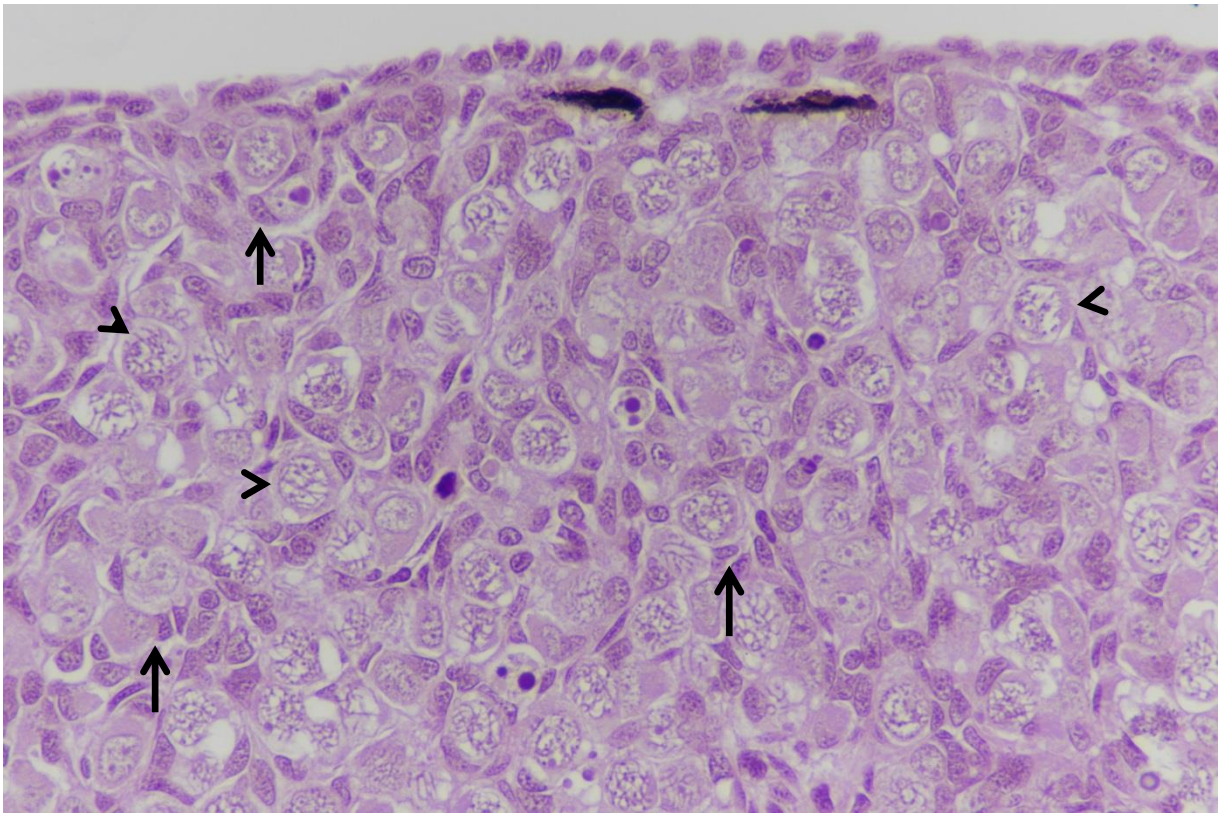

Figure 2. Control Frog (00D4) Testis

Gosner stage 46 (per contributor) In this section seminiferous tubules are immature and therefore indistinct. Robust spermatogonia (arrowheads) are interspersed among smaller, darker precursors of Sertoli cells (arrows). 40X PAS

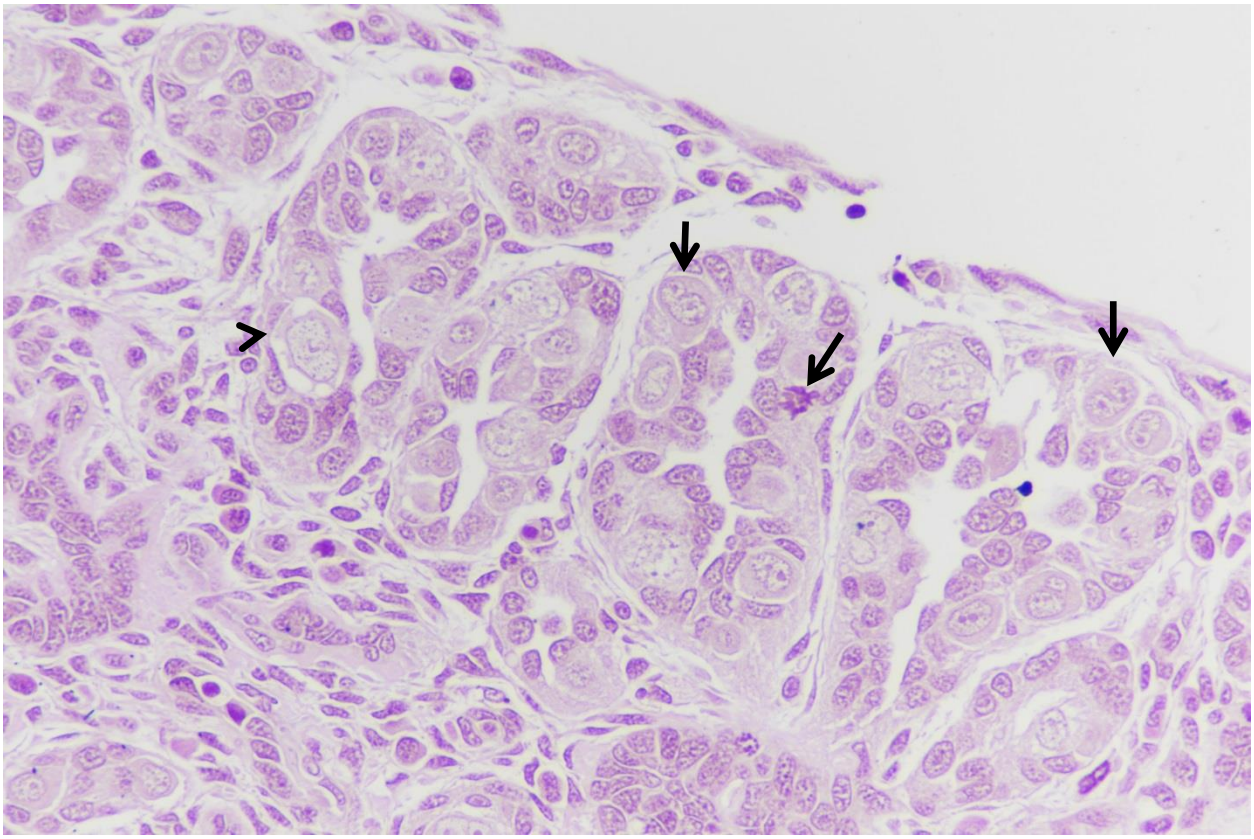

Figure 3. High-Concentration NTO Frog (2D01) Testis

Seminiferous tubules at the margin of the testis with smaller cells in the center of the tissue. Spermatogonia occasionally have vacuolated cytoplasm (arrowhead) and deeply indented nuclei or mitotic figures (arrows) 40X PAS

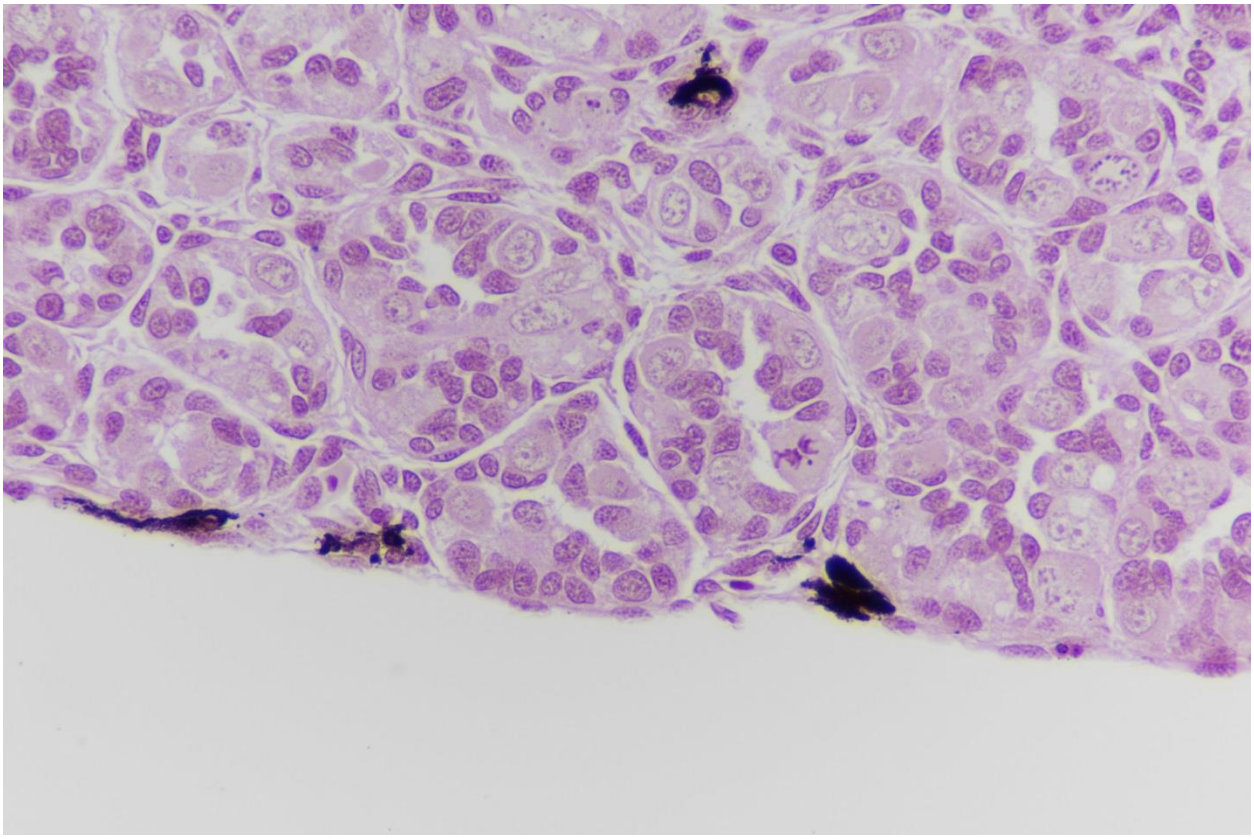

Figure 4. High-Exposure Frog (20B2) Testis

Seminiferous tubules are distinct with occasional mitotic figures of spermatogonia. Pigment is present, general at the tissue surface. 40X PAS

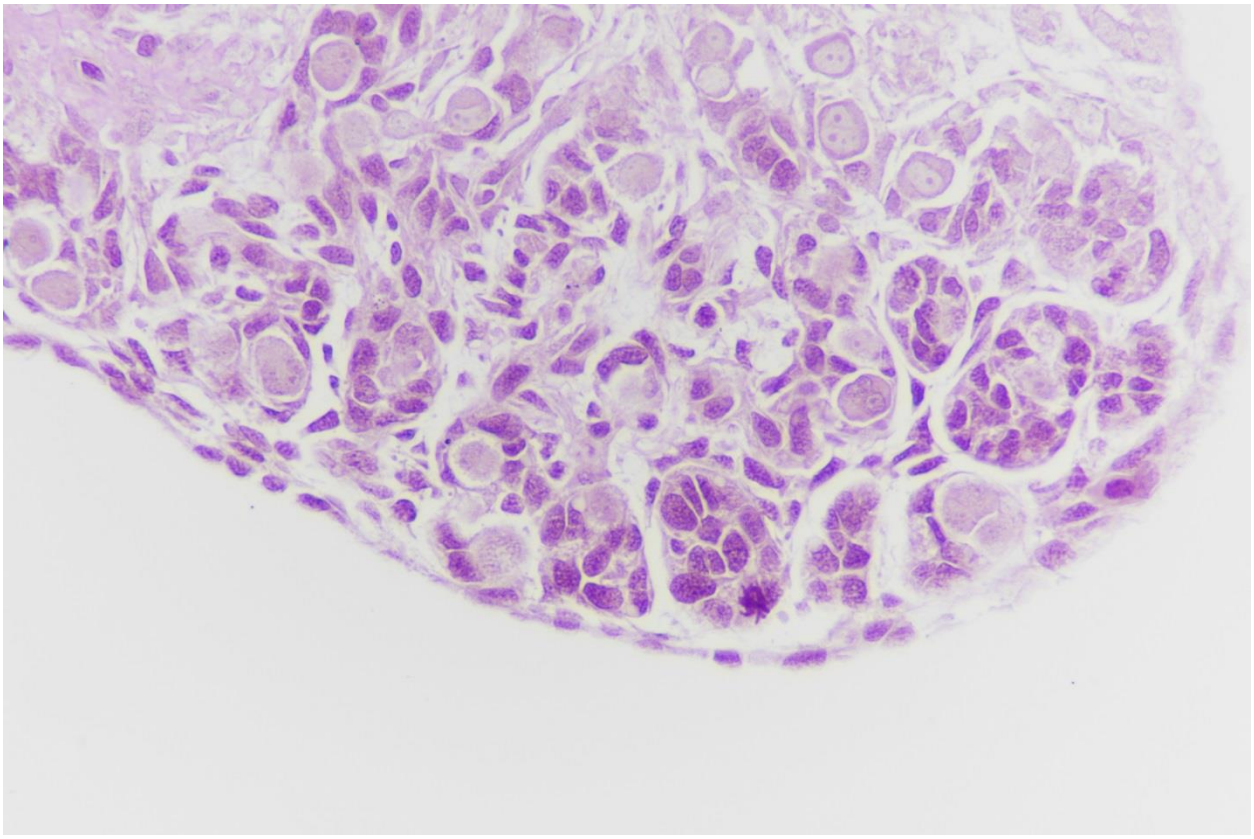

Figure 5. High-Concentration NTO Frog Testis

(20D2) This specimen has smaller seminiferous tubules, less robust Spermatogonia. 40X PAS.