Acute and Chronic Toxicity of Hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) in Deer Mice (*Peromyscus maniculatus*)

by

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ABSTRACT

Contamination of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) has been identified at areas of explosive manufacturing, processing, storage, and usage in a variety of environmental media. Conversion of RDX to anaerobic N-nitroso metabolites (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX)) has been demonstrated in the environment and *in vivo*, in the gastrointestinal tract of mammals. Thus, potential exists for human and wildlife exposure to these N-nitroso compounds. Few papers report acute or chronic toxicity of these N-nitroso metabolites, thus my research is to assess acute and chronic toxicity of these compounds.

Acute toxicity was assessed using acute oral median lethal dose (LD_{50}). This was determined in deer mice (*Peromyscus maniculatus*) of three age classifications (21 d, 50 d, and 200 d) for RDX, MNX, and TNX using the U.S. EPA Up-and-Down Procedure (UDP). Hexahydro-1,3,5-trinitro-1,3,5-triazine and N-nitroso metabolites caused similar overt signs of toxicity. Median lethal dose for 21 d deer mice were 136, 181, and 338 mg/kg for RDX, MNX, and TNX respectively. Median lethal dose for 50 d deer mice were 319, 575, and 999 mg/kg for RDX, MNX, and TNX respectively. Median lethal dose for 200 d deer mice were 158, 542, and 338 mg/kg for RDX, MNX, and TNX respectively. These data suggest that RDX is the most potent compound tested, and age dependent toxicity may exist for all compounds.

Chronic toxicity was evaluated with a reproductive study and a multigenerational study. Following exposure, reproductive toxicity of TNX was evaluated in three consecutive cohorts (F1A-C) of deer mice. TNX was administered *ad libitum* via drinking water at four exposure levels-control (0 µg/L), 1µg/L, 10 µg/L, and 100 µg/L. Endpoints investigated include: reproductive success, offspring survival, offspring weight gain, offspring organ weights, and liver TNX residues. Data from this study indicate that TNX bioaccumulates in the liver and is associated

with postpartum mortality, dose dependent decrease in body weight from birth to weaning, and decrease in kidney weight in deer mice offspring.

While exposed to TNX via drinking water *ad libitum*, deer mice were bred in a multigenerational fashion (parents produced offspring, which bred to produce more offspring) to produce three generations F1A-D, F2A-B, and F3A. TNX was administered at four exposure levels-control (0 µg/L), 10 µg/L, 100 µg/L, and 1 mg/L. Endpoints investigated include: reproductive success, offspring survival, offspring weight gain, and offspring organ weights. Data from this study indicate that TNX is associated with decreased litter size and increased postpartum mortality of offspring. No teratogenic effects were linked with exposure to TNX.

With tissue samples from both the reproductive and multigenerational studies, 12 microsatellite DNA loci were amplified and analyzed using both change in original parent allele frequencies and the parent/offspring approach of direct mutation rate calculation to assess genotoxicty of TNX *in vivo*. Findings demonstrate no dose dependent differences in deviation from parent microsatellite DNA allele frequencies or direct microsatellite mutation rate using the parent/offspring approach.

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CHAPTER I

INTRODUCTION

High Explosives

High explosives are defined as a compound that generates gas with extreme rapidity and has a shattering effect (1996). High explosives are used primarily for military purposes (warheads), and secondarily in civilian applications (mining & demolition). Explosives classified as high explosives include dynamite, trinitrotoluene (TNT) (CAS #118-96-7), pentaerythritol tetranitrate (PETN) (CAS #78-11-5), 1,3,5,7-tetranitro-1,3,5,7-tetrazocane (HMX) (CAS #2691-41-0), and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (CAS #121-82-4).

RDX

RDX was first synthesized in Germany in 1899 by Hans Hennings. Early uses of RDX include medicinal purposes and as a rodenticide. Around 1920, the explosive properties of RDX were discovered; and, by World War II, RDX was used as a common military explosive. RDX is typically used in combination with TNT or PETN in bombs, warheads, and other munitions. It is also used in plastic explosives, blasting caps, and percussion-type explosives.

Manufacturing, packaging, use, and disposal of RDX have contaminated a variety of environmental media including surface water, groundwater, soils, and sediments. The U.S. Environmental Protection Agency's (U.S. EPA) Comprehensive Environmental Response, Compensation, and Liability Information System Database identifies 19 sites on the National Priorities List that are contaminated with RDX. The U.S. Army has confirmed ground water explosive contamination at 583 sites within 82 installations in the United States and suspects 88 additional sites within 22 installations as possibly being contaminated (Davis *et al.* 2004). Concentrations of RDX in soil have been reported as high as 15,000 µg/kg at the Massachusetts Military Reservation (Falmouth, MA, USA) (Pennington *et al.* 2005). Concentrations of RDX

in an inactive lagoon at the Louisiana Army Ammunition Plant (Doyline, LA, USA) were as high as 28.9 mg/L (Spanggord *et al.* 1983).

RDX Toxicity

The toxic effects of RDX exposure have been well documented. In acute exposures, RDX causes central nervous system effects that included convulsions, seizures, and, with increasing dose, death (von Oettingen *et al.* 1949; Schneider *et al.* 1977; Burdette *et al.* 1988; Meyer *et al.* 2005). Typically seizures consist of clonic/tonic convulsions and hyperactivity. Other reported acute toxic effects include decreased food consumption, weight gain, egg production, and hypotriglyceridemia (Levine *et al.* 1990; Gogal *et al.* 2003).

Median lethal dose (LD_{50}) of RDX in Swiss-Webster mice ranges from 75 mg/kg/day in females to 86 mg/kg/day in males (Dilley *et al.* 1978). LD_{50} values in Spauge-Dawley rats have been reported as 71 mg/kg/day (Dilley *et al.* 1978) and 187 mg/kg/day (Meyer *et al.* 2005). B6C3F1 mice have reported LD_{50} values of 56 mg/kg/day in females to 97 mg/kg/day in males (Cholakis *et al.* 1980). In Fischer 344 rats, the LD_{50} was reported to be 118 mg/kg/day (Levine *et al.* 1981). Acute lethal doses to northern bobwhite (*Colinus virginianus*) were 187 and 280 mg/kg for females and males, respectively (Gogal *et al.* 2003).

In chronic exposures, RDX causes hepatotoxicity and also has harmful effects on blood (Levine *et al.* 1981; Levine *et al.* 1990). Weight loss, increased liver weights, and testicular degeneration have also been reported (Lish *et al.* 1984). The U.S. EPA has classified RDX as a possible human carcinogen (C). The basis for this classification is a study that detected hepatocellular adenomas and carcinomas in female B6C3F1 mice (Lish *et al.* 1984). In a recent revaluation of this classification, hepatocellular adenomas and/or carcinomas were again observed in female B6C3F1 mice (Parker *et al.* 2006), and the classification remained the status quo.

In a two generation reproduction study with Fischer 344 rats, elevated maternal mortality, neurotoxicity, and a higher percent of stillborn pups were observed at 50

mg/kg (the high dose group) (Cholakis *et al.* 1980). Rats from F0 and F1 generations were dosed during the 13 weeks prior to mating.

Developmental toxicity studies were conducted with Fischer 344 rats and New Zealand rabbits (Cholakis *et al.* 1980). Both rats and rabbits were dosed during the second trimester of gestation. Teratogenicity was not demonstrated at any dose tested. Based on embryotoxicity and maternal toxicity, the no adverse effect level (NOAEL) for both studies was 2 mg/kg/day.

Distribution and metabolism studies were conducted in Fischer 344 rats and swine (Schneider *et al.* 1977; Schneider *et al.*1978). RDX was distributed throughout the body, including the following: heart, blood plasma, brain, liver, and kidneys (Schneider *et al.* 1977; Schneider *et al.*1978). RDX concentrations in kidneys were highest of those tested. RDX concentrations in liver were most variable, since the liver is the site of RDX metabolism (Bradley 1977).

In freshwater invertebrates, RDX in sediments had no effect on survival (1,000 mg/kg) and caused significantly increased growth at lower concentrations (25 mg/kg) (Steevens *et al.* 2002). In saltwater invertebrates, RDX in sediments had no effect on survival (Lotufo *et al.* 2001). Reproduction was significantly reduced at the highest RDX concentration in sediment (~1,000 μg/g). RDX in water also had little impact on invertebrate survival in other studies (Peters *et al.* 1991). Concentrations of RDX in water did have a negative effect on reproduction with a NOAEL of 3.64 mg/L.

RDX had no effect on adult earthworm (*Eisenia fetida*) survival (Simini *et al.* 2004). However, it did decrease cocoon production-effective concentration of 20% of the population values (EC_{20}) are 1.2 mg/kg (freshly amended soil) and 19 mg/kg (weathered/aged soil). Juvenile production was also decreased-EC_{20} values were 1.6 mg/kg (freshly amended soil) and 4.8 mg/kg (weathered/aged soil).

Regulatory Values

Because of known toxicity of RDX, regulatory values have been established to protect the human population. The U.S. EPA has set the oral reference dose at 0.003

mg/kg/day (IRIS 1993). This is based on a chronic study where inflammation of the prostate in males was noted at a lowest observed adverse effect (1.5 mg/kg/day) with the no observed effect level (NOEL) being 0.3 mg/kg/day (Levine *et al.* 1983). The reference dose was established by multiplying the NOEL by an uncertainty factor of 100 an attempt to represent the uncertainty in the extrapolation of dose levels from laboratory animals to humans (10) and a threshold for sensitive humans (10) (IRIS 1993). The U.S. EPA has set a lifetime drinking water health advisory at 2 µg/L (FSTRAC 1994).

Transformation of RDX

Transformation of RDX occurs two ways: aerobically and anaerobically. Aerobically, RDX can be degraded (Binks *et al.* 1995; Coleman *et al.* 2002), but transformation occurs faster and more easily via anaerobic degradation (McCormick *et al.* 1981; Kitts *et al.* 1994; Young *et al.* 1997).

Aerobic transformation of RDX occurs through a denitration process of one or more of the N-nitro moieties. This pathway proceeds with the enzymatic cleavage of the $N\text{-}NO_2$ bond, at times mediated by cytochrome P450 enzymes (Coleman *et al.* 2002; Bhushan 2003). Through this process of denitration and spontaneous hydrolytic decomposition, 4-nitro-2,4-diazabutanal (NDAB) is formed. Other abiotic processes are thought to transform RDX into NDAB as well (Fournier *et al.* 2004).

Anaerobically, RDX can be degraded in a combination of two pathways (**Fig. 1.1**). One pathway of anaerobic transformation occurs via reduction of RDX N-nitro groups to form N-nitroso moieties. Progressive reduction of the three N-nitro groups of RDX produces the following compounds: hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) (CAS #5755-27-1), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) (CAS #80251-29-2), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) (CAS #13980-04-6) (McCormick *et al.* 1981; Kitts *et al.* 1994; Regan and Crawford 1994; Young *et al.* 1997; Boopathy *et al.* 1998; Kitts *et al.* 2000; Beller 2002). These N-nitroso compounds can continue this reduction process to form hydroxylamino

derivatives. This process is thought to be mediated by nitroreductase in various bacteria. These N-nitroso metabolites of RDX have been found in RDX contaminated groundwater beneath the Iowa Army Ammunition Plant (Middletown, IA, USA) with concentrations as high as 430 μg/L (Beller and Tiemeier 2002), suggesting that this reductive RDX transformation occurs in the environment. RDX concentrations in ground water at that same facility have been reported as high as 36 mg/L (Tucker *et al.* 1985). Formation of RDX N-nitroso metabolites has also been demonstrated in gastrointestinal tracts of deer mice (*Peromyscus maniculatus*) exposed to RDX in food (Pan *et al.* 2007b).

Another pathway of anaerobic RDX transformation is hydrolytic ring cleavage (Hawari *et al.* 2000). The parent compound (RDX) or any products from the N-nitro reductive pathway (N-nitroso metabolites of RDX or some hydroxylamino derivative of those compounds) can undergo this process. Products from RDX hydrolytic ring cleavage include methylnedinitramine (MEDINA) and bis(hydroxymethyl)nitramine. These products can undergo further hydrolysis to ultimately form methane, carbon dioxide, ammonia, nitrous oxide, and nitrogen gas.

Toxicity of N-nitroso Metabolites

While RDX toxicity has been studied and regulatory values have been established, little work has been completed with RDX N-nitroso metabolites. This is reason for concern; because, in general, N-nitroso compounds are recognized as potent carcinogens. N-nitroso compounds require bioactivation to exhibit carcinogenic properties (**Fig. 1.2**). This process entails first hydroxylation of the carbon in the α position, which then can induce carcinogenic effects through alkylation of macromolecules, such as DNA (Archer 1989). Hydroxylation is mediated by cytochrome P450 (Cyp) enzymes; typically Cyp 2E1 for small, short chained N-nitroso compounds such as N-nitrosodimethylamine, and Cyp 2A6 for large, bulky chained N-nitroso compounds such as N-nitrosomethylphenylamine (Kushida *et al.* 2000; Fujita and Kamataki 2001). N-nitroso compounds also have been shown to

cause centrilobular hepatic necrosis in acute exposure and liver cirrhosis in chronic exposure (Brown 1999).

While general N-nitroso compounds have shown carcinogenic properties and other various toxic responses, little research has been conducted with specific RDX N-nitroso metabolites. The only toxicological data for mammals is an Up-and-Down Procedure (UDP) determination of acute oral toxicity in Sprauge-Dawley rats, coupled with a fixed dose acute toxicity study with MNX (Meyer *et al.* 2005). Growth and survival in the earthworm (*Eisenia fetida*) was reduced with exposure to MNX and TNX (Zhang *et al.* 2006b). Without activation, TNX was shown to be genotoxic using a microsuspension modification of the *Salmonella* histidine reversion assay (George *et al.* 2001). Using S9 activation, MNX and TNX showed weak to moderate mutagenesis depending on the bacterial strain with the Ames Assay (Pan *et al.* 2007a).

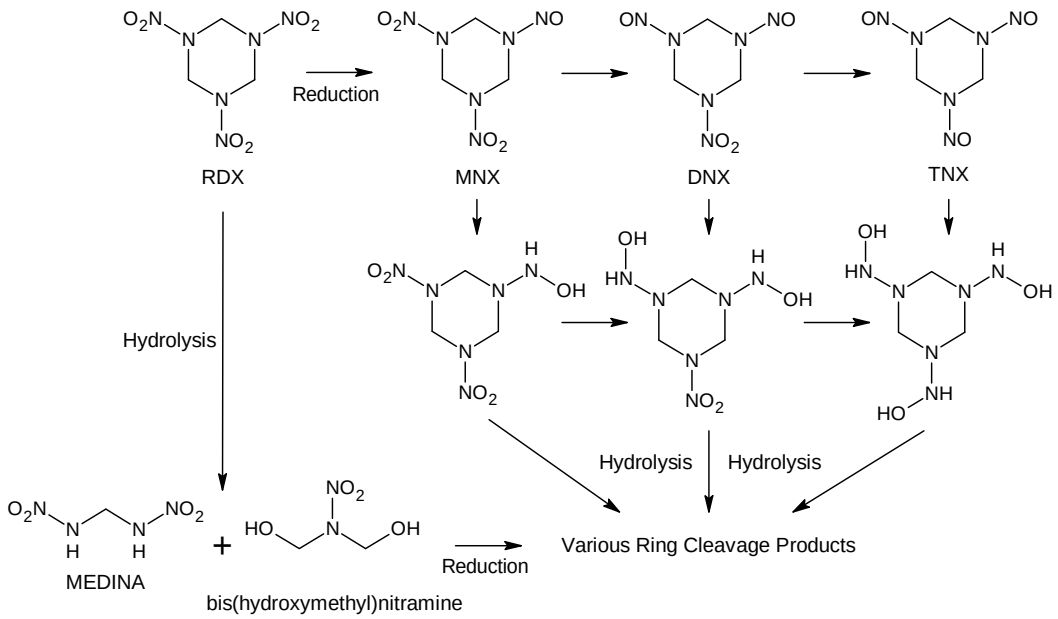
Research Rationale and Objectives

With the presence of RDX in the environment and the possibility of RDX N-nitroso metabolites being formed either environmentally or *in vivo*, there is potential for exposure of RDX N-nitroso compounds to human and wildlife. In addition to the possibility of these compounds forming naturally, RDX remedial practices involve anaerobic *in situ* degradation, meaning that these N-nitroso metabolites will be produced by extant remediation technologies (Radtke 2005). This is a problem considering the potential risk of N-nitroso compounds and the lack of toxicological data describing these specific compounds. Some bacterial, invertebrate, and mammalian toxicological data are available (George *et al.* 2001; Meyer *et al.* 2005; Zhang *et al.* 2006a; Zhang *et al.* 2006b; Zhang *et al.* 2006c; Pan *et al.* 2007a). However, they provide limited understanding of the nature of these compounds. RDX N-nitroso metabolites have been studied for genotoxicy in various strains of bacteria; however, no research has been conducted to assess genotoxicity of these N-nitroso compounds in multicellular organisms *in vivo*. Thus, the overall objective of my study is to explore the toxicity of these compounds.

To accomplish this goal, the toxicity of RDX N-nitroso metabolites was characterized. The deer mouse (*Peromyscus maniculaus bairdii*) was used as the animal model for all studies. The deer mouse is a novel (to studies of RDX exposure), yet ecologically relevant species. Deer mice are common throughout North America and are the most widely studied wide-type rodent species in the United States (Presti *et al.* 2004). TNX was selected as the primary toxicant of interest in this study, because it is the most nitrosolated of RDX N-nitroso compounds. Thus, if there are some differences in toxic responses based on chemical moiety (i.e., N-nitro versus N-nitroso), TNX should have the most profound effect.

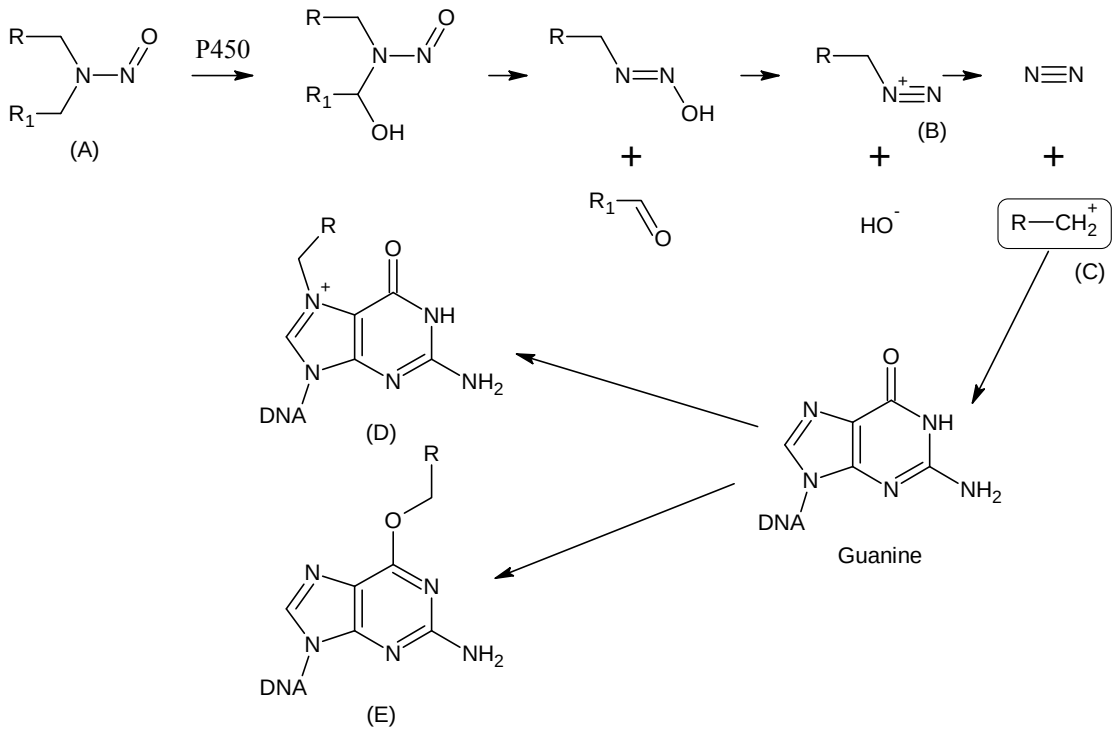
For a complete assessment, acute and chronic toxicity of RDX N-nitroso compounds were investigated. For acute toxicity (Chapter 2), RDX was compared to two of the N-nitroso compounds using median lethal dose (LD_{50}). Three different age classifications were tested as well to investigate potential age dependent toxicity. For chronic toxicity, two separate studies were completed. The first chronic study, a reproductive study (Chapter 3), was a range finding study in which breeding rodents were continuously exposed to TNX. Various endpoints (reproductive success, offspring survival, offspring growth, offspring organ weight, and toxicant uptake) were investigated on offspring of these rodents. The second study was a verification and expansion of the reproductive study. It was a multigenerational study (Chapter 4) in which breeding pairs of rodents were continuously exposed to TNX and allowed to produce offspring. Some of these offspring were bred and produced a second generation of offspring. This process was repeated for a third generation of offspring. Similar endpoints were investigated in these offspring. Microsatellite DNA was extracted from parents and offspring from both the reproductive study and the multigenerational study. Allele frequencies and mutation rates of microsatellite DNA were analyzed to assess potential genotoxicity of TNX (Chapter 5). Information obtained within this dissertation research will be useful for RDX and RDX N-nitroso metabolite ecological risk evaluation of terrestrial wildlife at RDX contaminated sites.

Figures



1.1 Anaerobic degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)

Two pathways of anaerobic degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). Reductive pathway is the formation of hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine; then proceeding further to hydroxylamino derivatives. Hydolysis pathway is the formation of methylenedinitramine (MEDINA) and bis(hydroxlymethyl)nitramine. Reductive products can also undergo ring cleavage via hydrolysis. Ring cleavage products of RDX can undergo reduction.



1.2 Enzymatic activation of N-nitroso compounds

General N-nitroso compound (A) undergoing hydroxylation at an α carbon, then rearrangement to form a diazonium ion (B). The diazonium ion can undergo further rearrangement to form a carbonium cation (C), which can then bind to a neucleophilic center, such as the N^7 (D) or O^6 (E) on the nucleic acid guanine, which would ultimately form a DNA adduct.

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CHAPTER II

AGE DEPENDENT ACUTE ORAL TOXICITY OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE (RDX) AND TWO ANAEROBIC N-NITROSO METABOLITES IN DEER MICE (*PEROMYSCUS MANICULATUS*)

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Abstract

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) transforms anaerobicly into N-nitroso compounds: hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). Exposure to these N-nitroso metabolites may occur in areas contaminated with explosives, as anaerobic degradation occurs via some bacteria and is one remediation strategy used for RDX. Few papers report acute oral toxicity and none have evaluated age dependent toxicity of RDX or its N-nitroso metabolites. Median lethal dose (LD_{50}) was determined in deer mice (*Peromyscus maniculatus*) of three age classifications 21 d, 50 d, and 200 d for RDX, MNX, and TNX using the U.S. EPA Up-and-Down Procedure (UDP). Hexahydro-1,3,5-trinitro-1,3,5-triazine and N-nitroso metabolites caused similar overt signs of toxicity. Median lethal dose for 21 d deer mice were 136, 181, and 338 mg/kg for RDX, MNX, and TNX respectively. Median lethal dose for 50 d deer mice were 319, 575, and 999 mg/kg for RDX, MNX, and TNX respectively. Median lethal dose for 200 d deer mice were 158, 542, and 338 mg/kg for RDX, MNX, and TNX respectively. These data suggest that RDX is the most potent compound tested, and age dependent toxicity may exist for all compounds and could play a role in RDX and RDX N-nitroso metabolite ecological risk evaluation of terrestrial wildlife at RDX contaminated sites.

Keywords: up-and-down procedure; hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine; hexahydro-1,3,5-trinitroso-1,3,5-triazine; RDX; MNX; TNX

Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (CAS #121-82-4) is an explosive primarily used for military purposes, and secondarily used in civilian applications. The U.S. Environmental Protection Agency's (U.S. EPA) Comprehensive Environmental Response, Compensation, and Liability Information System Database lists 19 sites on the National Priorities List that are contaminated with RDX. The U.S. Army has confirmed groundwater environmental explosive contamination at 583 sites within 82 installations, as well as suspecting 88 additional sites within 22 installations as possibly being contaminated within the United States (Davis *et al.* 2004). Soil RDX concentrations have been reported as high as 15,000 μg/kg at the Massachusetts Military Reservation (Falmouth, MA, USA) (Pennington *et al.* 2005).

Anaerobically, RDX biodegrades progressively (**Fig. 2.1**) into the following N-nitroso compounds: hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) (CAS #5755-27-1), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) (CAS #80251-29-2), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) (CAS #13980-04-6) (McCormick *et al.* 1981; Kitts *et al.* 1994; Regan and Crawford 1994; Young *et al.* 1997; Boopathy *et al.* 1998; Kitts *et al.* 2000; Beller 2002). These N-nitroso metabolites of RDX have been found in RDX contaminated groundwater beneath the Iowa Army Ammunition Plant (Middletown, IA, USA) with concentrations as high as 430 μg/L (Beller and Tiemeier 2002), suggesting that RDX transforms to N-nitroso metabolites in the environment. Formation of RDX N-nitroso metabolites has been demonstrated in the gut of deer mice (*Peromyscus maniculatus*) exposed to RDX in food (Pan *et al.* 2007). Additionally, when site remediation is warranted, anaerobic degradation is one common clean-up strategy of RDX via *in situ* degradation or use of bioreactors (Radtke 2005).

In acute exposures, RDX causes central nervous system toxicity in the form of seizure activity (von Oettingen *et al.* 1949; Schneider *et al.* 1977; Burdette *et al.* 1988; Meyer *et al.* 2005), and also manifests liver toxicity in chronic exposures (Levine *et*

al. 1981; Levine *et al.* 1990). Median lethal dose (LD_{50}) of RDX in mice ranges from 86 to 97 mg/kg (Dilley *et al.* 1978; Cholakis *et al.* 1980). The U.S. EPA has set a lifetime drinking water health advisory at 2 μg/L (FSTRAC 1994) and an oral reference dose at 0.003 mg/kg/day ((IRIS) 1993).

While RDX toxicity has been studied and regulatory values have been established, little work has been completed with RDX N-nitroso metabolites. Two papers have been published regarding toxicity of these N-nitroso metabolites in rodents. One is a reproductive toxicity study with deer mice (Smith *et al.* 2006) and the other is an Up-and-Down Procedure (UDP) determination of acute oral toxicity in Sprauge-Dawley rats, coupled with a fixed dose acute toxicity study with MNX (Meyer *et al.* 2005).

Anaerobic conditions necessary for RDX transformation to these compounds may occur in the environment or internally within the gut of the organism. As N-nitroso metabolites are produced, there is a possibility that although parent compound (RDX) concentration is significantly reduced, byproducts may not be any less toxic. Well known transformation products of particular parent compounds have been shown to cause equal or greater toxicity. Some of these transformations include aldicarb to aldicarb sulfoxide (Cobb *et al.* 2001), polycyclic aromatic hydrocarbons (PAH) to PAH-geminal (Conney 1982; Williams and Weisburger 1986), and hydroxylation of various polychlorinated biphenyl congeners (Bergman *et al.* 1994). Therefore, since toxicity data for these reduction products are scarce, more data are needed in order to ascertain risk.

Determination of acute oral toxicity is an initial step in evaluating toxic characteristics of a substance. Acute toxicity tests serve as the basis for a variety of hazard and risk assessment processes for human and environment health effects (OECD 2001; Rispin *et al.* 2002). Median lethal dose (LD_{50}) is one of the main acute oral toxicity endpoints and is defined as the dose in which 50% of animals exposed are expected to die. Recently, the U.S. EPA UDP maximum likelihood test guideline has been accepted as a replacement for traditional LD_{50} determination methods (Rispin *et*

al. 2002). Whereas traditional LD_{50} determinations use multiple animals at 5 to 7 defined dose intervals, the UDP tests one animal at a time dosing in a staircase fashion until a stopping criterion is met. This type of test significantly reduces the number of animals required to estimate LD_{50} values.

In the process of evaluating toxic characteristics of a substance, there has been a recent increase of interest in toxicant exposure to young organisms. Toxicity during early stages life may occur since absorbtion, distribution, metabolism, and excretion mechanisms develop at differents rates, thus age dependent toxic repsonses may exist (U.S. NRC 1993). For example, kidney glomerular filtration is poorly developed in infants, and therefore, increases the chance of toxicity via lack of excretion (Klienman 1982). The capacity of the liver to detoxify various compounds via conjugation also develops slowly, thus, an increased risk of toxicity due to underdeveloped metabolism (Vessell 1982). With a potential for increased toxicity among young organisms, age dependent toxicity is a crucial determinate.

Due to unknown characteristics of these N-nitroso compounds, one goal of this study was to provide additional acute oral toxicity data in the deer mouse (*Peromyscus maniculatus bairdii*), a novel, yet ecologically relevant species. Deer mice are common throughout North America and are the most widely studied wide-type rodent species in the United States (Presti *et al.* 2004). Toxicity of N-nitroso metabolites was determined with respect of RDX for two reasons: 1) RDX is the parent compound and 2) RDX toxicity is sufficiently well known in other species to provide a reference toxicant for deer mice and the UDP method. An additional goal for this study was to investigate the possibility of age dependent toxicity for RDX and its N-nitroso metabolites. To achieve these goals, LD_{50} values were determined in deer mice of three age classifications 21, 50, and 200 d for RDX, MNX, and TNX using the UDP. Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) was not used due to low purity (<70%) of commercially available DNX. While the primary focus of this paper is for ecological risk, age classifications were chosen to correlate with different human life stages for a clearer perspective. These human equivalencies are infant (1-12 months),

young adult (21-40 years), and mature adult (40-65 years) for 21, 50, and 200 d respectively (U.S. EPA 2002). Information obtained in this study will be useful for RDX and RDX N-nitroso metabolite ecological risk evaluation of terrestrial wildlife at RDX contaminated sites.

Materials and methods

Animals

Male deer mice of three age classifications (21 d, 50 d, & 200 d) were obtained from an in-house breeding colony at Texas Tech University (Lubbock, TX, USA). Stock mice of the Texas Tech University Breeding Colony were obtained from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA). Mice were acclimated for one week. Mice in 50 and 200 d age classifications were housed in groups of 6 according to age classification. Mice from the 21 d age classification were housed with their mother and litter until the dosing procedure was initiated. These mice were then weaned from their litters 3-4 hours prior to dosing. Mice were identified using ear notches. Housing used standard rodent cages (~ 29 x 18 x 14 cm) lined with laboratory-grade aspen bedding (Harlan Teklad, Madison, WI, USA). Cages were located in rooms with temperatures ranging from 19.8 to 25.6 ºC, 21 to 70% relative humidity, and 12:12 h light:dark period. Purina Certified Rodent Chow® 5002 (Purina Mills, St. Louis, MO, USA) and water were provided manually, checked daily, and available *ad libitum* to mice. Experiments and housing were conducted in compliance with Texas Tech University's Animal Use and Care Committee's guidelines. Mice were 21.7 ± 0.3 d (mean ± standard error) (n=20), 50.9 ± 0.8 d (n=20), and 199.5 ± 5.7 d (n=20) old and weighed 9.62 ± 0.28 g, 16.81 ± 0.26 g, and 19.47 ± 0.39 g for the 21 d, 50 d, and 200 d age classifications, respectively.

Chemicals

Hexahydro-1,3,5-trinitro-1,3,5-triazine was obtained from Accurate Energetic Systems LLC (McEwen, TN, USA). Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine

(>99 % pure) and hexahydro-1,3,5-trinitroso-1,3,5-triazine (>99 % pure) were obtained from SRI International (Menlo Park, CA, USA).

Vehicle used was poly(ethylene glycol) (PEG) (n~300) obtained from Sigma-Aldrich (St. Louis, MO, USA). Toxicants used were not soluble at concentrations dosed; hence, suspensions were created using magnetic stir bars and stir plates. Suspensions were agitated for at least ten minutes prior to obtaining the proper dosing volume. Hamilton Gastight® Syringes (Reno, NV, USA) were coupled with 20 gauge, 1.5 in., bent gavage needles (Popper & Sons: New Hyde Park, NY, USA) were used to deliver the dose to the rodents and obtain samples for dose verification. To minimize error, one person dispensed all doses and samples for verification.

Dose verification

Doses were verified using a Hewlett-Packard 1100 (Wilmington, DE, USA) reverse phase high performance liquid chromatography (HPLC) coupled with ultraviolet detection (wavelength: 254 nm). Water and acetonitrile (45:55) were used as the mobile phase which passed through a 25 cm X 4.6 mm X 5 μm Discovery® C18 column (Supelco, Bellefonte, PA, USA). Precision of the suspension delivery with the gavage syringes was tested in triplicate at the start dose for each toxicant prior to initiating dosing.

Up and Down Procedure

Three to four hours before dosing, feed was withdrawn from deer mice. A mouse was then administered the starting dose of one toxicant suspended in PEG via gavage. On the first day, all toxicants were administered to individual mice. Starting doses were 190 mg/kg for MNX and TNX, and 136 mg/kg for RDX. Doses were selected based on similarity to previously calculated LD_{50} values (Meyer *et al.* 2005). Dose volumes ranged from 7.58 to 10.00 μL/g body weight. After dosing, mice were observed continuously for a minimum of one hour (pending visible signs of toxicity) post dosing, periodically for the next 24 hr, and daily for 14 days post dosing. Food was reintroduced 1-2 hours post dosing. Moribund animals were removed and

euthanized using carbon dioxide gas as an asphyxiant. At necropsy, blood was obtained from all animals via heart puncture, and the following organs were removed: liver, kidney, testes, spleen, and brain.

The next sequential dose in the UDP was based on the 48 hr status of the first dosed animal. If the animal survived, the next dose increased by the pre-determined dose progression factor-a quarter log dose (1.778). If the animal did not survive, the dose decreased by the same dose progression factor. Another animal was then dosed with the new dose, and the process was repeated. This iterative process of dosing, adjusting the subsequent dose, and repeating was continued until the one of the following stop criteria were met: A) 3 consecutive animals survived the upper bound (2000 mg/kg) B) 5 reversals occurred in any 6 consecutive animals tested or C) after the first reversal, at least 4 animals were dosed and two likelihood ratios exceeded a critical value of 2.5 (OECD 2001; Rispin *et al.* 2002). Once a stopping criteria was met and dosing ceased, an LD_{50} value was calculated. This procedure was used for 3 toxicants-RDX, MNX, and TNX with three age classifications of deer mice-21 d, 50 d, and 200 d.

Statistical analysis

The Acute Oral Toxicity (Guideline 425) Statistical Program (AOT425) (Westat 2001) was used to calculate LD_{50} values and 95% confidence intervals. Software used for graphics and all other statistical analyses was R: A language and environment for statistical computing Version 2.1.0 (R 2004). Other statistical analyses included linear regression on organ weights relative to body weight, hematocrits, time to onset of toxicity, and time to death. An α value of 0.05 was used to determine significance of slopes equal to zero.

Results

Based upon analytical chemistry concentrations, doses were deemed acceptable if they were within 15% of the intended dose. Measured doses greater than the intended dose were adjusted volumetrically to the actual target dose. Actual

measured doses were entered into AOT425 software for LD_{50} and confidence interval computations. Gavage delivery precision was tested using coefficient of variation for the triplicate aliquots prior to dosing. Coefficients of variation were 4.8% for RDX, 3.8% for MNX, and 8.2% for TNX.

Excluding those mice that did not display overt toxicity during the initial observation period, onset of toxicity occurred 4-188 min for RDX, 4-185 min for MNX, and 3-309 min for TNX post dosing. Overt signs of toxicity included excessive twitching, trembling, salivating, lethargic activity, vacuous chewing, and spontaneous seizures. Typically, mice displayed a kindling effect type seizure. Kindling effect seizures generally initiated a tense, stiff tail; then proceeded with unilateral and bilateral forelimb clonus, head bobbing, pawing at mid air, rearing, and falling. The seizure usually terminated into a quiescent period or normal behavior. Sometimes these kindled effect seizures advanced into generalized motor seizures characterized by jumping, some running, and generalized motor dysfunction. Advanced seizures typically terminated with the animal falling to its side with tonic contractions of all limbs. This was followed by either recovery to a regular state or mortality in which rigor mortis immediately occurred in the tonic, contracted state.

One mouse did not display the same type of seizures as other mice. This mouse was a 200 d old mouse dosed with 1069 mg/kg MNX. It displayed extreme hyperactive type seizures that were characterized by uncontrolled running and jumping, similar to the 'running fit' described by Goddard *et al.* (1969). These seizures were different than other generalized motor seizures previously noted because they did not show any other signs of toxicity other than spontaneous bursts of extreme, intense running and other activity (sometimes 30+ sec.) followed by either stationary, lethargic behavior or rapid respiration.

Four mice that displayed seizures did not die. Three of these mice were dosed with 541.7 mg/kg MNX. This dose and toxicant also caused the single delayed death observed in this study. Overt signs of toxicity unique to MNX that were displayed by these mice and other MNX dosed mice included extremely lethargic behavior (laying

upright in cage corner) and eyes matted shut on day 1 post dosing, due to a buildup of dried ocular exudate. Other than these unique features, overt toxicity was undistinguishable among toxicants.

Lowest doses that caused mortality were 136 mg/kg for RDX, 106 mg/kg for MNX, and 338 mg/kg for TNX (**Fig. 2.2**). Of those mice that died, 65% of mortality occurred within 4.5 hr of dosing, and 88% of mortality within 24 hr of dosing. Calculated LD_{50} values ranged from 136-319 mg/kg for RDX, 181-574 mg/kg for MNX, and 338-999 mg/kg for TNX (**Table 2.1**). Adverse effects included seizure activity, eyes matted shut with dried ocular exudate, and death.

Linear regression models were fit to organ weights relative to body weight dependant on dose for mice of different age classifications, different toxicants, and different outcomes of dosing, i.e. survival and death. Four linear models showed slopes (relationships) that were significantly different from zero. Liver weights relative to body weight of 21 d mice dosed with TNX that died were lower (p=0.035). Kidney weights relative to body weight of 200 d mice dosed with RDX that survived were higher (p=0.034). Spleen weights relative to body weight of 200 d mice dosed with RDX that survived were lower (p=0.02). Spleen weights relative to body weight of 21 d mice dosed with MDX that survived were lower (p=0.0089). No other slopes were statistically different from zero for other organ weights relative to body weight or blood hematocrit.

Discussion

Noted overt signs of RDX toxicity (excessive salivation, kindled seizures, vacuous chewing) were similar to previous observations (von Oettingen *et al.* 1949; Schneider *et al.* 1977; Burdette *et al.* 1988; Meyer *et al.* 2005). Meyer *et al.* (2005) also noted these similar indications of toxicity for MNX and TNX. Kindling effect seizures are due to stimulation of the olfactory-limbic system, with the amygdala being the most sensitive (Goddard *et al.* 1969). Others have hypothesized that the limbic system is the target for RDX toxicity (Burdette *et al.* 1988). Since MNX and

TNX also produced this type of response, one can hypothesize that they display a similar toxic mechanism as does RDX. Extreme hyperactivity type seizures, as displayed by one MNX dosed mouse, have been seen due to stimulation of the brainstem and cerebellum (Goddard *et al.* 1969). These brain structures may also be a target of MNX.

Completion of UDP went fairly close to expectations: finding the general range of the LD_{50} value, then oscillating reversals around that value. The exception was found in MNX testing with 21 d mice. These mice displayed variable results over a wide range of doses. One possible explanation for this occurrence is a flat slope of the mortality dose curve. Raw data from the MNX fixed dose acute toxicity study in adult Sprauge-Dawley rats (Meyer *et al.* 2005) was used to fit a probit model (probit mortality dependant on log dose plus one). That model yielded a slope of 4.7. By comparison, according to the U.S. EPA Office of Pesticide Programs one-liner database, mortality slopes for terrestrial vertebrate responses to a wide range of compounds regulated under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) showed a mean of 5.3 (0.56-89.0) (n=500). Thus, the value of 4.7 would be considered low. The UDP design does not allow slope determination as would a standard dose response curve. Thus, slopes for MNX and TNX are unknown.

Another possible explanation of this inconsistent response is varying particle sizes. Particle size of RDX has been demonstrated to be directly linked to RDX concentrations in the blood, and hence, RDX toxicity (Schneider *et al.* 1977). Particle sizes of toxicants in suspensions were not investigated, but there is a possibility that particle sizes of toxicants were decreased through the duration of the study due to an ever increasing stirring time. This may be a reason for seemingly resistant mice early in the study and then sensitive mice in later stages.

Confidence intervals (95%) of determined LD_{50} values were rather large, sometimes yielding indeterminate values (0 to >20,000 mg/kg), which hinders statistical comparisons. Possible explanations for this are the UDP study design, possible flat slope of the mortality dose curve, and possible variance in toxicant

particle size. The purpose of the UDP is to calculate the LD_{50} point using a minimal number of animals, concentrating the sampled data points around the LD_{50} dose. This inherently leaves few tested doses that are more than one log dose from the LD_{50}. Thus, little information is available to predict tight confidence intervals. While this leads to larger than desired confidence intervals, it still provides a basic assessment of a chemical's acute toxicity.

Calculated median lethal dose for RDX in deer mice is similar to slightly elevated compared to what others have seen in rodents (**Table 2.2**). Calculated values for RDX N-nitroso metabolites are elevated when compared to other reported values (**Table 2.2**). The other RDX values in this table are from traditional LD_{50} protocols, except Meyer *et al.* (2005), which was an incomplete UDP study. It has been reported that the large variation in reported RDX LD_{50} values is due to variation of gavage vehicles (Yinon 1990). Poly(ethylene glycol) was used in this study due to its high viscosity and, hence, ability to keep particles homogenously suspended for longer times. Others have used small amounts of dimethylsulfoxide for dissolution, then added that solution to corn oil which produced a suspension for dosing (Schneider *et al.* 1977; Meyer *et al.* 2005). Addition of these carriers to vehicles may affect absorption rates of toxicants.

One potential source of error with suspended dosing solutions is differences nominal and verified dose concentrations. This was observed in our study. Nominal and measured concentrations differed by up to 60% following initial dose preparation, which required adjustments to the toxicant amounts added to the dose. As stated previously, doses were not deemed acceptable unless they were at or 15% below the target dose. These differences between nominal and measured dose concentration exist due to heterogeneity of the dosing suspensions. One potential source of heterogeneity is particles sinking in the suspension while being stirred with the magnetic stir bar. The vehicle (PEG) was chosen to minimize this effect due to high viscosity, but that viscosity may have also limited the gavage needle to intake particles

from bulk solution. Most LD_{50} studies omit verification procedures, including recent studies of MNX, DNX, and TNX (Meyer *et al.* 2005).

One of the most obvious potential differences in the two studies that report LD_{50} values for RDX N-nitroso metabolites is the species of animal model. Meyer *et al.* (2005) used Sprauge-Dawley rats as an animal model, while deer mice were used in this study. Gender differences may also play a role. Some studies have shown females to be more sensitive to RDX (Dilley *et al.* 1978; Cholakis *et al.* 1980), including the study driving the U.S. EPA potential carcinogen classification of RDX (Lish *et al.* 1984). Meyer *et al.* (2005) used females as well. Males were used in our study to reduce variation due to varying hormonal profiles.

For age classifications 21 d and 50 d, order of increasing potency is TNX, MNX, and RDX. For the 200 d classification, order of increasing potency is MNX, TNX, and RDX. Thus, in an acute oral dose, the parent compound appears to be the most toxic. This was also seen and noted by Meyer *et al.* (2005).

Age dependent toxicity was demonstrated for all of these compounds. While LD_{50} confidence intervals are large, and, in most cases, encompass other age classifications, all of the toxicant LD_{50} values follow the pattern of increasing sensitivity: 50 d, 200 d, and 21 d. When looking further at the mortality data for each toxicant, the minimum dose that caused mortality compared between 50 d mice (the most resistant) and 21 d mice (the most sensitive) is quite profound. For example, for MNX the lowest dose mortality in 50 d mice is 541 mg/kg and in 21 d mice is 106 mg/kg-three dosing steps difference. The other two toxicants, RDX and TNX, show similar patterns, but a two dosing step difference exists for both compounds. The most sensitive age classification, 21 d (human equivalency of infancy 1-12 mo.), is significant because populations themselves are dependent on the success of young. This information is also beneficial to help assess adequacy of regulation values and adjust accordingly.

While some statistically significant differences have been noted with organ weights relative to body weight, it is also note worthy that the number of significant slopes was approximately 5% of the total linear regressions evaluated for the study, the same percentage as our α value. Also, some of these significant regressions are derived with small samples sizes (n=3). If actual responses do occur, one would also expect similar significant differences or even trends of similar cohorts, i.e. with toxicant or age classification. This does occur in spleen weight relative to body weight of deer mice dosed with MNX and survived. All these mice show either a trend or a statistically significant dose dependant decrease in spleen weight to body weight (21 d age classification p=0.0089, 50 d age classification p=0.69, and 200 d classification p=0.070). This seems to suggest that MNX may have a negative impact on spleens. Meyer *et al.* (2005) demonstrated dose dependant deposition of ferric iron in spleens of rats in an MNX fixed-dose study, and hypothesized this was due to hemosiderosis caused by N-nitroso groups.

Our findings indicate that RDX and N-nitroso metabolites cause similar toxic responses. Median lethal dose for 21 d deer mice were 136, 181, and 338 mg/kg for RDX, MNX, and TNX respectively. Median lethal dose for 50 d deer mice were 319, 575, and 999 mg/kg for RDX, MNX, and TNX respectively. Median lethal dose for 200 d deer mice were 158, 542, and 338 mg/kg for RDX, MNX, and TNX respectively. These data suggest that RDX is the most potent compound tested, and age dependent toxicity may exist for all compounds. Although the UDP produced large confidence intervals, these results may play an important role in future policy decisions for remedial design and effectiveness at explosive contaminated sites; in that, RDX ecological risk estimates may be not be affected by inclusion of acute data from N-nitroso metabolites. Also, ecological uncertainty factors in risk assessments may be reduced by incorporating these data.

Acknowledgements

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Tables

2.1 Median lethal dose (LD_{50}) of deer mice (*Peromyscus maniculatus*) exposed to hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and two metabolites

Median lethal dose (LD_{50}) determined using the U.S. EPA Up-and-Down Procedure in deer mice (*Peromyscus maniculatus*) for three age classes (21, 50, and 200 d) and three toxicants (hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX)).

Compound	Age (d)	LD_{50} (mg/kg)	Lower CI[a]	Upper CI[a]
RDX	21	136	0	>20000
RDX	50	319	202	1530
RDX	200	158	0	>20000
MNX	21	181	0	>20000
MNX	50	575	344	1290
MNX	200	542	344	1290
TNX	21	338	165	833
TNX	50	999	627	4380
TNX	200	338	224	>20000

[a] 95% confidence intervals (CI) of LD_{50} values

2.2 Reported median lethal dose (LD_{50}) of deer mice (*Peromyscus maniculatus*) exposed to hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and two metabolites

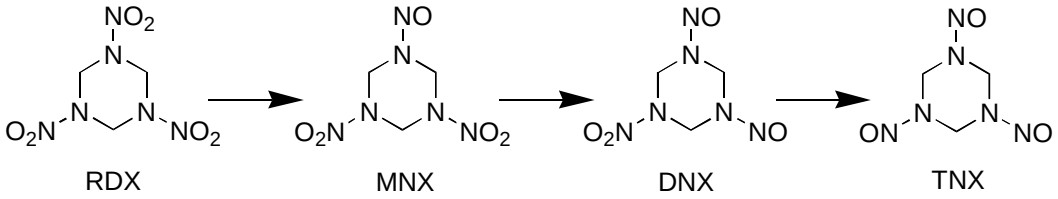
Literature median lethal dose (LD_{50}) values for rodents dosed with one of the following compounds: hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), or hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX).

Species	LD_{50} (mg/kg) Toxicant			Source
	RDX	MNX	TNX	
Sprague-Dawley Rats	187[a]	187[a]	400[ab]	Meyer *et al.* 2005
Sprague-Dawley Rats		187		Meyer *et al.* 2005
Sprague-Dawley Rats	71			Dilley *et al.* 1978
Fischer 344 Rats	119			Levine *et al.* 1981
Male Swiss-Webster Mice	86			Dilley *et al.* 1978
Female Swiss-Webster Mice	75			Dilley *et al.* 1978
Male B6C3F1 Mice	97			Cholakis *et al.* 1980
Female B6C3F1 Mice	56			Cholakis *et al.* 1980

[a] Determined using the U.S. EPA Up-and-Down Procedure

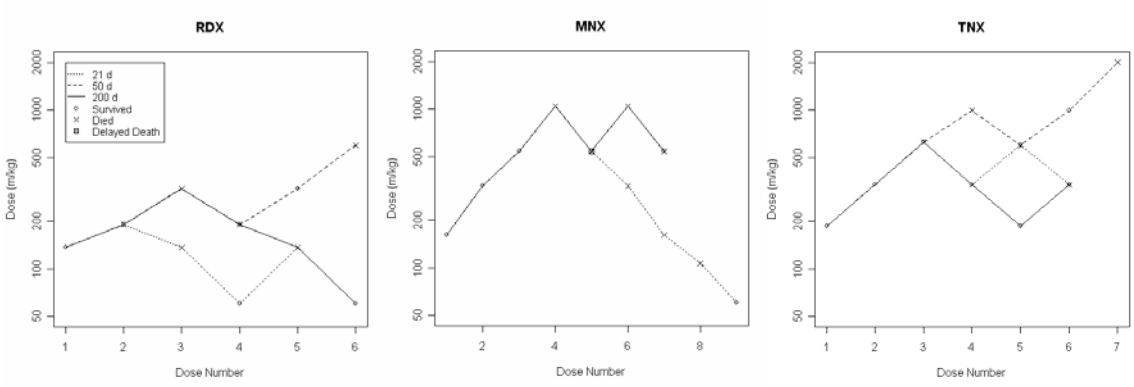
[b] Authors calculated from raw data available in manuscript

Figures



2.1 Reductive degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) progressive anaerobic degradation to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX).



2.2 Dose progression

Dose progression for deer mice (*Peromyscus maniculatus*) of three age classes (21, 50, and 200 d) dosed with hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) in determining median lethal dose (LD_{50}) using the U.S. EPA Up-and-Down Procedure.

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CHAPTER III

REPRODUCTIVE EFFECTS OF HEXAHYDRO-1,3,5-TRINITROSO-1,3,5-TRIAZINE IN DEER MICE (*PEROMYSCUS MANICULATUS*) DURING A CONTROLLED EXPOSURE STUDY

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Abstract

Contamination with hexahydro-1,3,5-trinitro-1,3,5-triazine (Royal Demolition Explosive [RDX]) has been identified at areas of explosive manufacturing, processing, storage, and usage. Thus, the potential exists for exposure to N-nitroso compounds, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine, hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine, and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX), formed via anaerobic transformation of RDX. Following exposure, reproductive toxicity of TNX was evaluated in three consecutive litters of deer mice (*Peromyscus maniculatus*). Hexahydro-1,3,5-trinitroso-1,3,5-triazine was administered *ad libitum* via drinking water at four doses: 0 (control), 1, 10, and 100 μg/L. Endpoints investigated included reproductive success, offspring survival, offspring weight gain, offspring organ weights, and liver TNX residues. Data from the present study indicate that TNX bioaccumulates in the liver and is associated with postpartum mortality, dose-dependent decrease in body weight from birth to weaning, and decrease in kidney weight of deer mice offspring.

Keywords: Deer mouse Royal demolition explosive Hexahydro-1,3,5-trinitroso-1,3,5-triazine Hexahydro-1,3,5-trinitro-1,3,5-triazine

Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX; Chemical Abstract service [CAS] 121-82-4) has been used as an explosive for both military purposes and civilian applications worldwide. The U.S. Army has confirmed environmental explosive contamination at 583 sites within 82 installations in the United States and suspects 88 additional sites within 22 installations as possibly being contaminated (Davis *et al.* 2004). The U.S. Environmental Protection Agency Comprehensive Environmental Response, Compensation, and Liability Information System Database has identified 20 Superfund sites that are contaminated with RDX. In areas where RDX is the primary contaminant of concern, anaerobic degradation currently is the preferred method of clean-up. Such degradation is conducted *in situ* or using bioreactors (Radtke 2005).

Using anaerobic sludge, McCormick *et al.* (1981) first showed RDX to biodegrade progressively (**Fig. 3.1**) into the following N-nitroso compounds: hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (CAS 5755-27-1), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (CAS 80251-29-2), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX; CAS 13980-04-6). Others have since confirmed and expanded our understanding of RDX degradation to include the formation of hydroxylamino derivatives, such as hydroxylamino-dinitroso-RDX (Kitts *et al.* 1994; Regan and Crawford 1994; Young *et al.* 1997; Boopathy *et al.* 1998; Kitts *et al.* 2000; Adrian and Chow 2001; Beller 2002). These compounds can undergo hydrolytic ring cleavage and degrade into a variety of compounds, ultimately forming formaldehyde, nitrous oxide, and hydrazines (McCormick *et al.* 1981; Hawari *et al.* 2000; Bhushan *et al.* 2002). Transformation of RDX occurs more easily via anaerobic degradation rather than by aerobic degradation (McCormick *et al.* 1981; Kitts *et al.* 1994; Young *et al.* 1997). Recently, N-nitroso metabolites of RDX have been found in groundwater beneath the Iowa Army Ammunition Plant (Middletown, IA, USA), with concentrations as high as 430 µg/L (Beller and Tiemeier 2002). As anaerobic degradation proceeds and N-nitroso degradates are produced, a possibility exists that although the concentration of the parent compound (RDX) is significantly reduced, the

by-products may not be any less toxic. Well-known transformational processes produce by-products that are equally or more toxic than the parent compound. Some of these compounds include aldicarb (Cobb *et al.* 2001), polycyclic aromatic hydrocarbons (Conney 1982; Williams and Weisburger 1986), and various polychlorinated biphenyl congeners (Bergman *et al.* 1994).

Few peer-reviewed articles have addressed the presence of RDX N-nitroso metabolites. Thus, it is difficult to predict the current extent or magnitude of N-nitroso metabolite contamination. However, the fact that best practices in RDX remedial actions involve anaerobic *in situ* degradation demonstrates that these N-nitroso metabolites will be produced by extant remediation technologies (Radtke 2005). Critical questions that need to be addressed are to what extent is remedial action needed, and when can RDX remediation cease (i.e., at the point where RDX reaches a regulatory threshold, or at a point where N-nitroso metabolites diminish below a no-observed-adverse-effect level). Whereas the toxicity of RDX has been well documented (Schneider *et al.* 1977; Schneider *et al.* 1978; Levine *et al.* 1981), little is known about the toxicity of RDX N-nitroso metabolites, and to our knowledge, no data have been published regarding their toxicity in rodents.

Other N-nitroso compounds (nitrosamines) have been shown to exhibit carcinogenic properties via bioactivation of the compound through oxidation (generally by cytochrome P450 enzymes), then carcinogenicity through alkylation of general macromolecules (Kotsonis *et al.* 2001). Although this effect most often is reported for straight chain N-nitroso compounds, cancer induction pathways have been demonstrated for nitrogenous heterocyclic compounds as well (Lijinsky and Kovatch 1993; Wong *et al.* 2003). N-nitroso compounds also have been shown to cause centrilobular hepatic necrosis in acute exposure and liver cirrhosis in chronic exposure (Brown 1999).

Because of the unknown characteristics of these compounds, the goal of the present study was to begin defining the potential toxic nature of these N-nitroso compounds. To achieve this goal, reproductive success, offspring survival, growth,

organ weight, and toxicant uptake were all studied with the hope that the results may play a role in future decisions for remediation of explosive contaminated sites.

Materials and Methods

Test chemical

Hexahydro-1,3,5-trinitroso-1,3,5-triazine (purity, >99%) was obtained from SRI International (Menlo Park, CA, USA). Dosing solutions were renewed every 3 d and analyzed with reverse-phase, high-performance liquid chromatography to ensure concentration. Dosing water bottles were weighed at the time of water change to monitor water consumption by deer mice (*Peromyscus maniculatus*).

Animals

Twenty-three breeding pairs of virgin male and female deer mice were obtained from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA). Mice were allowed to acclimate for one week. Breeding pairs were housed separately in standard rodent cages (~29 × 18 × 14 cm) lined with laboratory-grade aspen bedding (Harlan Teklad, Madison, WI, USA). Cages were located in rooms with temperatures ranging from 18.3 to 25.6°C, 25 to 75% relative humidity, and a 16:8-h light:dark period. Food and water were provided manually, checked daily, and available *ad libitum* to mice. Purina Certified Rodent Chow® 5002 (Purina Mills, St. Louis, MO, USA) was used as food. Experiments and housing were conducted in compliance with Texas Tech University Animal Use and Care Committee guidelines.

Experimental procedure

Once acclimated, deer mice were randomly paired. Six pairs were assigned to each exposure group (1, 10, and 100 µg/L), and five pairs were assigned to the control group. Hexahydro-1,3,5-trinitroso-1,3,5-triazine was administered *ad libitum* in drinking water. Deer mice bred and produced three litters of offspring (F1A, F1B, and F1C). Offspring from F1A and F1B litters were weaned on postnatal day (PND) 21 and were dosed until killed on PND 45. These offspring were weighed at weaning and

then again every 7 d until euthanized. Offspring in the F1C litter were weighed at PNDs 2, 5, 10, 15, and 20. These offspring were euthanized on PND 21 using the same procedure as for F1A and F1B mice.

Euthanasia was conducted using carbon dioxide gas as an asphyxiant. At necropsy, blood was obtained from all animals via heart puncture and used in biochemical analyses as part of an allied study. The following organs were removed: liver, kidney, gonads, and brain. Parent mice (F0) were euthanized on the same day as their F1C offspring. Endpoints investigated on the offspring included number born, survival, weight gain, organ weights, and liver TNX residue.

Liver samples were divided into two groups: one for TNX residue analysis, and one for cytochrome P4502B analysis in an allied study (results not yet available). Livers within litters from common dose groups were composited to produce sufficient sample masses (~1 g) for trace analyses. Liver samples were then homogenized and extracted using Accelerated Solvent Extraction (Dionex, Salt Lake City, UT, USA). The extract was then cleaned, first with a Florisil solid-phase extraction cartridge (Bellefonte, PA, USA) and then with a styrene-divinylbenzene cartridge (Supelco, Bellefonte, PA, USA). Gas chromatography was conducted using an electron-capture detector (Agilent, Palo Alto, CA, USA) and HP-5 column (length, 30 m; inner diameter, 0.25 mm; film thickness, 0.25 mm; Hewlett-Packard, Wilmington, DE, USA). The temperature profile was initiated at 90°C, maintained for 2 min, ramped to 130°C at a rate of 25°C /min, ramped to 200°C at a rate of 10°C /min, and finally, ramped to 250°C at a rate of 25°C/min. This method allows recovery of 88.1 ± 13.2% (relative standard deviation), with a detection limit of approximately 20 µg/L (Pan *et al.* 2005). Liquid chromatography–mass spectroscopy in negative-ion mode was used to verify the presence and concentration of TNX. Acetic acid was added to create a TNX–acetic acid complex (233 amu). A primary peak of 233 amu was monitored in the analysis.

Statistics

Litter size, body weights, relative organ weights, and absolute organ weights were evaluated using one-way analysis of variance coupled with the Tukey–Kramer test (Zar 1999). Offspring mortality was evaluated using a chi-square test of independence for overall and developmental period mortality. Developmental periods evaluated included birth (live vs stillbirths), birth to PND 4, PND 4 to weaning (PND 21), and weaning to death (PND 45). All statistical tests used an α value of 0.05. The software used to analyze data was R: A Language and Environment for Statistical Computing, Version 2.0.0 (R 2004).

Results

Results from high-performance liquid chromatographic verification of dosing solutions show TNX concentrations (mean ± standard error [SE]) to be not detectable (n = 45), 0.73 ± 0.02 µg/L (n = 38), 9.01 ± 1.10 µg/L (n = 39), and 107.14 ± 1.05 µg/L (n = 45) for the control, 1 µg/L, 10 µg/L, and 100 µg/L dose groups, respectively. Liver TNX residue analysis showed little to no accumulation in control (all nondetects) and low-dose (nondetect to 28 ng/g) groups, whereas the medium-dose (20–138 ng/g) and high-dose (nondetect to 140 ng/g) groups showed greater accumulation (**Table 3.1**).

Water consumption during cohabitation to birth ranged from 15.33 to 18.73 ml/d/breeding pair for all dose groups (**Table 3.2**). Consumption was increased to 21.25 to 27.30 ml/d/breeding pair with litters of offspring. Once weaned, offspring consumed 5.68 to 6.55 ml/d/individual.

The number of offspring per litter (mean ± SE) ranged from 3.00 ± 1.10 to 6.33 ± 0.79 for all litters and dose groups (**Table 3.3**). Results showed no significant differences among dose groups for the number of offspring born within every litter ($p \geq 0.355$) except F1B. Within that litter, a significant difference was observed between the low-dose (1 µg/L) and medium-dose (10 µg/L) groups ($p \geq 0.0055$). Other dose groups within this litter showed no significant differences among one another ($p \geq 0.247$).

Chi-square test of independence showed that F1A overall mortality ($\chi^2 = 11.4$, $p = 0.0097$) and F1B overall mortality ($\chi^2 = 11.5$, $p = 0.0095$) were dependent on dose of TNX. Dividing overall mortality into the aforementioned developmental periods revealed that F1A mortality from birth to PND 4 ($\chi^2 = 8.5$, $p = 0.0373$) and F1B birth mortality ($\chi^2 = 14.9$, $p = 0.0019$) also were dependent on dose (**Table 3.4**). From weaning to death, mice in the F1A litter approached the significance level for mortality dependence on dose ($\chi^2 = 6.9$, $p = 0.0754$). No other significant differences in overall or developmental period mortality ($p \geq 0.1679$) were found in any of the litters.

Results showed that F1A mice dosed with 100 µg/L of TNX approached significantly lower weight at PND 21 compared to F1A mice in the 1 and 10 µg/L dose groups ($p = 0.0609$–0.0996) (**Fig. 3.2**). All other periods demonstrated no significant differences in body weights among dose groups for F1A mice ($p \geq 0.1652$). Mice from the F1B litter in the 10 µg/L dose group had significantly lower body weight compared to all other F1B dose groups at PND 21 ($p \leq 0.0371$) as well as differences from F1B mice dosed with 1 µg/L of TNX ($p = 0.0486$) and 100 µg/L of TNX ($p = 0.0118$) at PND 28 (**Fig. 3.3**). All other periods and dose groups in F1B displayed no significant differences ($p \geq 0.1452$). Results showed a dose-dependent decrease in body weight of F1C mice from PND 2 to PND 21 (**Fig. 3.4**). Control F1C and F1C mice that received a TNX dose of 1 µg/L exhibited no significant differences in body weight during this time period ($p \geq 0.8550$); however, F1C mice dosed with 100 µg/L of TNX ($p \leq 0.0005$) had significantly lower body weight compared to F1C control mice. Mice from the F1C litter in the 10 µg/L dose group had significantly lower body weight from PND 2 to PND 5 when compared to F1C control mice ($p \leq 0.0446$). From PND 10 to PND 21, no significant difference was observed between F1C control and F1C mice dosed with 10 mg/ L of TNX ($p \geq 0.2060$).

Absolute brain weights of F1A mice dosed with 100 µg/L TNX effects on deer mice of TNX were significantly lower than those of F1A control mice ($p = 0.0167$) (**Table 3.5**). Relative weights (organ wt/body wt) of brain, kidneys, testes, and liver in

F1A and F1B litters showed no significant differences among dose groups ($p \geq 0.1556$). Absolute kidney weights of F1C mice dosed with 100 µg/L of TNX were significantly lower than those of all other dose groups ($p \leq 0.0007$), as were kidney weights normalized by brain weight (organ wt to brain wt ratio; $p \leq 0.0049$). Relative kidney weights showed no significant differences among the same mice ($p \geq 0.5271$). Mice in the F1C litter dosed with 100 µg/L of TNX showed a significant increase in relative brain weight when compared to F1C mice in all other dose groups ($p \leq 0.0304$). Absolute brain weights for the 100 µg/L dose group of F1C mice, however, showed no significant differences among dose groups ($p \geq 0.9574$). All other relative organ weights of F1C mice showed no significant differences among dose groups.

Discussion

Concentrations of TNX in the liver indicate that TNX was absorbed by the gastrointestinal tract of deer mice. Although small sample size precludes statistical analysis, the present results show that liver TNX concentration increases with increased dose. Other studies have shown that RDX, the parent compound, bioaccumulates in the liver (Schneider *et al.* 1977; Schneider *et al.* 1978). Bioaccumulation of N-nitroso compounds in the liver is toxicologically significant, because these compounds are bioactivated by cytochrome P450 enzymes into electrophilic alkylating agents via α-oxidation (Archer 1989). Liver is a primary location for cytochrome P450 enzymes; thus, the potential exists for toxic insult or potential carcinogenesis. All mice dosed with 10 µg/L of TNX showed liver TNX concentrations slightly greater than that in their water. Mice dosed with 100 µg/L of TNX showed liver TNX concentrations fairly close to aqueous dose concentrations.

Exposure of TNX to breeding deer mice did not affect the number of offspring born per successful litter; however, offspring mortality was increased. The majority of offspring mortality occurred at birth or within 4 d following birth. This suggests a direct toxic effect via interuterine and milk exposure, an indirect toxic effect via maternal toxicity, or a combination of the two. Toxicity during early stages of life is plausible, because absorption, distribution, metabolism, and excretion mechanisms

grow and develop at different rates and, thus, age-dependent toxic responses exist (U.S. NRC 1993). For example, kidney glomerular filtration is poorly developed in infants and, therefore, leads to an increased chance of toxicity (Klienman 1982). The capacity of the liver to detoxify various compounds via conjugation also develops slowly; thus, an increased risk of toxicity exists for these types of compounds (Vessell 1982). Indirect offspring toxicity may result from several maternal toxic responses, including decreased uterine blood flow, maternal anemia, altered nutritional status, altered organ function, autoimmune status, electrolyte/acid–base disturbances, decreased milk quantity or quality, and abnormal behavior (Chernoff *et al.* 1989; Daston 1994). Nutritional status and/or compromised metabolic pathways may be plausible effects, because food avoidance has been demonstrated with the parent compound (RDX) in birds (Gogal *et al.* 2003).

Results also indicate that TNX causes a dose-dependent decrease in body weight during the early life stage (birth to weaning or shortly thereafter). After weaning, high-dose groups have an increased growth rate. By the onset of puberty, high–dose group body weights converged with lower–dose group body weights. Dose-dependent differences in body weight of offspring are seen from approximately birth to weaning, which further supports the possibility that TNX may adversely affect the dam's mothering ability, milk production, or milk palatability rather than the possibility of a direct toxic effect on offspring. Alternatively, dose-induced decreases in body weight during early life stages may have resulted from direct toxicity to offspring, whereby offspring metabolism was increased and offspring were reliant on limited maternal food sources. Other studies have reported RDX, the parent compound, to cause hyperactivity in Fischer 344 rats (Levine *et al.* 1981; Levine *et al.* 1990).

At first glance, F1C differences in relative brain weights indicate a biological response of the brain to TNX; however, this response more likely is a function of high-dose group offspring having lower body weights compared with lower-dose group offspring. This idea is supported by the fact that absolute brain weights showed

no significant differences among same-dose groups. In turn, this suggests that brain growth was similar in dosed and untreated mice, whereas overall body weight of dosed mice lagged body weights of undosed mice, creating the observed increased relative brain weights in dosed mice.

Absolute kidney weights of F1C mice in the 100 µg/L dose group show a significant decrease compared to F1C mice in lower-dose groups, with relative kidney weights showing no significant differences in the same mice. When test material alters body weight, brain weights typically are not altered; thus, organ weights can be normalized by brain weight (Wilson and Hayes 1994). This situation is the case for F1C mice, in which a dose-dependent decrease in body weight was observed with no significant differences in brain weight. Kidney weights normalized to brain weight of F1C mice further emphasize decreased kidney weights, indicating that kidney growth in dosed mice lagged kidney growth in unexposed mice at a rate similar to the lag in body weight already noted.

Our findings demonstrate TNX bioaccumulation in the liver in a dose-dependent fashion, with concentrations in this organ being similar to TNX concentrations in water. Findings also indicate that TNX could be responsible for increased mortality and dose-dependent decreases in body and kidney weight from birth to weaning in developing deer mice. These results may play an important role in future decisions for remedial design and effectiveness at explosive-contaminated sites, in that risk estimates may be affected by inclusion of N-nitroso transformation products.

Acknowledgement

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Tables

3.1 Hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) concentrations in liver

Hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) concentrations[a] (ng/g) in liver tissue from deer mice offspring that were exposed to varying concentrations of TNX in drinking water.

Litter	Dose	Concentration[a]	*n*	Number of detects
F1A[b]	Control	Below detection	4	0
	1 µg/L	Below detection	2	0
	10 µg/L	49.03 ± 14.58	2	2
	100 µg/L	53.30 ± 10.54	2	2
F1B[c]	Control	Below detection	4	0
	1 µg/L	Below Detection	2	1
	10 µg/L	93.21 ± 10.70	9	9
	100 µg/L	87.93 ± 39.68	3	2
F1C[d]	Control	Below Detection	3	0
	1 µg/L	Below Detection	2	0
	10 µg/L	54.955 ± 8.47	3	3
	100 µg/L	121.78 ± 24.88	4	4

[a] Mean ± standard error

[b] First litter of mice, sacrificed on post natal day 45

[c] Second litter of mice, sacrificed on post natal day 45

[d] Third litter of mice, sacrificed on post natal day 21

3.2 Drinking water consumption

Drinking water consumption[a] (mL/d) that contains varying concentrations of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) by deer mice.

Dose	Water consumption[a]		
	Cohabitation to birth[b]	Birth to weaning[c]	Weaning to adolescence[d]
Control	15.33 ± 2.63	25.08 ± 3.26	5.72 ± 0.29
1 µg/L	16.96 ± 2.73	21.25 ± 4.21	6.55 ± 0.27
10 µg/L	18.73 ± 2.96	27.30 ± 3.96	5.69 ± 0.29
100 µg/L	17.91 ± 3.26	23.33 ± 3.65	5.68 ± 0.28

[a] Mean ± standard error

[b] Consumption per an adult pair of deer mice

[c] Consumption per an adult pair of deer mice plus an offspring litter

[d] Consumption per juvenile deer mouse

3.3 Offspring per litter

Number of offspring[a] per successful litter of deer mice exposed to varying concentrations of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). Capital superscripts represent similar statistical groups within litter.

Dose	Offspring[a]		
	F1A[b]	F1B[c]	F1C[d]
Control	4.75 ± 0.29^{A}	4.75 ± 0.55^{B}	4.33 ± 0.33^{E}
1 µg/L	3.00 ± 1.10^{A}	3.33 ± 1.07^{BC}	4.67 ± 0.88^{E}
10 µg/L	4.00 ± 1.03^{A}	6.33 ± 0.79^{BD}	5.50 ± 0.71^{E}
100 µg/L	3.67 ± 0.87^{A}	4.67 ± 0.30^{B}	5.17 ± 1.43^{E}

[a] Mean ± standard error.

[b] First litter of mice

[c] Second litter of mice

[d] Third litter of mice

3.4 Survival of deer mice offspring

Offspring survival of deer mice dosed with varying concentrations of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX).

Litter	Dose	Percent survival at post natal day			
		0	4	21	45
F1A[a]	Control	100	100	100	100
	1 µg/L	83.3	83.3	83.3	83.3
	10 µg/L	95.8	95.8	95.8	95.8
	100 µg/L	95.5	81.8	77.3	68.2
F1B[b]	Control	100	100	100	100
	1 µg/L	75	75	70	70
	10 µg/L	100	97.4	94.7	94.7
	100 µg/L	92.6	85.2	81.5	81.5
F1C[c]	Control	100	100	100	NA
	1 µg/L	100	85.7	85.7	NA
	10 µg/L	97	91	91	NA
	100 µg/L	100	84	84	NA

[a] First litter of mice, sacrificed on post natal day 45

[b] Second litter of mice, sacrificed on post natal day 45

[c] Third litter of mice, sacrificed on post natal day 21

3.5 Organ weights

Organ weights[a] of deer mice exposed to varying concentrations of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). Capital superscripts represent similar statistical groups within litter, organ, and type of organ measurement.

Litter	Dose	Organ Weights			
		Liver		Kidney	
		Absolute (g)	Relative	Absolute (g)	Relative
F1A	Control	0.703 ± 0.074^{a}	0.0425 ± 0.005^{a}	0.200 ± 0.020^{b}	0.0121 ± 0.001^{c}
	1 µg/L	0.696 ± 0.108^{a}	0.0432 ± 0.004^{a}	0.194 ± 0.020^{b}	0.0121 ± 0.001^{c}
	10 µg/L	0.686 ± 0.118^{a}	0.0423 ± 0.006^{a}	0.197 ± 0.019^{b}	0.0122 ± 0.001^{c}
	100 µg/L	0.671 ± 0.129^{a}	0.0414 ± 0.007^{a}	0.194 ± 0.019^{b}	0.0120 ± 0.002^{c}
F1B	Control	0.687 ± 0.103^{f}	0.0417 ± 0.004^{a}	0.208 ± 0.028^{g}	0.0127 ± 0.001^{c}
	1 µg/L	0.651 ± 0.144^{f}	0.0399 ± 0.007^{a}	0.208 ± 0.038^{g}	0.0127 ± 0.001^{c}
	10 µg/L	0.693 ± 0.084^{f}	0.0422 ± 0.004^{a}	0.199 ± 0.018^{g}	0.0121 ± 0.001^{c}
	100 µg/L	0.708 ± 0.099^{f}	0.0427 ± 0.004^{a}	0.213 ± 0.029^{g}	0.0129 ± 0.002^{c}
F1C	Control	0.349 ± 0.083^{jk}	0.0385 ± 0.006^{n}	0.143 ± 0.023^{l}	0.0159 ± 0.002^{n}
	1 µg/L	0.405 ± 0.058^{j}	$0.0424 \pm 0.004n^{o}$	0.146 ± 0.020^{l}	0.0151 ± 0.001^{n}
	10 µg/L	0.366 ± 0.058^{jk}	$0.0414 \pm 0.004n^{o}$	0.137 ± 0.014^{l}	0.0156 ± 0.001^{n}
	100 µg/L	0.324 ± 0.061^{k}	0.0425 ± 0.003^{o}	0.117 ± 0.020^{m}	0.0154 ± 0.002^{n}

Litter	Dose	Organ Weights			
		Liver		Kidney	
		Absolute (g)	Relative	Absolute (g)	Relative
F1A	Control	0.703 ± 0.074^{a}	0.0425 ± 0.005^{a}	0.200 ± 0.020^{b}	0.0121 ± 0.001^{c}
	1 µg/L	0.696 ± 0.108^{a}	0.0432 ± 0.004^{a}	0.194 ± 0.020^{b}	0.0121 ± 0.001^{c}
	10 µg/L	0.686 ± 0.118^{a}	0.0423 ± 0.006^{a}	0.197 ± 0.019^{b}	0.0122 ± 0.001^{c}
	100 µg/L	0.671 ± 0.129^{a}	0.0414 ± 0.007^{a}	0.194 ± 0.019^{b}	0.0120 ± 0.002^{c}
F1B	Control	0.687 ± 0.103^{f}	0.0417 ± 0.004^{a}	0.208 ± 0.028^{g}	0.0127 ± 0.001^{c}
	1 µg/L	0.651 ± 0.144^{f}	0.0399 ± 0.007^{a}	0.208 ± 0.038^{g}	0.0127 ± 0.001^{c}
	10 µg/L	0.693 ± 0.084^{f}	0.0422 ± 0.004^{a}	0.199 ± 0.018^{g}	0.0121 ± 0.001^{c}
	100 µg/L	0.708 ± 0.099^{f}	0.0427 ± 0.004^{a}	0.213 ± 0.029^{g}	0.0129 ± 0.002^{c}
F1C	Control	0.349 ± 0.083^{jk}	0.0385 ± 0.006^{n}	0.143 ± 0.023^{l}	0.0159 ± 0.002^{n}
	1 µg/L	0.405 ± 0.058^{j}	0.0424 ± 0.004^{no}	0.146 ± 0.020^{l}	0.0151 ± 0.001^{n}
	10 µg/L	0.366 ± 0.058^{jk}	0.0414 ± 0.004^{no}	0.137 ± 0.014^{l}	0.0156 ± 0.001^{n}
	100 µg/L	0.324 ± 0.061^{k}	0.0425 ± 0.003^{o}	0.117 ± 0.020^{m}	0.0154 ± 0.002^{n}

[a] Mean ± standard deviation

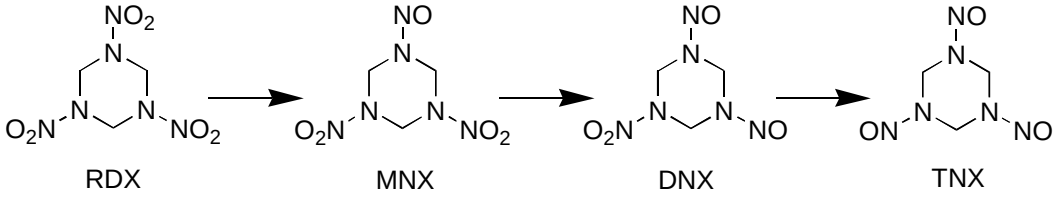
[b] Organ weight normalized to body weight

[c] First litter of mice, sacrificed on post natal day 45

[d] Second litter of mice, sacrificed on post natal day 45

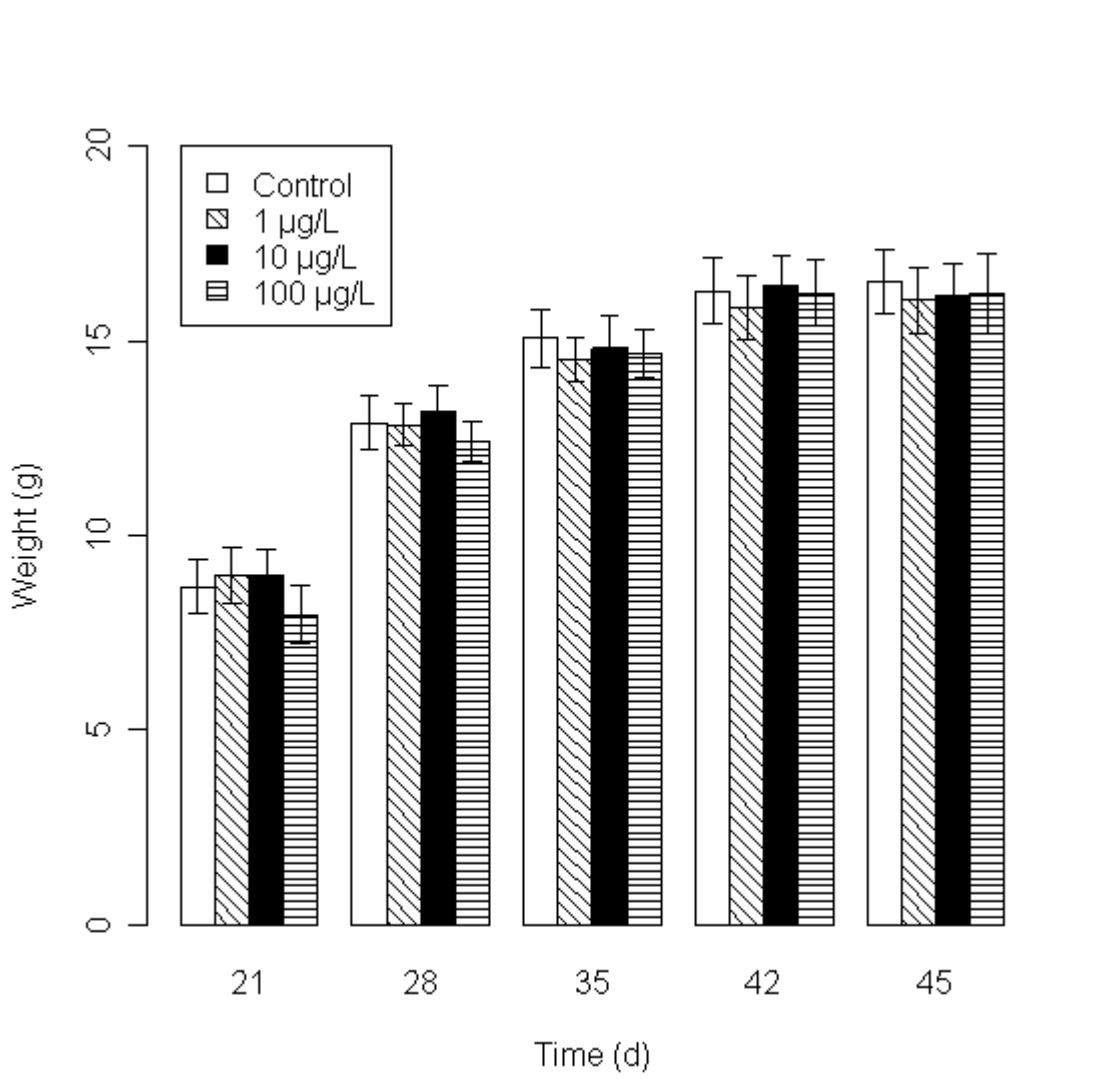
[e] Third litter of mice, sacrificed on post natal day 21

Figures



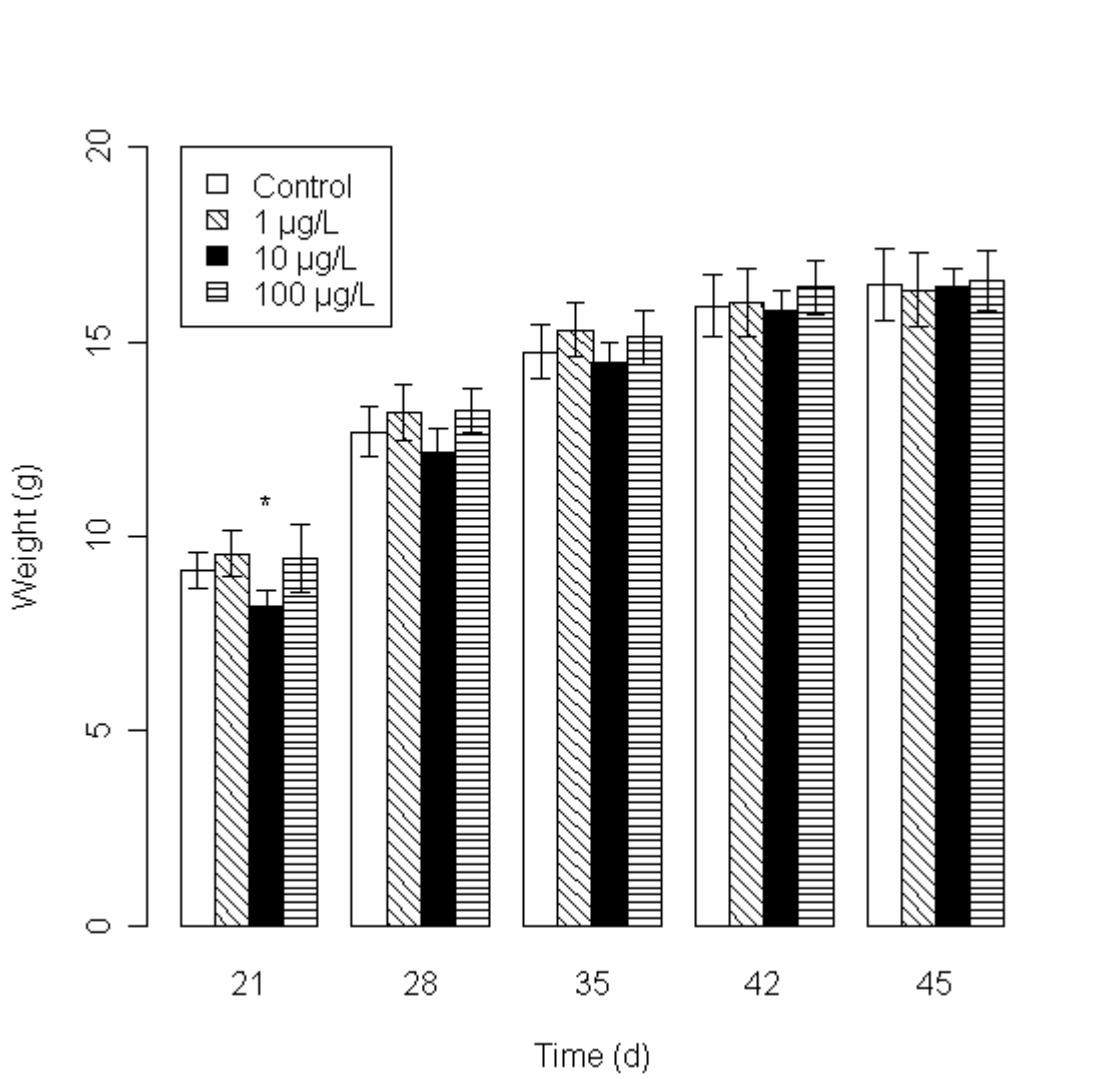
3.1 Reductive degradation of Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) progressive anaerobic degradation to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX).



3.2 F1A body weights

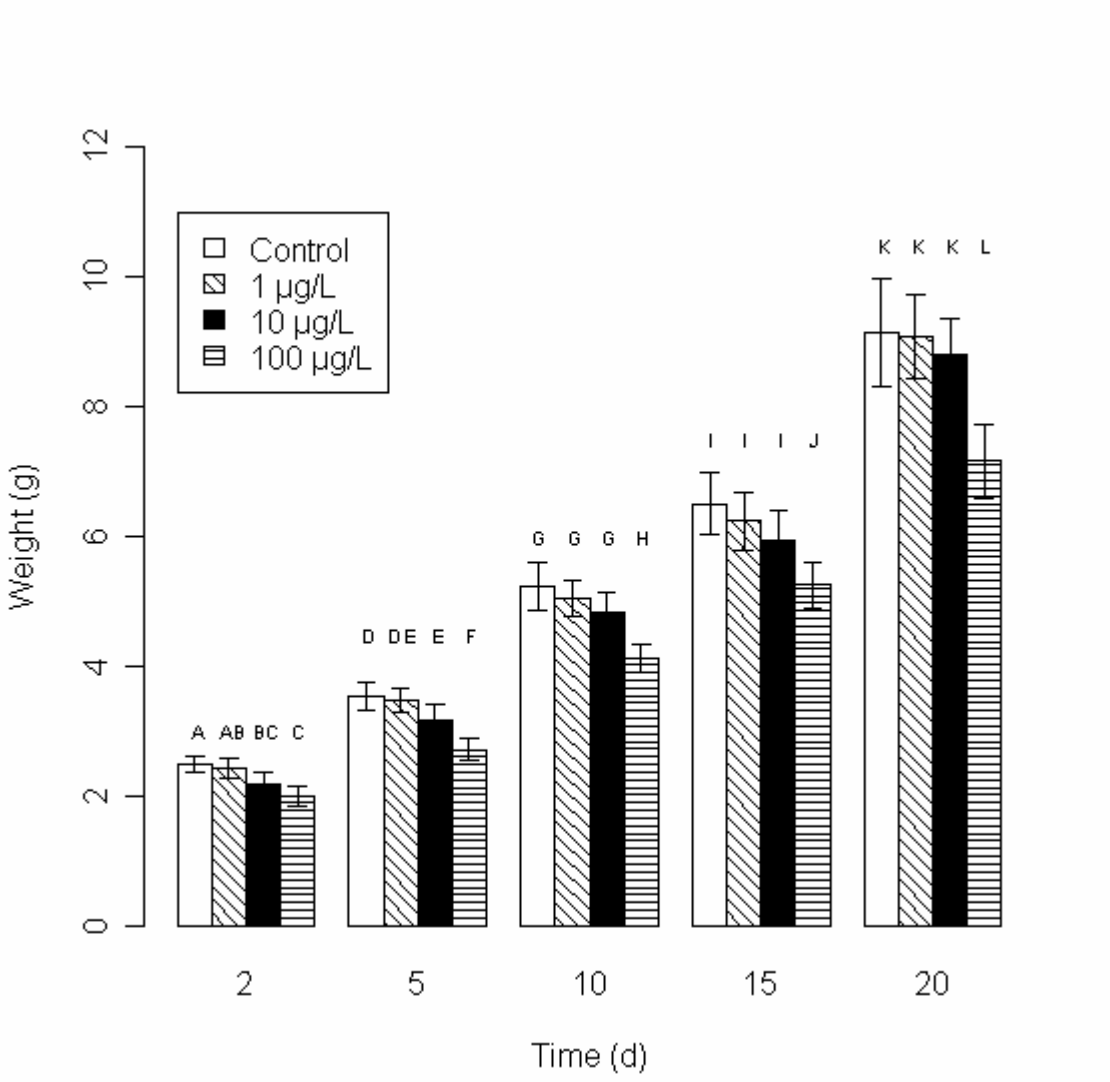
Weight (mean ± standard error) of first litter (F1A) deer mice offspring dosed with varying concentrations of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) in drinking water.



3.3 F1B body weights

Weight (mean ± standard error) of second litter (F1B) deer mice offspring dosed with varying concentrations of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) in drinking water.

* Indicates dose group weight is significantly different from the control weight for that day.



3.4 F1C body weights

Weight (mean ± standard error) of third litter (F1C) deer mice offspring dosed with varying concentrations of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) in drinking water.

Uppercase letters represent similar statistical groups within that day.

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CHAPTER IV

MULTIGENERATIONAL EFFECTS OF EXPOSURE TO HEXAHYDRO-1,3,5-TRINITROSO-1,3,5-TRIAZINE (TNX) IN DEER MICE (*PEROMYSCUS MANICULATUS*)

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Abstract

Contamination of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) has been identified at areas of explosive manufacturing, processing, storage, and usage. Conversion of RDX to anaerobic N-nitroso metabolites (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX)) has been demonstrated in the environment and in the gastrointestinal tract of mammals *in vivo*. Thus, potential exists for human and wildlife exposure to these N-nitroso compounds. While exposed to TNX via drinking water *ad libitum*, deer mice (*Peromyscus maniculatus*) were bred in a multigenerational fashion to produce cohorts F1A-D, F2A-B, and F3A. Hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) was administered at four exposure levels-control (0 µg/L), 10 µg/L, 100 µg/L, and 1 mg/L. Endpoints investigated include: reproductive success, offspring survival, offspring weight gain, and offspring organ weights. Data from this study indicate that TNX caused decreased litter size and increased postpartum mortality of offspring. No teratogenic effects were linked with exposure to TNX.

Key words: Deer mouse; Royal demolition explosive; Hexahydro-1,3,5-trinitroso-1,3,5-triazine, Hexahydro-1,3,5-trinitro-1,3,5-triazine, RDX, TNX

Introduction

The explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (CAS #121-82-4) has been used for military purposes and civilian applications worldwide since World War II. Manufacturing, packaging, use, and disposal of RDX have contaminated a variety of environmental media. The U.S. Environmental Protection Agency's (U.S. EPA) Comprehensive Environmental Response, Compensation, and Liability Information System Database identifies 19 sites on the National Priorities List that are contaminated with RDX. The U.S. Army has confirmed groundwater explosive contamination at nearly 600 sites; as well as, suspecting 90 additional sites as possibly being contaminated within the United States (Davis *et al.* 2004). Concentrations of RDX in soil have been reported as high as 15,000 µg/kg at the Massachusetts Military Reservation (Falmouth, MA, USA) (Pennington *et al.* 2005).

Transformation of RDX occurs more easily via anaerobic degradation rather than aerobic degradation (McCormick *et al.* 1981; Kitts *et al.* 1994; Young *et al.* 1997). Anaerobically, RDX biodegrades progressively into N-nitroso compounds: hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) (CAS #5755-27-1), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) (CAS #80251-29-2), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) (CAS #13980-04-6) (McCormick *et al.* 1981; Kitts *et al.* 1994; Regan and Crawford 1994; Young *et al.* 1997; Boopathy *et al.* 1998; Kitts *et al.* 2000; Beller 2002). These N-nitroso transformation products have been found in RDX contaminated groundwater beneath the Iowa Army Ammunition Plant (Middletown, IA, USA) with concentrations as high as 430 µg/L (Beller and Tiemeier 2002), suggesting this reductive transformation occurs environmentally. Transformation of RDX to N-nitroso metabolites may also occur *in vivo*, as Pan *et al.* (2007) demonstrated by formation of RDX N-nitroso metabolites in the gut of deer mice (*Peromyscus maniculatus*) exposed to RDX in food. Thus, with the presence of RDX and its N-nitroso metabolites in the environment, potential exists for RDX N-nitroso exposure to humans and wildlife species alike.

In acute exposures, RDX causes central nervous system seizures and, with increasing dose, death (von Oettingen *et al.* 1949; Schneider *et al.* 1977; Burdette *et al.* 1988). Median lethal dose (LD_{50}) of RDX in deer mice ranged from 136 to 319 mg/kg (Smith *et al.* 2007), depending on the age of the mouse. In chronic exposures, RDX causes hepatotoxicity and also has harmful effects on blood (Levine *et al.* 1981; Levine *et al.* 1990). The U.S. EPA has set a lifetime drinking water health advisory at 2 μg/L (FSTRAC 1994) and an oral reference dose at 0.003 mg/kg/day (IRIS 1993).

N-nitroso metabolites of RDX are infrequently studied compared to the parent compound. In acute doses, MNX and TNX have also demonstrated toxicity to the central nervous system in the form of seizure activity (Meyer *et al.* 2005; Smith *et al.* 2007) and eventual death, but these compounds are not as potent as RDX in an acute sense (MNX LD_{50} values: 181-574 mg/kg, TNX LD_{50} values: 338-999 mg/kg) (Smith *et al.* 2007). A reproductive study was completed with deer mice exposed to TNX (Smith *et al.* 2006). It demonstrated dose-dependent bioaccumulation in the liver, increased offspring mortality, and decreases in body and kidney weight from birth to weaning in developing deer mice.

The purpose of this multigenerational study was to verify results of the reproductive study (Smith *et al.* 2006), while expanding upon that study at the same time. To accomplish this goal, breeding pairs of deer mice produced offspring, which in turn, were bred to produce offspring of their own. This process was repeated once more to create a third generation of offspring. Endpoints investigated included: reproductive success, offspring survival, offspring growth, and organ weight analysis. Information obtained in this study will be useful for RDX and TNX ecological risk evaluation of terrestrial wildlife at RDX contaminated sites. In the remainder of this document, the current study will be referred to as the multigenerational study and the previous reproductive work within our group (Smith *et al.* 2006) will be referred to as the reproductive study.

Materials and methods

Test chemical

Hexahydro-1,3,5-trinitroso-1,3,5-triazine (>99 % pure) was obtained from SRI International (Menlo Park, CA, USA). Dosing solutions were created by dissolving the toxicant with nanopure water overnight in a 1 mg/L stock solution. Dilutions were withdrawn from the stock solution to create medium and low dose concentrations (100 µg/L and 10 µg/L). The control solution was pure nanopure water. Target doses were chosen as 10X levels from the reproductive study. Exposure levels in the reproductive study were chosen from environmentally relevant concentrations of TNX (Smith *et al.* 2006). Dosing solutions were renewed every 3 d and TNX concentrations were analyzed with liquid chromatography-mass spectroscopy (LCMS) in negative ion mode. Acetic acid was added to create a TNX-acetic acid complex (233 amu). A primary peak of 233 amu was monitored in the analysis. Dosing water bottles were weighed before and after the three day exposure period for each water change to monitor water consumption by deer mice.

Animals

Forty breeding pairs of virgin male and female deer mice were obtained from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA) in two separate shipments, three weeks apart. Mice were acclimated to laboratory conditions for one week. Breeding pairs were housed separately in standard rodent cages (~ 29 x 18 x 14 cm) lined with laboratory-grade aspen bedding (Harlan Teklad, Madison, WI, USA). Cages were located in rooms with temperatures ranging from 16.7 to 24.4° C, relative humidity ranging from 21 to 88%, and 16:8 h light:dark period. Feed and water were provided manually, checked daily, and available *ad libitum* to mice. Purina Certified Rodent Chow® 5002 (Purina Mills, St. Louis, MO, USA) was the feed used. Experiments and housing were conducted in compliance with Texas Tech University's Animal Use and Care Committee's guidelines Protocol # 03006-01.

Experimental procedure

Once acclimated, deer mice (F0) were randomly assigned to breeding pairs in two separate waves (twenty pairs of mice in each wave, all dose groups equally represented), 18 days apart. A total of ten pairs of deer mice were designated to each exposure level (0 µg/L, 10 µg/L, 100 µg/L, and 1 mg/L). Exposure was initiated at cohabitation, administered *ad libitum* in drinking water, and continued until the F0 mice were euthanized. Deer mice in the F0 group bred and produced four successive cohorts of deer mice offspring (F1A-D) (**Fig. 4.1**). Offspring were weighed at post-natal days (PND) 2, 5, 10, 15, 21, 28, 35, and 45. Offspring from the F1A-C groups were weaned at PND 21. Some offspring from F1A and F1B group were euthanized at PND 60 and 75 respectively. Offspring from the F1C group were euthanized at puberty (PND 45). Deer mice were weaned by first ear notching mice for identification. Then mice were segregated by gender and dose group and housed, up to six mice per cage. At PND 21, offspring from F1D cohort were not weaned, but instead euthanized along with their respective F0 parents.

Forty pairs of mice from F1A and F1B cohorts were not euthanized, but instead randomly paired within each dose group, cohort, and without siblings. These mice were allowed to breed and produced two successive cohorts (F2A and F2B). These mice were weaned at PND 21, and some were euthanized at PND 45. Parent mice from the F1 generation were euthanized at the time of F2B offspring weaning or shortly thereafter.

As before, forty pairs of mice from F2A and F2B cohorts were not euthanized, but randomly paired within each dose group, cohort, and without siblings instead. These mice were bred and produced one cohort of deer mice (F3A). All of these mice were weaned at PND 21 and euthanized at PND 45. Parents from the F2 generation were euthanized at the time of F3A weaning or shortly thereafter. All mice subsequent of F0 mice, were dosed *ad libitum* in drinking water for their entire lifetime: conception to euthanasia.

Euthanasia was conducted using carbon dioxide gas as an asphyxiant. At necropsy, blood was obtained from all animals via heart puncture. The following organs were removed: liver, kidney, testes, spleen, and brain. Endpoints investigated during this study were number born, survival, weight gain, and organ weights.

Statistics

Drinking water consumption, litter size, and hematocrit values were evaluated using the general linear model:

$$f(dose) = m * \log_{10} (dose + 1) + b$$

Slope (m) was evaluated for significance. Deer mice analyzed for drinking water consumption were subdivided into three categories of consumption: breeding pairs of deer mice without litters of offspring (cohabitation to birth for first litter and weaning to birth for subsequent litters), breeding pairs of deer mice with litters of offspring (birth to weaning), and weaned deer mice offspring. All three categories were analyzed separately.

Offspring mortality was evaluated using the Cox proportional hazard model (Cox 1972). Offspring body weight was evaluated by fitting different dose groups to the Gompertz nonlinear growth model (Gompertz 1825), which has the form:

$$f(day) = Asym * e^{(-b2 * b3^{day})}$$

Asym is a numeric parameter representing the upper asymptote (adult weight), b2 is a numeric parameter related to the value of the function at day = 0 (measure related to inverse birth weight), and b3 is a numeric parameter related to the scale the x axis (measure related to inverse growth rate). Parameters were evaluated using confidence intervals.

For organ weights, offspring were divided into two groups: offspring euthanized near the time of puberty (~PND 45) and offspring euthanized at weaning (~PND 21, F1D cohort). Since each individual organ weight was, in a sense, a pseudo-replicate, organ weights were evaluated using mean values for each individual

litter, since litter is the true experimental unit. Absolute organ weight and organ to body weight ratio for each group were analyzed using the general linear model:

$$f(dose) = m * \log_{10}(dose + 1) + b$$

Slope (m) was evaluated for significance. All statistical tests used an α value of 0.05. The software used to analyze data was "R: A language and environment for statistical computing" Version 2.4.1 (R 2004).

Results

Results from LCMS verification of dosing solutions showed TNX concentrations to be 10.8 ± 0.5 µg/L (*n*=37) 101.1 ± 2.3 µg/L (*n*=37), 997.6 ± 25.4 µg/L (*n*=36) (mean ± standard error (SE)) for 10 µg/L, 100 µg/L, and 1000 µg/L dose groups, respectively.

Water consumption of all breeding pairs of deer mice without litters of offspring ranged from 14.93 to 16.83 ml/d/breeding pair for all dose groups (**Fig. 4.2**) and showed a dose dependent decrease (m = -0.42, $p = < 0.001$, $R^2 = 0.007$). Breeding pairs of deer mice without offspring subdivided into F0 (m = -0.37, $p = < 0.001$, $R^2 = 0.006$) and F1 (m = -0.73, $p = < 0.001$, $R^2 = 0.02$) generations also all displayed similar patterns, but the F2 ($p = 0.40$, $R^2 = 0.001$) generation did not. Breeding deer mice with offspring consumed more water (21.71 to 23.96 ml/d/breeding pair) than those without offspring (**Fig. 4.3**), and also showed a dose dependent decrease (m = -0.79, $p = < 0.001$, $R^2 = 0.02$). Breeding pairs of deer mice with litters of offspring subdivided into F0 (m = -0.56, p = < 0.001, $R^2 = 0.008$) and F1 (m = -1.24, $p = < 0.001$, $R^2 = 0.04$) generations also showed significant differences, but the F2 generation (m = -0.42, $p = 0.073$, $R^2 = 0.005$) only approached significance. Weaned deer mice offspring consumed 5.60 to 5.95 ml/d/individual (**Fig. 4.4**) and showed a significant dose dependent decrease (m = -0.068, $p = 0.027$, $R^2 = 0.001$).

Cumulative analysis of all cohorts showed a significant dose-dependent decrease (m = -0.42, $p = < 0.001$, $R^2 = 0.05$) in number of offspring born (**Fig. 4.5**). Mice subdivided into F1 (m = -0.42, $p = 0.009$, $R^2 = 0.05$) and F2 (m = -0.41, $p =$

0.023, $R^2 = 0.07$) generations showed similar decreases, whereas mice from the F3 generation did not ($p = 0.30$, $R^2 = 0.03$).

Hematocrit values (45.5 ± 0.1) fell into the normal range, and linear regression models showed no dose dependent relationships ($p = 0.69$).

Survival analysis of all offspring, using the Cox proportional hazard model, yielded exposure groups 10 µg/L ($p = 0.038$) and 1 mg/L ($p = 0.013$) as having significantly lower survival rates (**Fig. 4.6**) than the control group. The 100 µg/L exposure group showed no significant differences ($p = 0.67$) when compared to the control group.

Using 95% confidence intervals, parameters of Gompertz nonlinear growth models of deer mice offspring body weight (**Fig. 4.7**) showed the 1 mg/L dose group had a significantly larger b2 parameter (numeric parameter related to the value of the function at x = 0) than the 10 µg/L dose group (**Fig. 4.8**). This was also the case for the F1 generation, but not F2 or F3 generations. In all offspring, 10 µg/L approached a significantly (using 90% confidence intervals) larger b2 parameter than the 100 µg/L dose group. This was also the case for the F1, but not F2 or F3 generations. The control group approached a significantly larger b2 parameter compared to the 1 mg/L dose group in the F1 generation. The control group approached a significantly larger b3 parameter compared to the 10 µg/L and the 1 mg/L dose group for all offspring. The control group also approached a significantly larger b3 parameter compared to the 1 mg/L dose group in the F1 generation. No significance differences were found for F2 and F3 generations. No other significant differences in other parameters were found.

Linear regression models of mean absolute organ weights for deer mice euthanized near the time of puberty showed testes as having a significant dose dependent increase ($m = 5.8 \times 10^{-3}$, $p = 0.04$, $R^2 = 0.03$). Mean absolute brain weights approached significance ($m = 3.4 \times 10^{-3}$, $p = 0.07$, $R^2 = 0.02$) for increased brain

weight. Mean absolute kidney ($p = 0.12$) and liver weights ($p = 0.30$) both showed no dose dependent relationships.

Linear regression models of mean organ weight to body weight ratios for deer mice euthanized near the time of puberty showed kidneys as having a significant dose dependent increase ($m = 1.2 \times 10^{-4}$, $p = 0.04$, $R^2 = 0.03$). Mean testes weight to body weight ratios approached an increased significance ($m = 2.6 \times 10^{-4}$, $p = 0.06$, $R^2 = 0.03$). Mean kidney ($p = 0.30$) and brain weight ($p = 0.13$) to body weight ratios both showed no dose dependent relationships.

Discussion

This mulitgenerational study of breeding deer mice exposed to TNX had several similarities to the previous reproductive study (Smith *et al.* 2006), but also had some different results as well. Overall, both studies showed a significant decrease in survival of deer mice offspring exposed TNX. Decreases in body weight from birth to weaning in the reproductive study were not observed in this study. However, decreased litter size was observed in this study, while the litter sizes in the reproductive study showed no dose dependent differences.

It is interesting to see similar dose dependent decreases in water consumption for both periods of litter status (with or without offspring) of cohabitating parent deer mice. Additionally, weaned offspring show a similar trend of decreased water consumption. Conversely, there may not be any relationship at all. Ranges of multigenerational consumption means are within the ranges of means seen in the reproductive study (Smith *et al.* 2006). Significance could be a result of a high sample size, as *n* for the entire data set is in excess of 15,700. This could be further supported by small R^2 values. If decreases in consumption truly exist, there could be several possibilities for the cause including: response to toxic insult, poor palatability of TNX solution, or decreased biological demand for water intake.

Linear regression models show a dose dependent decrease for number of offspring born in this study. The reproductive study did not show similar results

(Smith *et al.* 2006), however, there may be a threshold effect occurring in the multigenerational study. Using one-way analysis of variance coupled with the Tukey–Kramer test (Zar 1999), the 1 mg/L exposure group is the only exposure level that showed a significant decrease from the control ($p = 0.004$). Thus, a threshold may be occurring near the 1 mg/L exposure level. The reproductive study supports this possibility, since the highest exposure level in that study was 100 µg/L, and there were no significant differences.

This decrease in litter size along with the demonstrated dose dependent decrease in survival of offspring born indicates a toxic effect of TNX. As in the reproductive study (Smith *et al.* 2006), most of the offspring mortality occurred within the first few days postpartum. This suggests either a direct toxic effect to the offspring via interuterine and milk exposure; an indirect toxic effect to the offspring via maternal induced toxicity and/or stress; or some combination of the two potential effect routes.

One of 35 births in the 100 µg/L dose group from the F1B cohort was born without any hind feet. Otherwise, no other birth defects were observed in any mice ($n = 859$ total births). That particular female mouse seemingly lived normal until euthanasia at PND 75.

The significance of the 10 µg/L dose group having a larger b2 parameter than the 1 mg/L dose group for all offspring indicates the 10 µg/L had smaller birth weights than the high dose group. Other significant and approaching significant b2 indices marginally support a possible dose related increase in birth weight. In the reproductive study, early life weights (PND 2 to weaning) were significantly lower in F1C mice (the only cohort measured) (Smith *et al.* 2006), which is opposite than the possible effect observed in this study. The approaching significance of the control group having a greater b3 parameter than two of the higher dose groups indicates there may be a decreased growth rate of control mice. Offspring adult weight Asym parameters showed no significant differences or approaching significant differences.

These data indicate that any potential differences in early life weights and growth rates that may exist due to dose have little bearing on adult body weight.

Linear regression models for mean absolute organ weights and mean organ weight relative to body weights seem to show dose dependent increases in mean absolute kidney weights and mean testes weight relative to body weights. However, looking at the linear models, the effect size (i.e. slope) is small. This means that even though significant differences existed, the differences were small. Furthermore, the R^2 values (coefficient of determination or the amount of variance described by the model) are also pretty low. This means there are probably other factors that are affecting the variance of these organ weights besides dose. In comparison to the reproductive study (Smith *et al.* 2006) the multigenerational ranges of absolute organ weights were within those of the reproductive study. Thus, there is probably little effect of dose on kidneys and testes.

In a two generation reproduction study with Fischer 344 rats as the animal model and RDX as the toxicant, elevated maternal mortality, neurotoxicity, and a higher percent of stillborn pups were observed at 50 mg/kg (the high dose group) (Cholakis *et al.* 1980). In that study, dosing was not continuous; instead parents from F0 and F1 generations were dosed during the 13 weeks prior to mating. The overall no adverse effect level (NOAEL) for this study was 5 mg/kg/day. In our study, TNX demonstrated adverse effects (decreased offspring survival) at an exposure level of 10 μg/L. Using actual TNX concentrations, drinking water consumption of parent deer mice prior to parturition, and average weight of deer mice parents one can estimate the dose to approximate 0.0039 mg/kg/day in this study. If you use the same values with the U.S. EPA estimate (EPA 1993) of drinking water consumption (0.19 g of water/g body weight/day) instead, the dose approximates at 0.0021 mg/kg/day. Both values are much lower than the NOAEL of the RDX multigenerational study (Cholakis *et al.* 1980); however as mentioned, the RDX study did not have continuous dosing.

Our findings demonstrate decreased litter size of deer mice due to the exposure to TNX. Mortality of deer mice offspring was increased with increased exposure of

TNX to parent deer mice. No teratogenic effects were linked with exposure to TNX. This information may be useful for RDX and TNX ecological risk evaluation of terrestrial wildlife at RDX contaminated sites.

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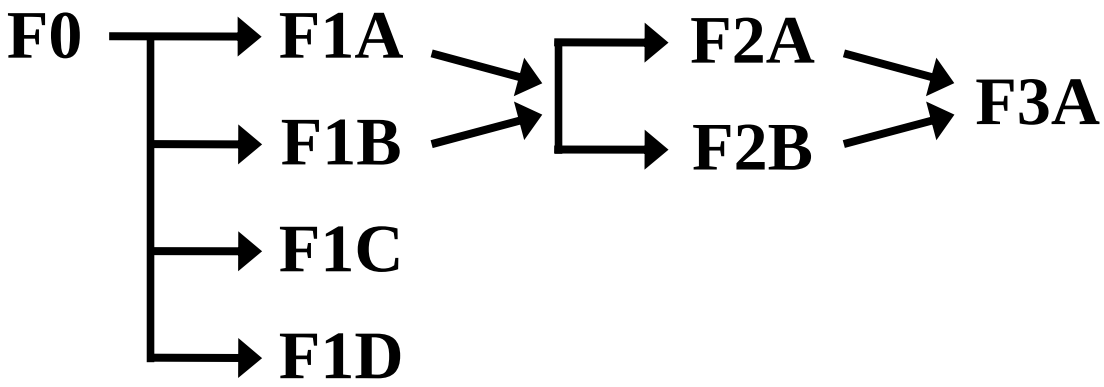
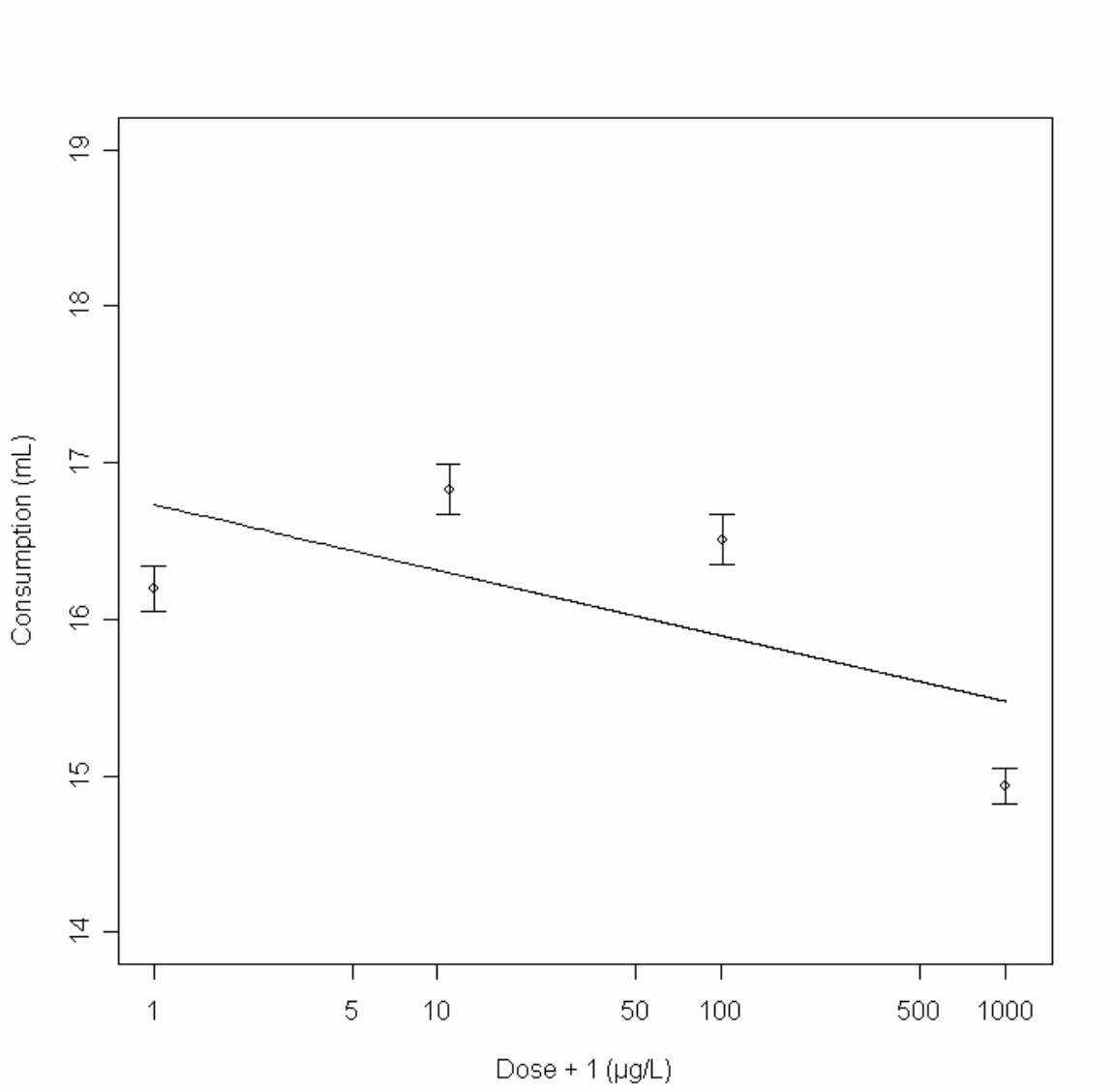
Figures



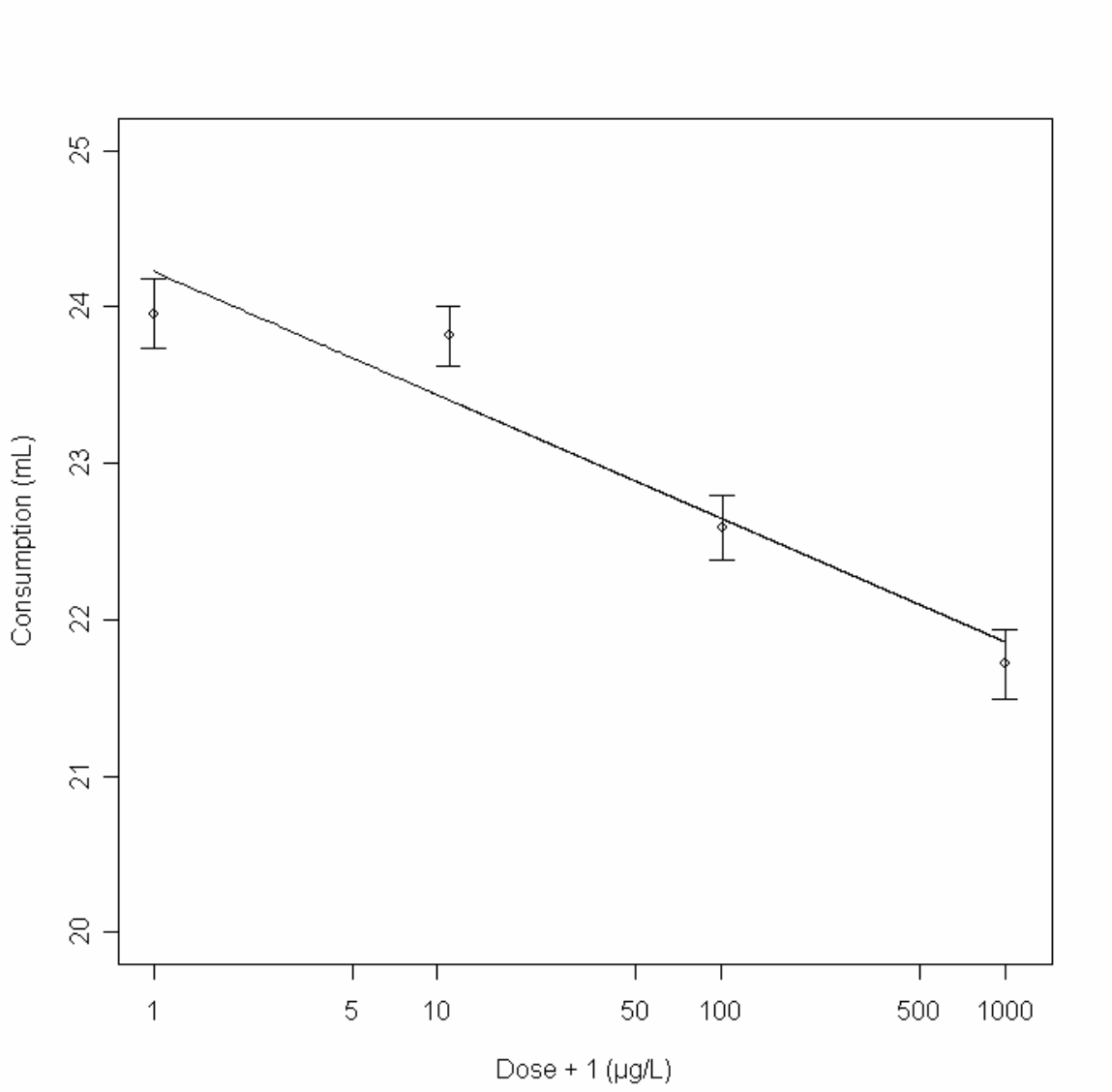
4.1 Multigenerational breeding protocol

Multigenerational breeding protocol of deer mice (*Peromyscus maniculatus*) exposed to hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). The number after the F indicates the generation, and the letter indicates the cohort.



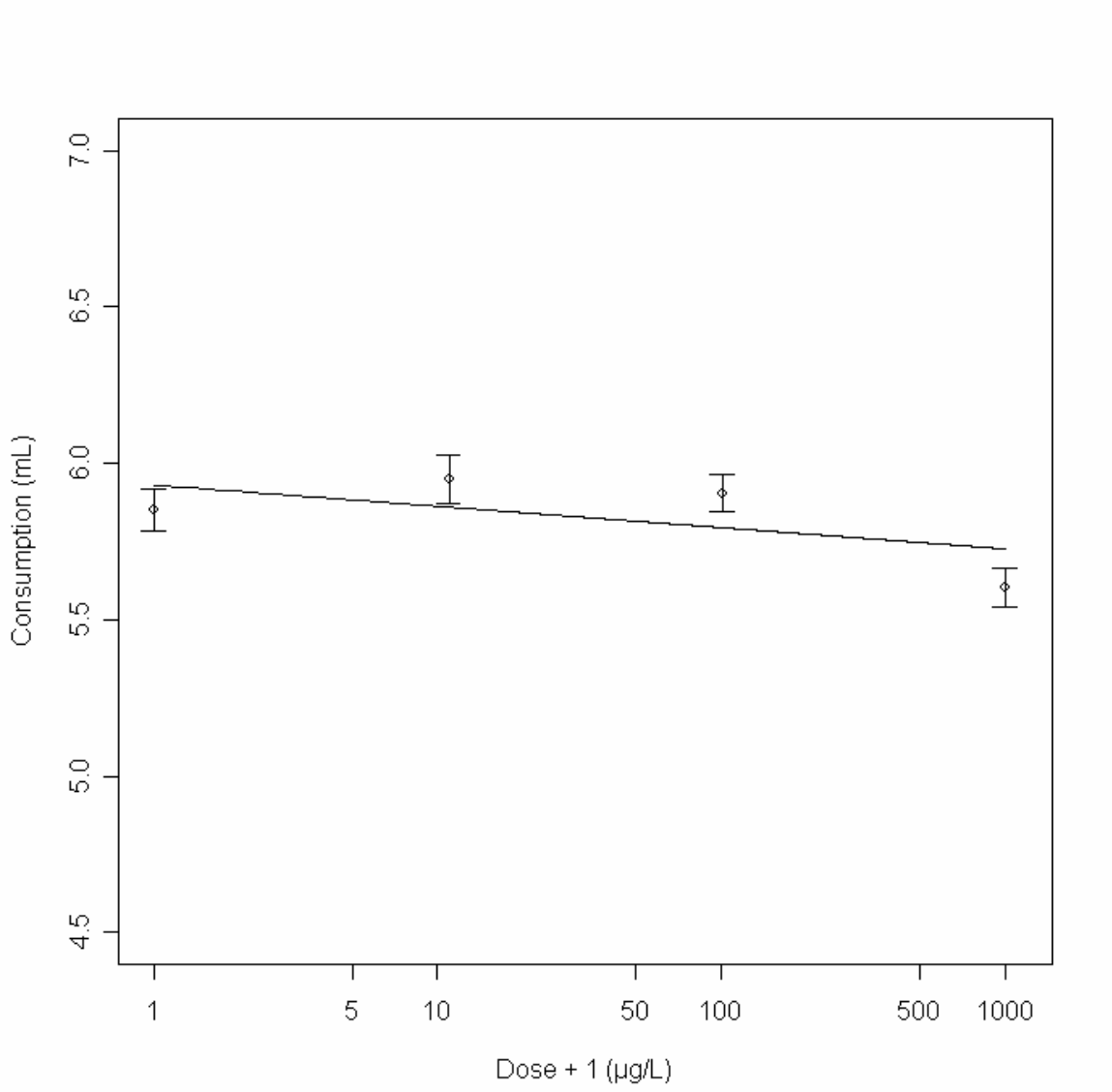
4.2 Water consumption of parent mice without offspring

Daily drinking water consumption (mean ± standard error) by breeding pairs of deer mice (*Peromyscus maniculatus*) without litters of offspring exposed to varying concentrations of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). Linear regression model: ($m = -0.42$, $p = 8.42e\text{-}11$, $R^2 = 0.007$).



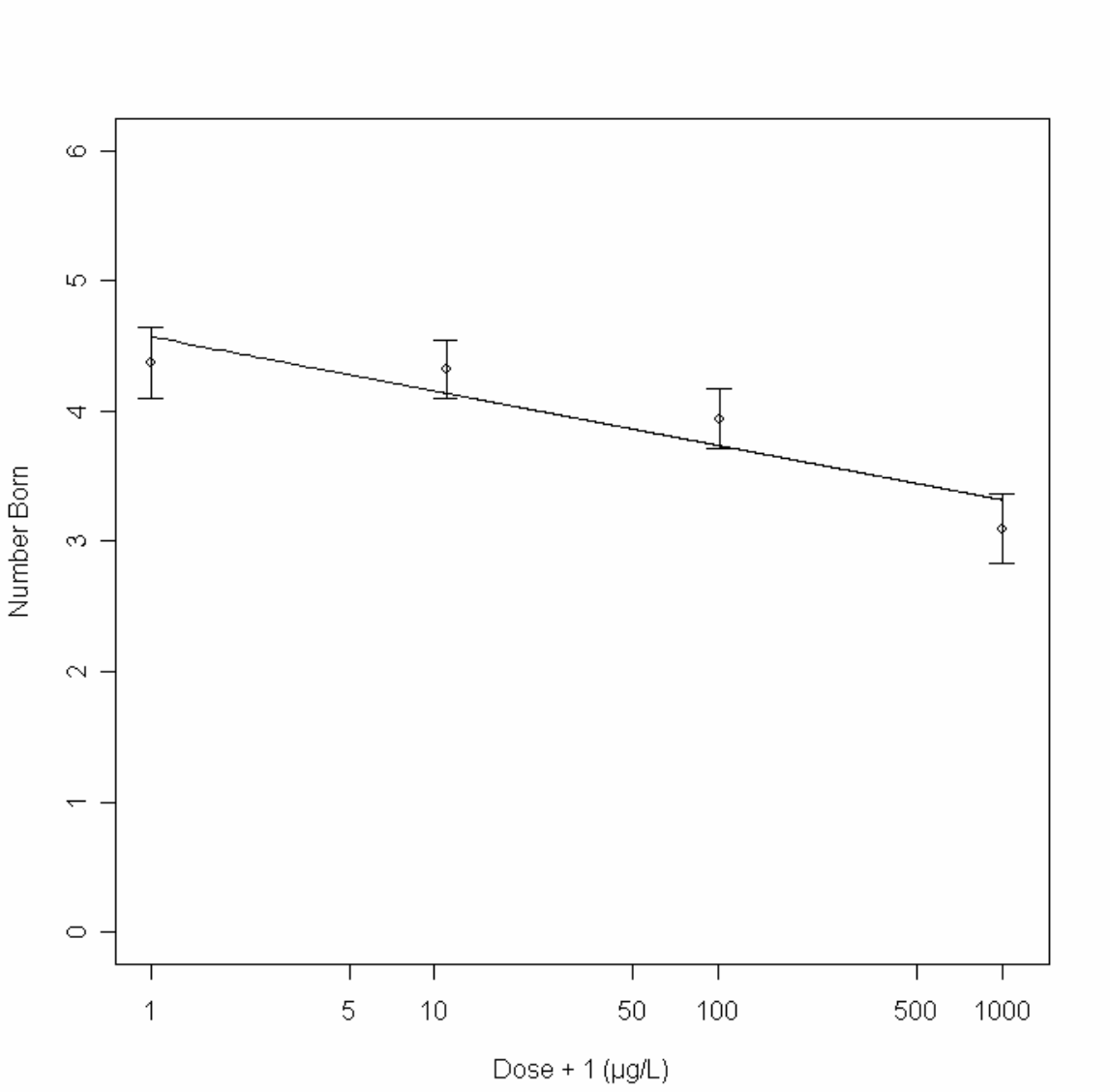
4.3 Water consumption of parent mice with offspring

Daily drinking water consumption (mean ± standard error) by breeding pairs of deer mice (*Peromyscus maniculatus*) with litters of offspring exposed to varying concentrations of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). Linear regression model: (m = -0.79, p = 2.26e-16, R^2 = 0.016).



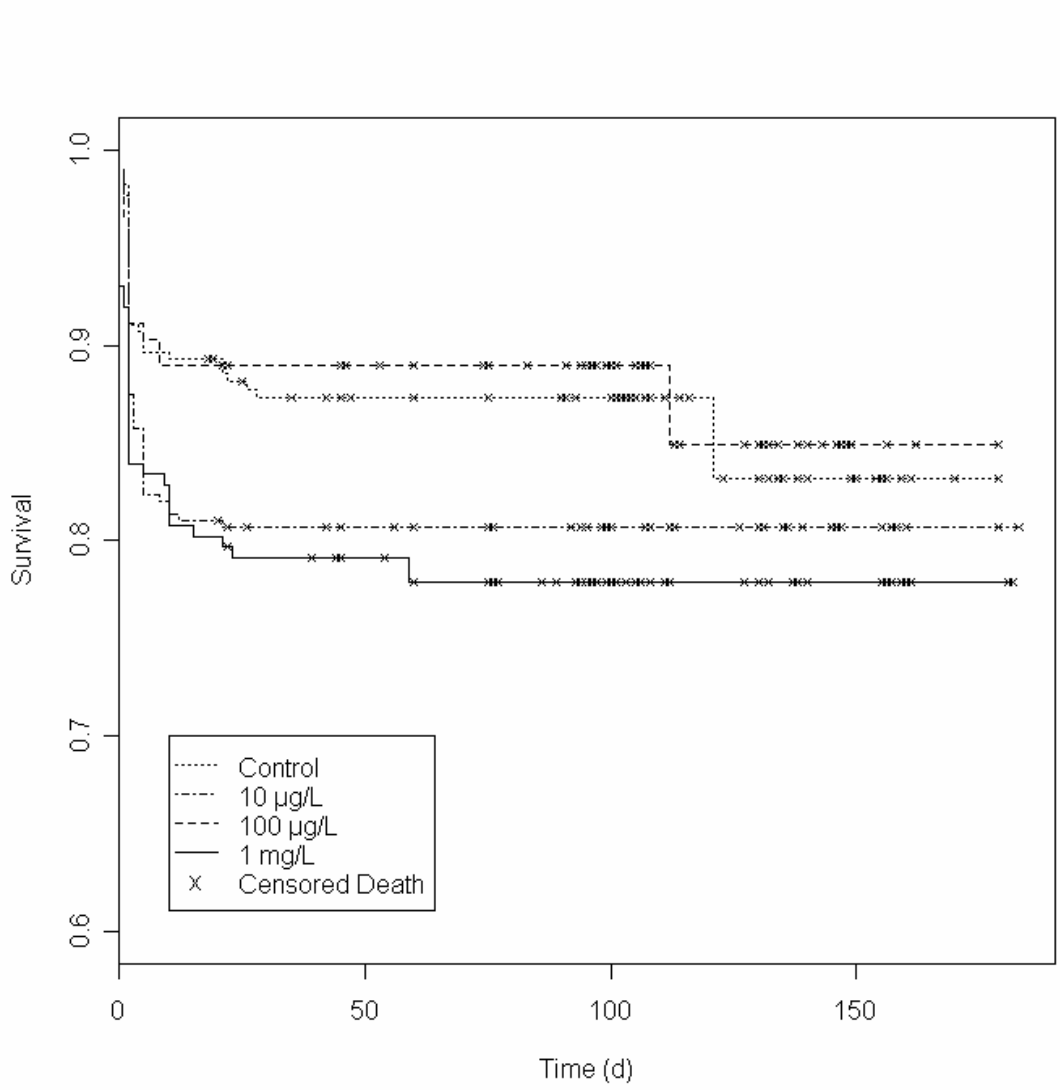
4.4 Water consumption of offspring

Daily individual drinking water consumption (mean ± standard error) of weaned deer mice (*Peromyscus maniculatus*) offspring exposed to varying concentrations of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). Linear regression model: ($m = -0.068$, $p = 0.027$, $R^2 = 0.0009$).



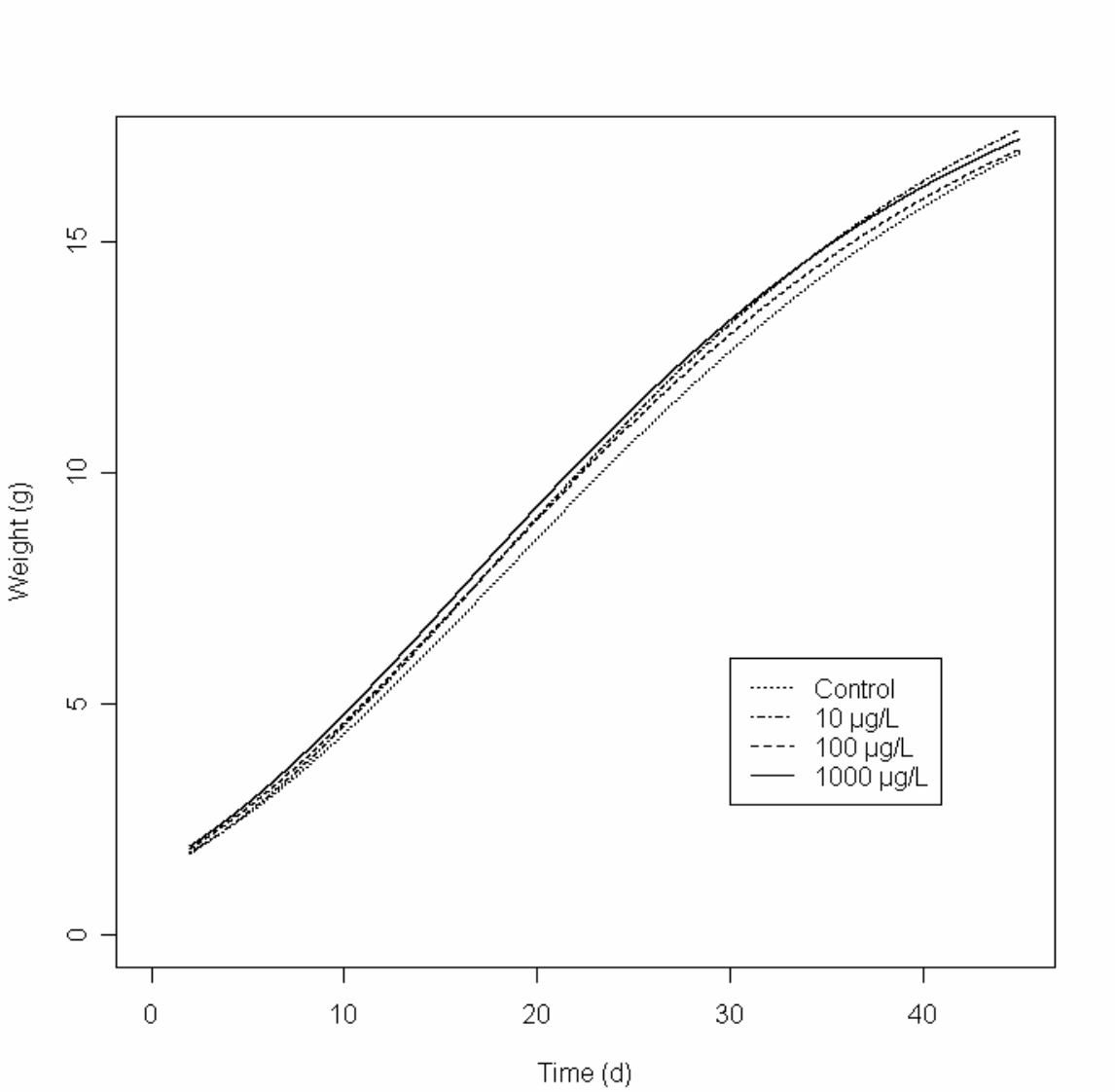
4.5 Offspring per litter

Number of offspring born (mean ± standard error) per litter of deer mice (*Peromyscus maniculatus*) exposed to varying concentrations of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) in drinking water. Linear regression model: ($m = -0.42$, $p = 0.0006$, $R^2 = 0.054$).



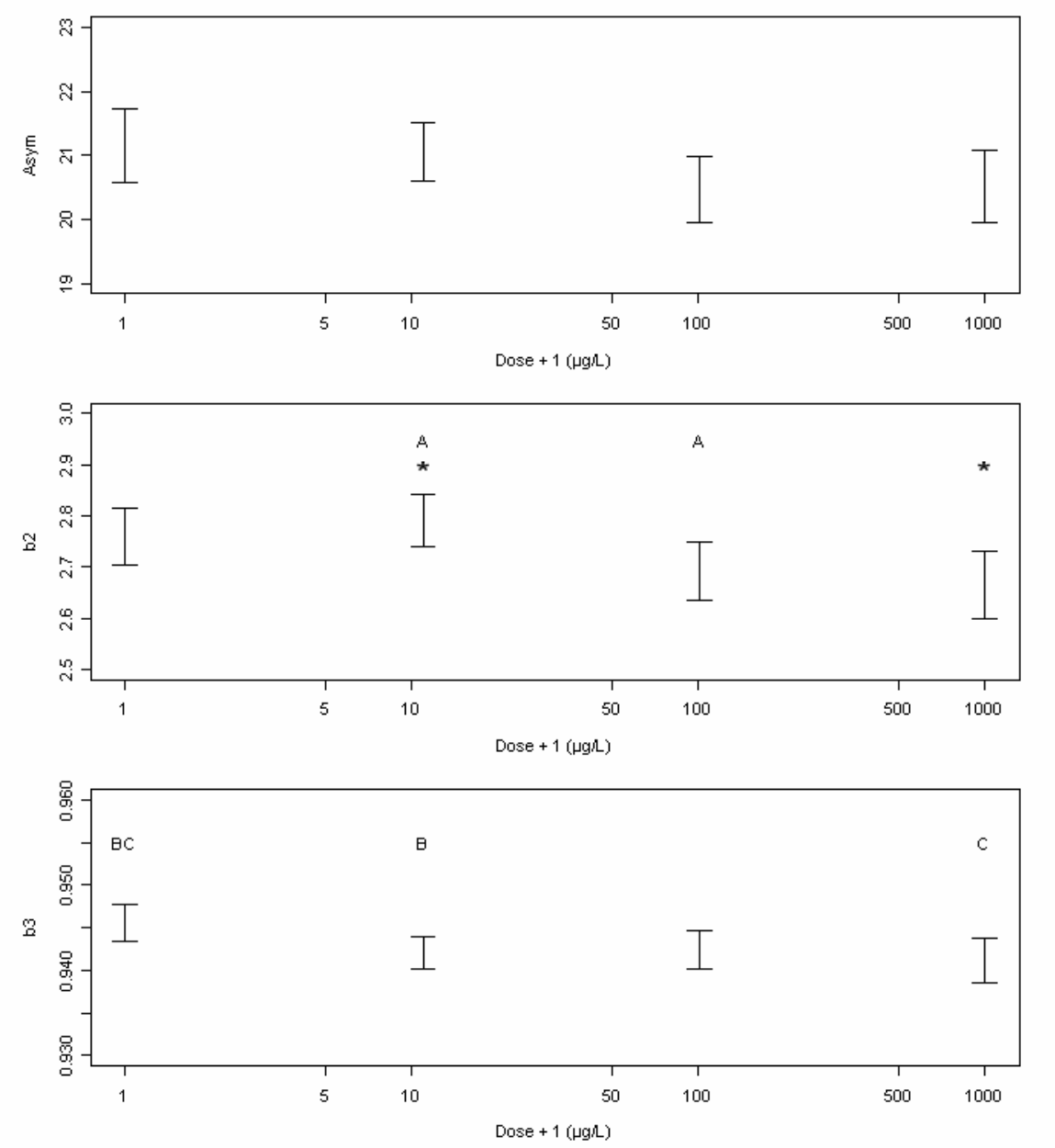
4.6 Survival of offspring

Survival of deer mice (*Peromyscus maniculatus*) offspring exposed to varying concentrations of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) in drinking water for the duration of their lifetime. Censored death indicates that particular death was due to a euthanization in the study protocol.



4.7 Gompertz growth models of offspring

Gompertz nonlinear growth models of deer mice (*Peromyscus maniculatus*) offspring body weight exposed to varying concentrations of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) in drinking water.



4.8 Confidence intervals of Gompertz parameters

Confidence intervals (95%) of three parameters from Gompertz nonlinear growth models of deer mice (*Peromyscus maniculatus*) offspring body weight exposed to varying concentrations of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) in drinking water. An asterisk indicates significant differences between parameters calculated using 95% confidence intervals. Uppercase letters indicates approaching significant differences between parameters calculated using 90% confidence intervals.

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CHAPTER V

MICROSATELLITE MUTATION RATE OF DEER MICE (*PEROMYSCUS MANICULATUS*) EXPOSED TO AN ANAEROBIC METABOLITE OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE (RDX)

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Abstract

Contamination of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) has been identified at areas of explosive manufacturing, processing, storage, and usage in a variety of environmental media. Conversion of RDX to anaerobic N-nitroso metabolites (hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX)) has been demonstrated in the environment and in the gastrointestinal tract of mammals *in vivo*. Thus, potential exists for human and wildlife exposure to these N-nitroso compounds. N-nitroso compounds are known carcinogens, and these compounds exhibited genotoxicity using the Ames assay with S9 activation.

In two dosing studies, a reproductive study and a multigenerational study, TNX was exposed via drinking water to deer mice (*Peromyscus maniculatus*). In the reproductive study, mice bred and produced three consecutive cohorts of offspring (F1A-F1C). TNX was administered at four doses-control (0 µg/L), 1µg/L, 10 µg/L, and 100 µg/L. In the multigenerational study, mice bred in a multigenerational fashion (parents produced offspring, which bred to produce more offspring) to produce the following cohorts in three generations: F1A-D, F2A-B, and F3A. TNX was administered at four exposure levels-control (0 µg/L), 10 µg/L, 100 µg/L, and 1 mg/L.

With tissue samples from both the reproductive and multigenerational studies, 12 microsatellite DNA loci were amplified. Amplified DNA fragments were analyzed using both change in original parent allele frequencies and the parent/offspring approach of direct mutation rate determination to assess genotoxicity of TNX *in vivo*. Findings demonstrate no dose dependent differences in deviation from parent microsatellite DNA allele frequencies or microsatellite mutation rate.

Key words: deer mouse; RDX, TNX, microsatellite, hexahydro-1,3,5-trinitroso-1,3,5-triazine, hexahydro-1,3,5-trinitro-1,3,5-triazine

Introduction

Since World War II, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (CAS #121-82-4) has been used as an explosive for military purposes and civilian applications worldwide. Manufacturing, packaging, use, and disposal of RDX have contaminated a variety of environmental media including surface water, groundwater, soils, and sediments. The U.S. Environmental Protection Agency's (U.S. EPA) Comprehensive Environmental Response, Compensation, and Liability Information System Database identifies 19 sites on the National Priorities List that are contaminated with RDX. The U.S. Army has confirmed groundwater explosive contamination at nearly 600 sites; as well as, suspecting 90 additional sites as possibly being contaminated within the United States (Davis *et al.* 2004). Concentrations of RDX in soil have been reported as high as 15,000 μg/kg at the Massachusetts Military Reservation (Falmouth, MA, USA) (Pennington *et al.* 2005).

Anaerobic transformation of RDX may occur in the environment, as suggested by presence of metabolites in ground water contaminated with RDX (Beller and Tiemeier 2002), or *in vivo* (Pan *et al.* 2007b), as suggested by presence of metabolites in the gut of deer mice (*Peromyscus maniculatus*) exposed to RDX in food. Anaerobic transformation occurs via reduction of RDX N-nitro groups to form N-nitroso moieties. Progressive reduction of the three N-nitro groups of RDX produces the following compounds: hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) (CAS #5755-27-1), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) (CAS #80251-29-2), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) (CAS #13980-04-6) (McCormick *et al.* 1981; Kitts *et al.* 1994; Regan and Crawford 1994; Young *et al.* 1997; Boopathy *et al.* 1998; Kitts *et al.* 2000; Beller 2002). With the presence of RDX and its N-nitroso transformational products in the environment, potential exists for RDX N-nitroso exposure to humans and wildlife species alike.

In general, N-nitroso compounds are recognized as potent carcinogens. N-nitroso compounds require bioactivation to exhibit carcinogenic properties. This process entails first hydroxylation of the carbon in the α position, then carcinogenicy

through alkylation of general macromolecules, such as DNA (Archer 1989). Hydroxylation is mediated by cytochrome P450 (Cyp) enzymes; typically Cyp 2E1 for small, short chained N-nitroso compounds such as N-nitrosodimethylamine, and Cyp 2A6 for large, bulky chained N-nitroso compounds such as N-nitrosomethylphenylamine (Kushida *et al.* 2000; Fujita and Kamataki 2001). While general N-nitroso compounds have shown carcinogenic properties, little research has been conducted with specific RDX N-nitroso metabolites in regards to genotoxicity. Without activation, TNX was shown to be genotoxic using a microsuspension modification of the *Salmonella* histidine reversion assay (George *et al.* 2001). Using S9 activation, MNX and TNX showed weak to moderate mutagenesis depending on the bacterial strain used in the Ames Assay (Pan *et al.* 2007a). No research has been conducted to assess genotoxicity of N-nitroso compounds in multicellular organisms *in vivo*.

The objective of this study was to investigate the potential genotoxicity of RDX N-nitroso metabolites *in vivo*. To achieve this objective, our group completed two separate dosing studies: a reproductive study (Smith *et al.* 2006) and a multigenerational study (Smith *et al.* 2007). In the reproductive study, breeding pairs of deer mice (F0) were exposed to varying concentrations of TNX. Three cohorts of offspring (F1A-F1C) were produced, and TNX demonstrated dose-dependent bioaccumulation in the liver, increased offspring mortality, and decreases in body and kidney weight from birth to weaning in developing deer mice. The multigenerational study expanded on the reproductive study, in which breeding pairs of deer mice (F0) produced offspring (F1A-F1D), which in turn, were bred to produce offspring of their own (F2A-F2B). This process was repeated once more to create a third generation of offspring (F3A). Findings from this study demonstrated decreased litter size of deer mice and increased mortality of deer mice offspring.

With tissue samples from both the reproductive and multigenerational studies, microsatellite DNA was analyzed using both change in original parent allele frequencies and the parent/offspring approach to assess genotoxicity. Microsatellites

are generally assumed to be neutral markers; and for a neutral marker, the degree of polymorphism is proportional to the underlying mutation rate (Ellegren 2004). Examination of allele frequencies in populations allow inferences to be made about how these parameters could have been influenced by contaminants. In a controlled laboratory setting, one can isolate dependent variables to the contaminant of interest.

The parent/offspring approach is a method of determining mutations based on the inheritance of alleles. According to Mendelian genetics, every organism has two allelic sets of genes in their genome. One allele is inherited from the mother, and the other allele is inherited from the father. With this in mind, one can assess genetic alterations (mutations) by comparing parents' alleles with corresponding offspring's alleles. If one of the mother's or father's alleles is present in the offspring's genome, we can assume there is no mutation for that particular allele. However, if neither one of the mother's or father's alleles are present in the offspring's genome; it is assumed a mutation has occurred to that particular inherited allele. This method was selected because it allows for a direct determination of the mutation rate.

Microsatellite DNA was analyzed from two dosing studies to assess potential genotoxicity of TNX *in vivo*. Endpoints investigated are change in parent allele frequency and direct determination of the microsatellite mutation rate using the parent/offspring approach. Information obtained in this study will be useful for RDX and TNX genetic ecological risk evaluation of terrestrial wildlife at RDX contaminated sites.

Materials and Methods

Animal model and toxicant exposure

Virgin breeding pairs of deer mice (*Peromyscus maniculatus bairdii*) were obtained from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA). According to protocols in two separate studies, deer mice were paired, bred, and produced offspring while being exposed to various levels of TNX in

drinking water provided *ad litbitum*. These studies are referred to as the reproductive study (Smith *et al.* 2006) and the multigenerational study (Smith *et al.* 2007).

In the reproductive study (Smith *et al.* 2006), deer mice were exposed to TNX in the following exposure groups: control, 1 µg/L, 10 µg/L, and 100 µg/L. These initial breeding pairs of deer mice (F0) produced three consecutive cohorts of offspring in one generation (F1A-F1C).

In the multigenerational study (Smith *et al.* 2007), TNX was exposed to deer mice in the following exposure groups: control, 10 µg/L, 100 µg/L, and 1 mg/L. These deer mice produced four cohorts of offspring (F1A-F1D) in a one generation. Deer mice offspring from the F1A and F1B cohorts were randomly selected, grown to maturity, randomly paired, bred, and produced two cohorts of offspring in a second generation (F2A-F2B). Again, deer mice from these two cohorts of offspring were randomly selected, grown to maturity, randomly paired, bred, and produced a final cohort of offspring (F3A) in a third generation.

For both studies, at the time of euthanasia, livers were obtained from both parents and their respective offspring in both studies. In some cases, female parent mice were pregnant when euthanized. If these embryos were large enough, they were harvested from female parent mice. Tissues were frozen until further analysis. Approximately 325 offspring from the reproductive study (Smith *et al.* 2006) were analyzed, and approximately 680 mice from the multigenerational study (Smith *et al.* 2007) were analyzed.

DNA extraction, amplification, and fragment analysis

Total DNA was extracted using a sample from the frozen liver or embryo tissue. Extraction of DNA was conducted using the Puregene® DNA Purification Kit (Gentra Systems Inc., Minneapolis, MN, USA), and DNA was stored in TE buffer (Tris-HCl, EDTA, pH 8.0). Total DNA was quantified using an Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany), and from that data, aliquots of uniformly concentrated DNA (100 ng/µL) were prepared in 96 well plates.

Amplification of 12 microsatellite DNA loci was conducted with polymerase chain reaction (PCR). The following parameters were used for PCR reactions: 95°C for 3 minutes, 32 cycles at 95°C for 30 seconds, 55°C for 10 seconds, 72°C for 30 seconds; followed with a final extension at 72°C for 10 minutes. Amplifications of DNA were performed using 1 unit final concentration of Master Taq-Eppendorf, 0.7X TaqMaster (enhancer), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer and 100 ng of genomic DNA. *Peromyscus* microsatellite primers used were Pml 02, Pml 03, Pml 04, Pml 05, Pml 06, and Pml 12 (Chirhart *et al.* 2000); as well as PO 09, PO 21, PO 26, PO 40, PO 368, and PO 385 (Prince *et al.* 2002) **(Table 5.1)**. Forward primers were labeled with either HEX, NED, or 6-FAM florescent tag.

Fragment analysis of amplified microsatellite loci was conducted using an 3100-*Avant* Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) or an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). This analysis used a 36 cm capillary array, Pop-4 polymer, and ROX 400HD size standard.

Fragment analysis output was analyzed with GeneMapper V3.0 software (Applied Biosystems, Foster City, CA, USA) to calculate fragment length. Genotyping of each individual was based on the microsatellite DNA fragment lengths (Ellegren 2004). Using GeneMapper, automatically generated "bins" were used for assigning whole number genotypes to data. Subsequently, genotyping was manually checked for errors. In order to ensure unbiased calls, manual genotyping was performed without knowledge of the pedigree structure. Genotypes were imported into a pedigree format in Excel.

Once pedigrees were established, parent to offspring inheritance was calculated based on Mendelian genetics. Length polymorphisms of alleles inherited from parents to offspring were considered mutations; in other words, if an offspring had an allele that was not present in either parents' genome, it is considered a mutation (Weber and Wong 1993; Ellegren 2004). All mutations were verified by repeating the protocol, in order to eliminate errors due to strand slippage during PCR.

Two approaches were used to make mutation calls. The first approach included all Mendelian discrepancies observed. The second approach included only those Mendelian discrepancies that yielded a novel allele, an allele that was not previously present in the population. This second, more conservative approach was used in an attempt to minimize potential errors that could artificially inflate the mutation rate such as presence of null alleles (Pemberton *et al.* 1995), short allele dominance (Wattier *et al.* 1998), misscoring of allele size (Delmotte *et al.* 2001), and cross-contamination of DNA (especially for analysis of small embryos, since maternally derived tissue could have been present in offspring embryo tissue samples). Since the purpose of this study is to determine if exposure to TNX effects microsatellite mutation rates in deer mice, the authors feel it is appropriate to use both approaches of mutations calls for an unbiased assessment of potential genotoxicity.

Statistics

Each microsatellite locus in each study was tested for Hardy-Weinberg equilibrium using functions in the Genetics package of "R: A language and environment for statistical computing" Version 2.4.1 (R 2004).

Deviations from original parent allele frequencies were calculated for each microsatellite locus and dosing study. Within each dose group, contingency tables of allele counts were created using the following horizontal columns: the parent allele count (F0), then the respective generations of offspring allele counts (F1, F2, etc.) depending on the study being analyzed. Contingency tables were tested for allele frequency continuity using the log-likelihood ratio test (g-test) (Wilks 1935).

Mutation rate was calculated by simply dividing the number of Mendelian discrepancies (using both approaches of calling mutations) by the total number of parent-offspring transmissions. To assess the dependence of the directly measured mutations rate, contingency tables of number of mutated alleles and non-mutated alleles were constructed using dose group as the horizontal columns. These contingency tables were also analyzed using the log-likelihood ratio test. All

statistical tests used an α value of 0.05. The software used to analyze data was "R: A language and environment for statistical computing" Version 2.4.1 (R 2004).

Results

All microsatellite loci in both studies were within Hardy-Weinberg equilibrium except for PO 40 in the reproductive study ($p < 0.001$) and PO 385 in the multigenerational study ($p < 0.001$). Heterozygous (Hu) frequencies of Pml microsatellite loci ranged 0 to 0.08 from the reproductive study and 0 to 0.18 for the multigenerational study (**Table 5.2**). Heterozygous frequencies of PO microsatellite loci ranged 0 to 0.50 for the reproductive study and 0 to 0.49 for the multigenerational study (**Table 5.3**). Of the 12 total microsatellite loci analyzed for the reproductive study, 5 were completely homozygous. And of the 11 total microsatellite loci analyzed for the multi generational study, 3 were completely homozygous. The number of alleles per loci ranged from 1 to 3 in the reproductive study and 1 to 4 in the multigenerational study.

There were two cases in which offspring allele frequency deviated from the parents' allele frequency. These included: the multigenerational study, control dose group, microsatellite locus PO 385 locus ($p = 0.001$) and the multigenerational study, 10 µg/L dose group, PO 21 locus ($p = 0.03$).

Using the first approach of calling mutations, ten mutations were discovered in the reproductive study and nine mutations were found in the multigenerational study. Overall mutation rate for the reproductive study was 1.35×10^{-3} mutated alleles/total parent-offspring transmissions and 6.28×10^{-4} mutated alleles/total parent-offspring transmissions for the multigenerational study. Mutations were found to be independent of dose ($p = 0.30$ for reproductive study, and $p = 0.73$ for the multigenerational study). Mutation rate of individual generations within the multigenerational study also were independent of dose (F1: $p = 0.44$, F2: $p = 0.48$, and F3: $p = 0.50$).

Using the second approach of calling mutations, two novel alleles were discovered in the reproductive study and six novel alleles were found in the multigenerational study. Overall mutation rate for the reproductive study was 2.71 x 10^{-4} mutated alleles/total parent-offspring transmissions and 4.18 x 10^{-4} mutated alleles/total parent-offspring transmissions for the multigenerational study. Mutations were found to be independent of dose ($p = 0.25$ for reproductive study, and $p = 0.38$ for the multigenerational study). Mutation rate of individual generations within the multigenerational study also were independent of dose (F1: $p = 0.43$, F2: $p = 0.39$, and F3: $p = 0.50$).

Discussion

Only 11 microsatellites were analyzed from the multigenerational study, because the PO 26 locus had very illogical results: inheritance of parent alleles to offspring non-mutated alleles matched up poorly for all dose groups, thus PO 26 was omitted from analysis.

Using the same microsatellite DNA loci, the number of alleles per locus was lower than those of wild *Peromyscus* (Chirhart *et al.* 2000) and those of captive *Peromyscus maniculatus sonoriensis* (a closely related subspecies) also from the Peromyscus Genetic Stock Center (Prince *et al.* 2002). Wild *Peromyscus* values for number of alleles per locus, using Pml microsatellite loci, ranged from 10 to 16 (Chirhart *et al.* 2000). *P. maniculatus sonoriensis* values for number of alleles per loci, using PO microsatellite loci, ranged from 5 to 9 (Prince *et al.* 2002). Heterozygosity values of mice in this study were also lower compared to the same aforementioned wild *Peromyscus* and captive *P. maniculatus sonoriensis*. In wild *Peromyscus*, ranges of Pml microsatellite loci were 0.75 to 0.95 (Chirhart *et al.* 2000); and in captive *P. maniculatus sonoriensis*, heterozygosity ranged from 0.25 to 0.875 for PO microsatellite loci (Prince *et al.* 2002). This reduced number of alleles per locus and heterozygosity indicates higher levels of inbreeding within *P. maniculatus bairdii* raised in captivity in comparison with wild-type deer mice and captive *P. maniculatus sonoriensis*. This is somewhat expected since *P. maniculatus bairdii* are

coming from a captive breeding colony established in 1948, and the *P. maniculatus sonoriensis* colony was established in 1995 (USC 2007). Breeding population size is greatly reduced in captive mice; and with increased time, more generations can increase inbreeding. The University of South Carolina *Peromyscus* Genetic Stock Center estimates the inbreeding coefficient of parent *P. maniculatus bairdii* to exceed 0.30 (USC 2007).

Previous genotoxicity studies of RDX exhibited no genotoxic effects in various *in vitro* and *in vivo* systems (Reddy *et al.* 2005). However, RDX has been listed as a potential carcinogen by the U.S. EPA based on the presence of hepatocellular adenomas and/or carcinomas in female B6C3F1 mice (Lish *et al.* 1984). In a recent re-evaluation of this status, hepatocellular adenomas and/or carcinomas were again observed in female B6C3F1 mice (Parker *et al.* 2006). Since it has been demonstrated that RDX can convert to N-nitroso intermediates in the gastrointestinal tract (Pan *et al.* 2007b), one can hypothesize that RDX N-nitroso metabolites may be related to the formation of hepatocellular adenomas and/or carcinomas.

Evaluation of RDX N-nitroso metabolites genotoxicity *in vitro* have been conducted in two studies. One study employed a variation of the Ames Assay for MNX and TNX using *Salmonella* strains TA98 and TA100 (George *et al.* 2001). Without activation, TNX was shown to be genotoxic using the TA100 strain. Using strains TA97a, TA98, TA100, and TA102; MNX and TNX were shown to be mutagenic using S9 activation in the TA100 strain (Pan *et al.* 2007a). The *Salmonella* TA100 strain is primarily mutated in GC sites and is commonly used to detect mutagens that caused base-pair substitution or frameshift mutations (Maron and Ames 1983). The *Salmonella* TA97a strain carries a frameshift mutation that is sensitive to mutations at a run of C as well as GC base pair substitution (Maron and Ames 1983; Levin *et al.* 1985). Other N-nitroso compounds have shown a strong affinity for methlyating or alklyating guanine nucleotides (Archer 1989; Souliotis *et al.* 2002).

Using both approaches of calling mutations, *in vivo* results from this study show no major differences among dose groups in either microsatellite DNA allele

frequencies variations from original parent allele frequencies or directly measured microsatellite DNA mutation rates. This indicates that exposure to TNX had A.) no genotoxic effect or B.) a genotoxic effect that could not be measured. In the literature, there is no uniform microsatellite DNA mutation rate-it is variable by loci, allele, and perhaps species (Ellegren 2004). Baseline microsatellite DNA mutations rates measured in this study are within the range of what others have seen in mammals (10^{-3} to 10^{-5}) (Schug *et al.* 1997).

There could be several reasons that a genotoxic effect was not detected, yet this compound my still be genotoxic. First, TNX may not have been bioactivated into the ultimate genotoxicant, thus not having any resulting toxic insult. As mentioned, N-nitroso compounds require bioactivation to exhibit carcinogenic properties: hydroxylation of the α carbon usually by a Cyp P450 enzyme (Archer 1989). In the Ames Assay, it was found that TNX was mutagenic, but only using the highest level of S9 bioactivation (Pan *et al.* 2007a). Perhaps cytochome P450 enzyme levels (probably CYP 2E1 and CYP 2A6) in these mice were not high enough to bioactivate sufficient quantities of toxicant. Thus, little ultimate toxicant may be available to initiate a toxic insult.

Mutation processes are thought to be a balance between rates of DNA damage and DNA repair. When the rate of DNA damage exceeds the rate of DNA repair, DNA lesions may become prevalent, and mutations may occur during the DNA replication process. Thus, another possibility for lack of observed mutations from a potential genotoxicant is that concentrations of the ultimate toxicant were not high enough to induce DNA damage at a rate that would overcome DNA repair rates. Dosing levels for these studies were chosen to be environmentally relevant with RDX N-nitroso metabolite concentrations found (Smith *et al.* 2006; Smith *et al.* 2007) at the Iowa Army Ammunition Plant (Middleton, IA, USA) (Beller and Tiemeier 2002). RDX N-nitroso compounds were found at concentrations up to 430 μg/L.

Since the measurement of direct microsatellite mutation rate is a measure of microsatellite length polymorphisms, we are really looking for insertion/deletion type

mutations. N-nitroso compounds (N-methyl-N'-nitro-N-nitrosoguanidine) have caused microsatellite length polymorphisms in other studies (Bardelli *et al.* 2001; Slebos *et al.* 2002); however, it is possible that these specific N-nitroso compounds may not be causing insertion/deletion type mutations in microsatellite DNA. Instead, alkylation or methylation of DNA bases maybe occurring with other potential downstream effects, such as base substitutions. Thus, this study may have missed this type of genotoxic insult. A potential marker that would provide evidence for or against such a hypothesis would be quantification of DNA adducts. There are no specific adducts known for RDX N-nitroso metabolites-it may be a simple methylation or perhaps a larger alkylation, all depending on environmental degradation, *in vivo* digestion, and *in vivo* metabolism of TNX to whatever the ultimate toxicant may be.

To assess genotoxicity of TNX *in vivo*, 12 microsatellite DNA loci were amplified and analyzed for two dosing studies using both change in original parent allele frequencies and the parent/offspring approach of direct mutation rate determination. Our findings demonstrate no dose dependent differences in deviation from parent microsatellite DNA allele frequencies or direct microsatellite mutation rate using the parent/offspring approach. Exposure of reproducing deer mice to TNX at environmentally relevant concentrations caused no alterations to microsatellite DNA lengths. Information obtained in this study will be useful for RDX and TNX genetic ecological risk evaluation of wildlife at RDX contaminated sites.

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Tables

5.1 Motifs of microsatellite DNA

Repeating motifs of microsatellite DNA loci amplified in deer mice (*Peromyscus maniculatus*) exposed to varying levels of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX).

Microsatellite Locus	Repeat Unit	Source
Pml 02	$(CA)_{20}$	(Chirhart *et al.* 2000)
Pml 03	$(CA)_{22}$	(Chirhart *et al.* 2000)
Pml 04	$(CA)_{27}$	(Chirhart *et al.* 2000)
Pml 05	$(CA)_{21}$	(Chirhart *et al.* 2000)
Pml 06	$(CA)_{24}$	(Chirhart *et al.* 2000)
Pml 12	$(CA)_{20}$	(Chirhart *et al.* 2000)
PO 09	$(AC)_{20}N_{14}(AC)_8\ N_{16}(AC)_{20}$	(Prince *et al.* 2002)
PO 21	$(AG)_7$	(Prince *et al.* 2002)
PO 26	$(AG)_{13}\,(ACAGAG)_4$	(Prince *et al.* 2002)
PO 40	$(AG)_{15}AC(AG)_{13}$	(Prince *et al.* 2002)
PO 368	$(TG)_{22+}$	(Prince *et al.* 2002)
PO 385	$(AC)_{21}$	(Prince *et al.* 2002)

5.2 Pml microsatellite DNA allele count and frequency

Pml microsatellite DNA allele count and frequency in deer mice (*Peromyscus maniculatus*) exposed to varying levels of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) in two separate studies: a reproductive study (Smith *et al.* 2006) and a multigenerational study (Smith *et al.* 2007).

[a] Heterozygosity (Hu)

[b] Alleles that were formed by a mutation

Microsatellite Locus	Reproductive Study			Multigenerational Study		
	Allele	Count	Frequency	Allele	Count	Frequency
Pml 02	243	380	1.00	243	1467	1.00
				247[b]	1	0.00
	Hu[a]		0.00	Hu[a]		0.00
Pml 03	222	688	1.00	222	1510	1.00
	Hu[a]		0.00	Hu[a]		0.00
Pml 04	218	692	1.00	216[b]	1	0.00
				218	1452	1.00
	Hu[a]		0.00	Hu[a]		0.00
Pml 05	211	29	0.04	211	147	0.10
	213	711	0.96	213	1355	0.90
				216[b]	2	0.00
	Hu[a]		0.08	Hu[a]		0.18
Pml 06	164	9	0.01	164	65	0.04
	206	716	0.99	206	1457	0.96
	208[b]	1	0.00	200[b]	1	0.00
				212[b]	1	0.00
	Hu[a]		0.03	Hu[a]		0.08
Pml 12	157	680	1.00	157	1364	1.00
	Hu[a]		0.00	Hu[a]		0.00

5.3 PO microsatellite DNA allele count and frequency

PO microsatellite DNA allele count and frequency in deer mice (*Peromyscus maniculatus*) exposed to varying levels of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) in two separate studies: a reproductive study (Smith *et al.* 2006) and a multigenerational study (Smith *et al.* 2007).

[a] Heterozygosity (Hu)

[b] Alleles that were formed by a mutation

Microsatellite Locus	Reproductive Study			Multigenerational Study		
	Allele	Count	Frequency	Allele	Count	Frequency
PO 09	162[b]	1	0.00	164	1459	0.98
	164	737	1.00	166[b]	1	0.00
				168	26	0.02
	Hu[a]		0.00	Hu[a]		0.04
PO 21	121	481	0.65	121	1081	0.76
	123	257	0.35	123	337	0.24
	Hu[a]		0.45	Hu[a]		0.36
PO 26	174	734	0.98			
	179	12	0.02			
	Hu[a]		0.03	Hu[a]		
PO 40	247	395	0.55	247	845	0.59
	272	329	0.45	272	579	0.41
	Hu[a]		0.50	Hu[a]		0.48
PO 368	255	688	1.00	255	1518	1.00
	Hu[a]		0.00	Hu[a]		0.00
PO 385	188	386	0.52	188	632	0.44
	255	360	0.48	255	802	0.56
	Hu[a]		0.50	Hu[a]		0.49

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CHAPTER VI

SUMMARY AND CONCLUSIONS

The objective of this dissertation project was to explore the toxicity of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) N-nitroso metabolites. To accomplish this task, I assessed toxicity in two separate exposure length scenarios-acute and chronic using deer mice (*Peromyscus maniculatus bardii*) as the animal model.

Acute toxicity was evaluated using acute oral median lethal dose (LD_{50}) (Chapter 2). LD_{50} values were determined using the U.S. EPA Up-and-Down Procedure (UDP) for three toxicants-RDX, hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). To assess potential age dependent toxicity, LD_{50} values were determined in three age classifications of deer mice as well: 21 d, 50 d, and 200 d. Results indicate RDX N-nitroso metabolites caused similar overt signs of toxicity: tremors, convulsive seizures, and death. Median lethal dose for 21 d deer mice were 136, 181, and 338 mg/kg for RDX, MNX, and TNX respectively. Median lethal dose for 50 d deer mice were 319, 575, and 999 mg/kg for RDX, MNX, and TNX respectively. Median lethal dose for 200 d deer mice were 158, 542, and 338 mg/kg for RDX, MNX, and TNX respectively. These data suggest that RDX is the most potent compound tested, and age dependent toxicity may exist for all compounds.

Chronic toxicity was evaluated with an initial reproductive study and a follow-up multigenerational study. In the reproductive study (Chapter 3), TNX was administered *ad libitum* via drinking water at four exposure levels (control (0 µg/L), 1µg/L, 10 µg/L, and 100 µg/L) to breeding deer mice. Those mice produced three consecutive cohorts (F1A-C) of offspring. Endpoints investigated include: reproductive success, offspring survival, offspring weight gain, offspring organ weights, and liver TNX residues. Data from this study indicate that TNX bioaccumulates in the liver and is associated with postpartum mortality, dose

dependent decrease in body weight from birth to weaning, and decrease in kidney weight in deer mice offspring.

In the multigenerational study (Chapter 4), TNX was administered *ad libitum* via drinking water at four exposure levels (control (0 µg/L), 10 µg/L, 100 µg/L, and 1 mg/L) to breeding deer mice. Those mice produced four consecutive cohorts of offspring (F1A-F1D). Deer mice from F1A and F1B cohorts were randomly selected, paired, and bred. Those mice produced two cohorts of offspring (F2A-F2B). Like the last generation, deer mice from those two cohorts were randomly selected, paired, and bred. They produced an F3A cohort of deer mice. Endpoints investigated include: reproductive success, offspring survival, offspring weight gain, and offspring organ weights. Data from this study indicate that TNX is associated with decreased litter size and increased postpartum mortality of offspring. No teratogenic effects were linked with exposure to TNX.

To assess genotoxicity *in vivo* from exposure to TNX, microsatellite DNA was analyzed (Chapter 5). With tissue samples (either liver or embryo) from both the reproductive and multigenerational studies, 12 microsatellite DNA loci were amplified, genotyped, and analyzed using both change in original parent allele frequencies and the parent/offspring approach of direct mutation rate calculation. Findings demonstrate no dose dependent differences in deviation from parent microsatellite DNA allele frequencies or direct microsatellite mutation rate using the parent/offspring approach.

Data from this dissertation will provide valuable information for regulatory and remediation decisions regarding RDX and N-nitroso metabolites of RDX. In an ecological model, RDX was demonstrated to be the most potent compound tested in an acute dose. In chronic exposures, RDX N-nitroso metabolites (TNX) demonstrated adverse effects (decreased offspring survival) at lower exposures (10 µg/L) than similar studies with RDX. Thus, it could be important to include RDX N-nitroso metabolites in aforementioned decisions of explosive contaminated sites.