Interspecific Effects of Amino-Dinitrotoluene Exposure

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Amino-Dinitrotoluene (A-DNT)

- Rapidly formed from TNT
- Primary anaerobic reduction metabolite of TNT
  - Bacteria facilitated
- In vivo bioaccumulation of A-DNT from TNT exposures
  - Earthworms
  - Salamanders
- Low water solubility and volatility
- Moderate mobility in soil
- Plants - highest concentration in roots
Transformation Pathway

2,4,6- Trinitrotoluene (TNT)

2HA-DNT

2-amino-4,6- dinitrotoluene (2A-DNT)

4HA-DNT

4-amino-2,6- dinitrotoluene (4A-DNT)
Concentrations of TNT and primary breakdown products*

**Soil**

- TNT
- 2A-DNT
- 4A-DNT

**Worms**

*Johnson et al. 2000*
Soil concentrations of TNT (parent) and reduction products (Bazar et al. 2008)
TNT transformation

- A nitro group on the TNT ring is rapidly reduced to an amine (2A-DNT or 4A-DNT)
- In vivo and in situ
  - In situ it is concentration dependant
  - In vivo it occurs at exposure point
    - TNT parent rarely found in tissue
    - Rate limiting secondary metabolic step
- 2A-DNT or 4A-DNT most prevalent in tissue of animals exposed to TNT
- Little plant uptake of parent or metabolites
2A-DNT Effects

Mammals - Acute Only in Rats and Mice (LD50 rats 959-2240 mg/kg; mice 1342-1722 mg/kg). Central nervous system excitability and/or depression, exaggerated reflexes, ataxia, delayed deaths to 10d after dosing, yellow-orange urine; 50% absorbed (Ellis et al. 1980).


Earthworms - Toxicity 4A-DNT > TNT > 2A-DNT. 2A-DNT bioaccumulated most and needs consideration when evaluating overall TNT toxicity (Lachance et al. 2004).

Birds - Northern Bobwhite (Colinus virginianus)
Reptiles – Western fence lizard (Sceloporus occidentalis)
Amphibians – red-backed salamander (Plethodon cinereus)
Soil amphibian model

- *Plethodon cinereus*
- Lungless
- Thin integument
- Terrestrial
- Long-lived
- Small home range
  - (0.16-0.33m$^2$ (Petranka 1998))
Salamander Toxicity Test

- 28-d exposure
- Blood parameters evaluated
- Histopathological examination
- Biomarkers

- Soils spiked at 4 concentrations + control
- Fed *Drosophila*
- Weighed weekly
- Fed every other day
- Observed daily
A-DNT soil exposures to *P. cinereus*

- **10d range finding study**
- **2A-DNT and 4A-DNT**
  - 2A-DNT more toxic
    - No mortality to 10k mg/kg, but greater occurrence of overt symptoms.
- **28d subchronic 2A-DNT study**
  - <0.05, 34, 173, 603, 1533 mg/kg (dry)
  - No mortality*
  - Lethargy, unresponsiveness, inapparentance, adverse behavior at 603-1533 mg/kg; adverse hematology at 1533 mg/kg.

*One death at 603 mg/kg not thought to be compound related (Bazar et al. *in prep*).
Sceloporus as a Reptile Model

- > 70 species from northern U.S. to Panama
- Sea level to > 4000 m, deserts to subalpine forests
- Terrestrial habitats vary geographically
- Size varies from 4 to 60gm (15 - 25g, 20 – 25cm total length)
- Diurnal, hibernates / aestivates
- Invertivore – insects - beetles, flies, ants, spiders, snails
U.S. Range Maps
Western & Eastern Fence Lizards
(Sceloporus occidentalis / undulatus)

Basic Study Design

- Acute lethal (LD_{50})
- Subacute (14-Day)
- Subchronic (60-Day)
Technical Approach

**Acute** → \( LD_{50} \) → **Sub-acute** → **Sub-chronic** → 60d study (N=120)

Sequential stagewise probit analysis (N=24)

Range-finding (14d) study (N=32)

5-treatments
(4 doses+1 control)
12/sex/treatment
Acute Toxicity

Cumulative Animal Mortality
LD$_{50}$ - no sex difference
Males = 1406 (947, 2087)
Females = 1867 (1076, 3237)

Depression, weakness, anorexia, weight loss, yellow vent and feces

Percent days survived decreased with increasing dosage - no sex difference.

Average (days):
Males = 9.29 ± 1.10
Females = 9.91 ± 0.99

Percent days survived decreased with increasing dosage - no sex difference.
Subacute (14-Day)

Significant effects on survival at ≥ 95 mg/kg-d. Two highest groups survived approx. 7 and 6 days.

Loss of BW was dose-related in the lower exposures due to approximate 14-d survival times.
Survival time and number of lizards per group decreased significantly at \( \geq 15 \) mg/kg-d in the 60-day experiment.
Reduced feeding rates were statistically significant at ≥ 15 mg/kg-d.

Net loss in BW at 15 mg/kg-day, coincident with a daily cricket intake of 2.5. Change in BW compared to Day 0.
Subchronic (60-Day)

No significant differences in measured hematologic endpoints.
# Subchronic (60-Day)

## 2A-DNT Dose (mg/kg-d)

<table>
<thead>
<tr>
<th>Test</th>
<th>0</th>
<th>5</th>
<th>15</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALB (g/dL)</td>
<td>2.01 +/- 0.20</td>
<td>1.64 +/- 0.23</td>
<td>1.40 +/- 0.18</td>
<td>1.60</td>
</tr>
<tr>
<td>ALKP (U/L)</td>
<td>34.00 +/- 4.04</td>
<td>25.57 +/- 5.38</td>
<td>34.67 +/- 10.18</td>
<td>45.00</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>104.17 +/- 11.42</td>
<td>64.14 +/- 11.67</td>
<td>80.67 +/- 19.72</td>
<td>121.00</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>2.67 +/- 0.21</td>
<td>2.86 +/- 0.14</td>
<td>3.75 +/- 0.48</td>
<td>6.00</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.53 +/- 0.08</td>
<td>0.64 +/- 0.09</td>
<td>0.73 +/- 0.29</td>
<td>1.10</td>
</tr>
<tr>
<td>Phosphorus (mg/dL)</td>
<td>10.26 +/- 0.64</td>
<td>8.45 +/- 0.47</td>
<td>13.83 +/- 2.88</td>
<td>11.80</td>
</tr>
<tr>
<td>Uric Acid (mg/dL)</td>
<td>1.31 +/- 0.26</td>
<td>1.33 +/- 0.25</td>
<td>1.90 +/- 0.99</td>
<td>2.00</td>
</tr>
</tbody>
</table>

Values : Mean ± SEM
Subchronic (60-Day)

No difference in liver mass. Kidney enlarged at $\geq 15$ mg/kg-d.
Non-significant differences in total numbers, motility, or progression of male gametes.
Summary

• *S. occidentalis* is a useful laboratory model for toxicological investigation in reptiles.

• Clinical signs were non-specific and best characterized by anorexia, cachexia and yellow-orange discolored vent.

• Kidney effects; liver effects at levels where mortality occurred.

• LOAEL / NOAEL were determined at 15 / 5 mg/kg-d
  (Based on survival, body weight, food intake, kidney effects)
Northern Bobwhite

• Oral acute (stagewise probit)

• Oral subacute (14d range finding)
  – 0, 50, 125, 265, 550, 1000 mg/kg-d

• Oral subchronic (60d gavage)
  – 0, 0.5, 3, 14, 30 mg/kd-d
Acute Study - results

LD50 = 1167 mg/kg
Fieller’s 95%CI (356,1466)
Delta 95% (942, 1445)
Sub-acute Study - methods

Daily gavage, 14 d

Treatment levels – 0, 50, 125, 265, 550, 1000 mg/kd-d

Measurements:

- mortality
- body weights
- organ weights
- gross observations
Sub-acute Study - results

# days survived

0 50 125 265 550 1000

2A-DNT (mg/kg-d)
Sub-acute Study - results

• No significant differences in body weights among treatments.

• Significant differences in liver and spleen : brain weight ratios among treatments for females only.

<table>
<thead>
<tr>
<th>organ</th>
<th>treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>brain</td>
<td>no</td>
</tr>
<tr>
<td>heart</td>
<td>no</td>
</tr>
<tr>
<td>liver</td>
<td>yes (f)</td>
</tr>
<tr>
<td>kidneys</td>
<td>no</td>
</tr>
<tr>
<td>spleen</td>
<td>yes (f)</td>
</tr>
<tr>
<td>gonads</td>
<td>no</td>
</tr>
</tbody>
</table>
Sub-acute Study - results

Liver : Brain Weight Ratios

![Chart showing liver to brain weight ratios for different 2A-DNT (mg/kg-d) levels, with annotations indicating significant differences between groups.](chart.png)
Sub-acute Study - results

Spleen : Brain Weight Ratios

![Graph showing spleen to brain weight ratios with labels a, b, c and comparisons between males and females at different 2A-DNT (mg/kg-d) concentrations.](image-url)
Sub-acute Study - results

Consistent signs upon necropsy:

- enlarged gall bladder
- green food contents in gizzard
- no food in lower GI, although crop full
- scant white feces (urates only)
Sub-chronic Study - results

Significant differences in feed consumption in both genders.
No significant differences in body weights among treatments.
Significant differences in liver : brain weight ratios among treatments for both genders.

<table>
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<th>organ</th>
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<tr>
<td>brain</td>
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</tr>
<tr>
<td>liver</td>
<td>yes</td>
</tr>
<tr>
<td>kidneys</td>
<td>no</td>
</tr>
<tr>
<td>spleen</td>
<td>no</td>
</tr>
<tr>
<td>gonads</td>
<td>no</td>
</tr>
</tbody>
</table>
Sub-chronic Study - results

Weekly Feed Consumption

male weekly feed consumption

0.5, 3, 14 mg/kg-d > 0 & 30 mg/kg-d
Sub-chronic Study - results

Weekly Feed Consumption

female weekly feed consumption

lowest feed consumption @ 0 mg/kg-d
Sub-chronic Study - results

**Weekly Body Weights**

### Male Weekly Body Weights

<table>
<thead>
<tr>
<th>Days Exposed</th>
<th>Body Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>195</td>
</tr>
<tr>
<td>4</td>
<td>200</td>
</tr>
<tr>
<td>11</td>
<td>205</td>
</tr>
<tr>
<td>18</td>
<td>210</td>
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<tr>
<td>25</td>
<td>215</td>
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<tr>
<td>32</td>
<td>220</td>
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<tr>
<td>39</td>
<td>225</td>
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<tr>
<td>46</td>
<td>230</td>
</tr>
<tr>
<td>53</td>
<td>235</td>
</tr>
</tbody>
</table>

### Female Weekly Body Weights

<table>
<thead>
<tr>
<th>Days Exposed</th>
<th>Body Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>195</td>
</tr>
<tr>
<td>4</td>
<td>200</td>
</tr>
<tr>
<td>11</td>
<td>205</td>
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<tr>
<td>18</td>
<td>210</td>
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<tr>
<td>25</td>
<td>215</td>
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<tr>
<td>32</td>
<td>220</td>
</tr>
<tr>
<td>39</td>
<td>225</td>
</tr>
<tr>
<td>46</td>
<td>230</td>
</tr>
<tr>
<td>53</td>
<td>235</td>
</tr>
</tbody>
</table>

**Legend**:
- ▲ 0
- □ 0.5
- △ 3
- × 14
- ● 30
Sub-chronic Study - results

Weekly Body Weights

male weekly body weights

female weekly body weights
Sub-chronic Study - results

Liver / Brain Weight Indices

![Graph showing liver/brain weight indices with bars labeled A,B, a,b, A, b, A,B, B, and a].

- Liver/brain weight ratio (g)
- 2A-DNT (mg/kg-d)
- Males vs. Females
Sub-chronic Study - results

Hematology results:

<table>
<thead>
<tr>
<th></th>
<th>Effect?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>no</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>no</td>
</tr>
<tr>
<td>Total solids</td>
<td>no</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>no</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>yes - females only</td>
</tr>
</tbody>
</table>

Image of a blood smear.
Sub-chronic Study - results

Total leukocytes (WBC)

![Graph showing total leukocytes (WBC) with sub-chronic exposure to 2A-DNT](image-url)

- a
- a,b
Sub-chronic Study - results

Blood chemistry results:

<table>
<thead>
<tr>
<th></th>
<th>Effect?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>no</td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td>yes - males only</td>
</tr>
<tr>
<td>Aspartate aminotransferase</td>
<td>no</td>
</tr>
<tr>
<td>Calcium ion</td>
<td>no</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>no</td>
</tr>
<tr>
<td>Total protein</td>
<td>no</td>
</tr>
<tr>
<td>Globulin</td>
<td>no</td>
</tr>
<tr>
<td>Albumin</td>
<td>no</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>no</td>
</tr>
<tr>
<td>Phosphate</td>
<td>no</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>yes, males only</td>
</tr>
<tr>
<td>Uric acid</td>
<td>no</td>
</tr>
<tr>
<td>Sodium ion</td>
<td>no</td>
</tr>
<tr>
<td>Potassium ion</td>
<td>no</td>
</tr>
<tr>
<td>Chlorine ion</td>
<td>no</td>
</tr>
</tbody>
</table>
Sub-chronic Study - results

Alanine aminotransferase (ALT)

![Bar chart showing ALT levels with a,b indicated for different groups and doses.](chart.png)
Sub-chronic Study - results

Triglycerides (TRIG)
### Summary subchronic LOAELs/NOAELs

<table>
<thead>
<tr>
<th>Species</th>
<th>TNT</th>
<th>A-DNT</th>
<th>Targets (TNT/ADNT)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amphibians</strong></td>
<td>373</td>
<td>603</td>
<td>Blood Behavior</td>
</tr>
<tr>
<td></td>
<td>472</td>
<td>173</td>
<td></td>
</tr>
<tr>
<td><strong>Reptiles</strong></td>
<td>25</td>
<td>15</td>
<td>Blood Kidney</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><strong>Birds</strong></td>
<td>70</td>
<td>30</td>
<td>Kidney/liver Liver</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>
Summary

• ADNT is primary TNT metabolite
• Effects from ADNT exposure are different from TNT
  – Differences between species
  – ADNTs are less toxic from acute exposures than TNT, however,
  – ADNTs are more toxic than TNT from subchronic exposures
    • Different sequelae, different endpoints
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*Development of Toxicity Data for Munition Compounds to Support Toxicity Reference Value Derivations for Wildlife.*
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