TOXICOLOGICAL EFFECTS OF LANDFARMED OIL REFINERY WASTES ON COTTON RAT (*SIGMODON HISPIDUS*) LIVER.

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Bachelor of Science

University of North Texas

Denton, Texas

1999

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 2001

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PREFACE

This research project was designed to increase understanding of terrestrial toxicant bioavailability and hepatic effects of chronic exposure to landfarmed petroleum wastes, and to determine the suitability of cotton rats as bioindicator organisms.

I wish to express my appreciation to my major advisor, Dr. David Janz for his guidance, friendship and time. I wish to thank Dr. Karen McBee for her advice, encouragement and editorial comments and Dr. Roman Lanno for his insight. I would also like to thank Dr. Weber for her friendship and advice. I would like to express my sincere appreciation for the support and love of my husband, mother, father and brother. Finally, I would like to thank the Department of Zoology for travel funding and the EPA for funding this research.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

NOMENCLATURE

Ah	aryl hydrocarbon
CYP	cytochrome P450
EROD	ethoxyresorufin O-dealkylation
GSH	glutathione
GSSG	glutathione disulfide
GST	glutathione s-transferase
H&E	hematoxylin and eosin
MROD	methoxyresorufin O-dealkylation
PAH	polyaromatic hydrocarbons
PROD	pentoxyresorufin O-dealkylation

INTRODUCTION

Under natural conditions of exposure, polyaromatic hydrocarbons (PAH) have a high bioavailability in mammals, and there is considerable interest in the use of small mammal populations in ecological risk assessment of contaminated terrestrial ecosystems (Elangbam et al 1989; Flickinger and Nichols 1990; McBee et al 1987, 1991; McBee and Bickham 1988, Fouchecourt et al 1999; Lochmiller et al 1999; Pfau et al 2001). Possible routes of PAH exposure in wild terrestrial mammals include percutaneous absorption, inhalation of fine particles, absorption in the gastrointestinal tract and ingestion of soil while digging, foraging or grooming (Garten 1980; Roos et al 1996). Some stressors such as climatic change, disease, reproduction, territory defense and dietary restriction are natural components of ecosystems. These stressors are interactive and extremely difficult to reproduce in studies using laboratory strains of mammals (Porter et al 1984; McMurry et al 1999). Nutritional stress, for example, may alter magnitude and duration of a dose-dependent xenobiotic response (Boyd and Campbell 1983). Biomonitoring has been defined as the use of organisms to monitor contamination and to imply possible effects to biota (Philips 1977). Characteristics of a desirable bioindicator organism are a relatively large size, ease of identification, ecological importance, well understood biology, widely distributed, and robustness (not killed by very low levels of contaminants) (Beardsley et al 1978; Peakall 1992a, b).

COTTON RATS

Cotton rats (*Sigmodon hispidus*) are common throughout the southeast and south-central United States into north-central Mexico and Central America in grass-dominated habitats (Cameron and Spencer 1981, Peppers and Bradley 2000). Cotton rats are plentiful and indigenous, distinct enough from other rodents to be easily identified, have a range of movement of less than a hectare, tend not to cross roads, have rapid generation times and are ecologically important as prey for birds, mammals, snakes and even occasionally catfish (Cameron and Spencer 1981, Swihart and Slade 1984; Caire et al 1989; Schroder et al 1999). Cotton rats are most frequently found in grass-dominated habitats with grass height and density being important (Cameron and Spencer 1981). Home ranges are seasonally sensitive (largest during winter and summer), positively correlated with body mass and negatively correlated to population density. Seasonal shifts in habitat may be due to seasonal alterations in the nutrient landscape (Cameron and Spencer 1981), since the preferred plant species in the diets of cotton rats change seasonally (Schetter et al 1998). Male cotton rats have a larger home range than females. Females are more selective and choose mixed habitats with better resources, while males select home ranges based more on the locations of females (Cameron and Spencer 1981). In addition to climate, nutritional stress, territoriality and reproductive stress, cotton rats are parasitized by a variety of internal and external parasites (Cameron and Spencer 1981). Cotton rats are generalist herbivores that supplement their diet with eggs, insects, crayfish, crabs and small mammals when available (Howell

1954; Cameron and Spencer 1981; Nowak and Paradiso 1983; Whitaker 1989). They are active during both day and night (Cameron and Spencer 1981). Nest building plasticity (surface or burrow) in response to varying climatic conditions has been reported (Cameron and Spencer 1981). Cotton rats do not cache food (Cameron and Spencer 1981) resulting in year round foraging (Elfler and Slade 1998). Cotton rats have periodic fluctuations in density both within and between years linked to food availability (Lochmiller et al 1998b). Extreme population fluctuations do occur, and entire population turnover in Oklahoma has been found to range from five to twelve months (Caire et al 1989). Within New World rats and mice, the cotton rat's constitutive and inducible forms of cytochrome P450-dependent detoxification enzymes are the most extensively characterized in laboratory studies (Qualls et al 1998). Therefore, cotton rats likely are good candidates for suitable bioindicator species.

BIOMONITORING

Toxicity occurring in wild species outside of laboratory conditions tends to result from exposure to complex mixtures of toxicants, metabolites, and degradation products (Rowley et al 1983). The use of wild mammals in biomonitoring has the disadvantages of high levels of variation in response as a result of either uneven distribution of contaminants or small area of contamination within a varying home range that overlaps the contaminated area, and age, sex, genetic and seasonal variation in sampled populations. Because biomonitoring is cost effective compared to chemical analyses of soil, water and vegetation, this allows for monitoring of contaminant migration and provides a

better idea of the biological impacts of contaminants on wild populations. These advantages may outweigh disadvantages (Lindamood 1991; Dickerson et al 1994; Lochmiller et al 1998b).

OIL WASTE/LANDFARMING

A significant problem in today's more regulated and ecologically aware, yet petroleum powered, society disposal of oil refinery waste. A complicating factor when studying petroleum wastes is the variable nature of petroleum. Hydrocarbon and metal composition and weathering characteristics vary among grades of petroleum (Engelhardt 1984). Landfarming is an economical means of disposal of oil sludge. Landfarming entails tilling of waste under the soil followed by site-specific irrigation and fertilizer application and then abandonment to allow microbial degradation (Baker and Herson 1994; Schroder et al 1999). Addition of exogenous microbes has not been found to increase degradation (Baker and Herson 1994). Possible fates for waste components include leaching, hydrolysis, photodecomposition, adsorption, desorption, oxidation, and uptake and metabolism by plants and microorganisms (American Petroleum Institute 1984). PAHs have been found both on the surface and in the internal tissues of plants (Eisler 1987). Some components of oil sludge, such as aromatics with more than four rings, have been found to be resistant to degradation (American Petroleum Institute 1984; Loehr et al 1992; Huesemann 1994). Metals (e.g. Cr, Cu, Pb, Zn) tend to accumulate in the treatment zone (American Petroleum Institute 1984) because they cannot be degraded. Salts also accumulate in certain landfarms (American Petroleum Institute 1984). High salt concentrations, heavy metals

(e.g. Pb, Cr, As, Cd, Ni), total hydrocarbon levels higher than 10% (wt) and low-molecular weight aromatics (petrochemical hydrocarbons) may inhibit microbial activity (Huesemann 1994). Factors that can affect degradation of petroleum hydrocarbons include soil pH, temperature, aeration, nutrient status (nitrogen and phosphorus), waste characteristics, and microbes naturally present (Huesemann 1994). Treatment depth for landfarms is 30 to 45cm from the surface, and any waste deeper may have to be removed or mixed into the treatment zone (Huesemann 1994, American Petroleum Institute 1984). The physical composition of refinery wastes can vary tremendously (American Petroleum Institute 1984). Waste application rate, soil amendments, and storm water management vary on landfarms (American Petroleum Institute 1984). Variation in toxicity to wild rodents at various sites has been reported (Propst et al 1999; Rafferty et al 2001). At petrochemical waste sites, it is very likely that no two sites will have the same complex mixture of contaminants present (Propst et al 1999). Polycyclic aromatic hydrocarbons (PAHs) are a major concern on such sites, because of their potential toxicity (Propst et al 1999; Rafferty et al 2001).

BIOMARKERS

The National Academy of Sciences has defined biomarkers as xenobiotically induced alterations in cellular or biochemical components or structures, functions or processes that are measurable in a biological system or sample (Dickerson et al 1994). Biomarkers can have the advantage over chemical analysis of eliminating the estimate of bioavailability (Dickerson et al 1994). A biomarker is a molecular, biochemical, physiological or histological

indicator of exposure to, or effects of toxicants. A good biomarker will be specific, sensitive, easy to measure, reproducible and reliable, cheap, noninvasive, mechanistically based, and applicable to field studies. (Dickerson et al 1994).

LIVER

Analysis of liver detoxification enzymes has been used as a biomarker in fish, birds and small mammals exposed to PAH (Payne 1976; Elangbam et al 1989b; Leighton 1995). Possible target organs for PAH toxicity are diverse, but the liver is often the first organ to be challenged by contaminants and is the main organ for xenobiotic metabolism both in terms of detoxification and bioactivation (Eisler 1987; Payne et al 1987). Generally, responses of detoxification enzymes due to toxicant exposure are sensitive and precede cellular and tissue level damage (Elangbam et al 1989b). Liver detoxification of lipophilic toxicants such as PAHs involves two phases.

PHASE 1

Phase I of hepatic detoxification involves nonsynthetic reactions, most commonly oxidation. Located on the smooth endoplasmic reticulum, cytochrome P450 (CYP) enzymes catalyze a large part of the first phase of detoxification in the liver (Guengerich and Liebler 1985). CYP enzymes work in concert with NADPH-cytochrome P450 reductase (Lindamood 1991). There are multiple CYP isozymes with overlapping substrate specificities. CYP1A1 and CYP1A2 are the major PAH-inducible isozymes, but CYP1A2 is induced to a much lesser extent (Whitlock 1986). Both CYP1A and CYP2B are present in the liver at low

constitutive levels under physiological conditions, but are highly inducible following exposure to certain chemicals (Ioannides and Parke 1990). The classical inducer of CYP2B is phenobarbital, but CYP2B can be induced in concert with high induction of CYP1A activity or in the presence of certain organochlorines (Whitlock 1986; Ioannides and Parke 1990). Specific enzymatic markers for CYP1A1, CYP1A2 and CYP2B are the dealkylation of ethoxyresorufin (EROD), methoxyresorufin (MROD), and pentoxyresorufin (PROD), respectively. Determination of hepatic EROD, MROD and PROD are sensitive and reliable biochemical markers of exposure to variety of environmental toxicants (Rattner et al 1989; Beebe et al 1992; Lubet et al 1992; Nims and Lubet 1995; Roos et al 1996; Fouchecourt et al 1999).

The general reaction catalyzed by CYP enzymes is the addition or exposure of a polar functional group to the exposure compound. The main reaction catalyzed is oxidation, donating one oxygen to the substrate and one to oxidize NADPH or NADH (Guengerich and Liebler 1985). Addition of polar groups increases hydrophilicity, but the metabolite is generally not hydrophilic enough to excrete at this stage, and these electrophilic intermediates have the potential to cause oxidative stress unless they are deactivated by phase II of detoxification (Ioannides and Parke 1990). Reactive intermediates can interact with macromolecules and nucleophiles such as proteins, enzymes and DNA resulting in covalent binding, toxicity and carcinogenicity (Ioannides and Parke 1990). Besides its protective function, cytochrome P450 enzymes play an important function in the synthesis and catabolism of cholesterol, steroid

hormones, fatty acids, eicosanolds and bile acids (Ioannides and Parke 1990). Cytochrome P450 also is involved in metabolism of vitamin D_3 (Ioannides and Parke 1990).

Cotton rats and Sprague-Dawley rats (*Rattus norvegicus*) have similarities and differences in the substrate specificity of their CYP systems depending on the inducing agent used (Novak and Qualls 1989; Elangbam et al 1989b; Qualls et al 1998). Male and female cotton rats have differences in some of their CYP isozymes (Novak and Qualls 1989; Elangbam et al 1991). Male cotton rats collected from a petrochemical waste site had significant induction of total cytochrome or EROD, MROD, and PROD, but females had a nonsignificant pattern for the same enzymes when compared to animals from a matched reference site (Elangbam et al 1989a; Lochmiller et al 1999 respectively). This was thought to be influenced by the much higher variability found in the female enzyme levels (Lochmiller et al 1999).

ARYL HYDROCARBON RECEPTOR

A gene battery is a group of genes that, through intricate interrelationships and cross-talk, can coordinate up and down regulation of proteins (Nebert et al 2000). The aryl hydrocarbon (Ah) battery contains six known genes with many more hypothesized to regulate cell growth and differentiation and other functions (Okey et al 1993; Nebert et al 2000). The genes known to be linked to the Ah gene battery are CYP1A1, CYP1A2, glutathione transferase, NAD(P)H: quinone oxidoreductase, aldehyde dehydrogenase and UDP glucuronosyltransferase (Nebert et al 2000). The mechanisms responsible for activation of CYP2B are

less understood, and are not part of the Ah gene battery (Whitlock 1986, Lindamood 1991). The Ah battery can be coordinately induced by both PAHs and dioxins (Nebert et al 2000). The hydrocarbon response elements are thought to be upstream of dioxin responsive genes (Nebert et al 2000). The Ah receptor is found in the cytosolic fraction of prepared cells of both mammalian and nonmammalian species (Landers and Bunce 1991; Okey et al 1993). Multiple forms of the Ah receptor have been found and Ah receptor content varies across tissues and developmental stages (Landers and Bunce 1991). The Ah receptor is associated with heat shock proteins 90 and 70 (Landers and Bunce 1991; Okey et al 1993). Heat shock protein 90 maintains the untransformed receptor in the inactive state (Landers and Bunce 1991). When a ligand binds to the Ah receptor the heat shock protein 90 is dissociated and the receptor becomes active with the formation of a heterodimer between Ah receptor ligand subunit and Ah receptor nuclear translocator protein (Landers and Bunce 1991; Sarasasquete and Segner 2000). The active heterodimer is translocated into the nucleus where it binds to the DNA with Ah receptor response elements, transcription, translation and increased activity from the newly created enzymes result (Sarasasquete and Segner 2000). Ah battery genes have the capacity to both promote and prevent (thru activation of electrophile response element which induces oxidative stress-detoxifying enzymes, such as glutathione transferase) oxidative stress (Nebert et al 2000).

Oxidative stress is damage to living tissue caused by oxygen, free radicals or reactive intermediates (Ioannides and Parke 1990; Nebert et al 2000).

Oxidative stress can cause DNA damage, perturb ion efflux, cell volume, and intracellular pH, and is a major signal in precipitating apoptosis (Nebert et al 2000). Glutathione provides protection against all forms of oxidative stress by scavenging free radicals and electrophiles and reducing cysteine groups of transcription factors via a transcription factor (redox factor), which activates genes that function to halt oxidative damage once the oxidative stress signal transcription cascade is in full swing (Nebert et al 2000). Ah receptor governs the aryl hydrocarbon receptor repressor gene leading to negative feedback control (Nebert et al 2000).

PHASE II

Phase II of hepatic detoxification involves conjugation of the xenobiotic with highly polar endogenous compounds in the cell, such as sugars, amino acids and glutathione. Phase II reactions are important in the destruction of oxygen intermediates, such as epoxides and free radicals formed during phase I metabolism. Glutathione (GSH) conjugation is an example of an important phase II reaction. Glutathione (γ-L-glutamyl-L-cysteinyglycine) is a tripeptide containing several sulfhydryl groups (Mannervik et al 1989). Glutathione is the most concentrated intracellular nonprotein thiol in liver cells and makes up approximately 25% of the thiol of Old World rat (*Rattus norvegicus*) livers (Smith and Mitchell 1989, Goethals et al 1990). Glutathione is important as an antioxidant, a possible route of metal excretion, and as a storage site for cysteine (Akerboom and Sies 1990; Gonzalez and Esteller 1990; Tateishi 1990). Examples of metals bound by glutathione include cadmium, methyl mercury,

zinc, silver, and copper (Gonzalez and Esteller 1990). During conjugation, glutathione can be oxidized into glutathione disulfide (GSSG). GSSG can, in turn, be enzymatically reduced to GSH by glutathione reductase (Anderson 1989; Romero and Galaris 1990). Besides catabolism of reactive oxygen species, glutathione also is important because of its involvement in the synthesis of proteins, regulation of enzyme activity, participation in glucose metabolism, involvement in rejoining of radiation-induced DNA breakage, and amino acid transport (Redegeld and Galaris 1990; Akerboom and Sies 1990; Saez et al 1990). Additionally, hepatic glutathione may be involved in the regulation of cytochrome P450 enzymes (Gonzalez and Esteller 1990). The glutathione status of the liver also may be important to other organs and systems that uptake GSH exported by the liver (Akerboom and Sies 1990). Significant but low amounts of GSH have been detected in the plasma. Other organs, primarily the kidney, have the ability to uptake GSH from the blood (Meister and Anderson 1983; Smith and Mitchell 1989; Akerboom and Sies 1990). Approximately two-thirds of glutathione in plasma is excreted through the renal system (Smith and Mitchell 1989).

Glutathione is a cofactor for the Phase II biotransformation enzyme glutathione S-transferase (GST). There are multiple forms of GST with overlapping substrate specificity (Meister and Anderson 1983; Mannerik and Danielson 1988). Like the structural genes for CYPIAI and CYPIA2, GST genes are part of the Ah locus (Lindamood 1991). GST makes up as much as 10% of extractable protein of the liver (Kosower and Kosower 1989). Primarily through

the action of GST, with a small contribution of non-enzymatic processes; GSH conjugates electrophilic xenobiotics or their metabolites to form predominately nontoxic conjugates that are easily excreted (Levine 1983; Ishikawa and Sies 1989). GST induction has been reported following exposure to phenobarbital, 3-methylcholanthrene, and 2-4-benzo(a)pyrene (Gonzalez and Esteller 1990). GST, like glutathione, is found in both cytosolic and microsomal portions of the cell, with the majority of both found in the cytosol (Mannervik and Danielson 1988; Romero and Galaris 1990).

Glutathione can be depleted in animals that are challenged with significant amounts of toxicants. Protection from some compounds is so efficient that almost no damage is incurred until virtually all GSH is gone (Smith and Mitchell 1989). Following GSH depletion, the potential for liver injury by reactive intermediates increases (Wendel et al 1990). Increased hepatic lipid peroxidation has been found with decreasing GSH levels (Levine 1983). Severe oxidative stress can lead to acute cell death, which is preceded by loss of glutathione (Nicotera and Orrenius 1994).

LIVER MORPHOLOGY

Alterations of phase I and phase II hepatic detoxification processes can result in liver histopathology. Markers of sublethal cell injury include alterations of the nuclear envelope, chromatin, and nucleoli (Marzella and Trump 1991). Inducers of CYP enzymes can cause proliferation of smooth endoplasmic reticulum and cell hypertrophy (Eustis et al 1990). Proliferation of smooth endoplasmic reticulum, if visible by light microscopy, has a ground glass

appearance (Cullen and Ruebner 1991). Increased fibrous tissue can result from chronic exposure to toxicants or toxins (Eustis et al 1990). Following toxicant exposure, the most common histological change in liver is an association of hepatocellular swelling and large droplet fatty change (Cullen and Ruebner 1991). This association is a precursor to necrosis (Cullen and Ruebner 1991). Changes in the structure of nuclei also indicate cell necrosis (Marzella and Trump 1991).

EFFECTS OF OIL ON MAMMALS

Effects measured in cotton rats and other wild mammals inhabiting abandoned oil refinery sites include altered liver mass, altered immune system function with decreased immunity and resistance, decreased proportion of juveniles, low population densities, elevated mutation frequencies, alteration of cytochrome P450 activity, alteration of glutathione S-transferase activity, chromosomal aberrations, bioaccumulation of lead and fluoride in bones, tooth fluorosis and dental lesions (Lower et al 1983; Elangbam et al 1989a; Rattner et al 1993; Paranjpe et al 1994; McMurry et al 1999, Lochmiller et al 1999; Schroder et al 1999, 2000; Wilson et al 2000; Kim et al 2001a, b). Extent of dental lesions, lead and fluoride accumulation in bones, hepatocellular hypertrophy and cytochrome P450 induction were found to be greater in winter compared to other seasons (Rattner et al 1993; Lochmiller et al 1999; Schroder et al 2000; Kim et al 2001a, b). Female cotton rats inhabiting some of the landfarm sites in this study had significantly increased rates of ovarian and thymic cell apoptosis and a lower number of uterine scars (Savabieasfahani et al

1999). Heavy metal concentrations, especially lead, were elevated in the kidney of rats from these study sites. Contaminated sites also showed lower species diversity (Wilson et al 2000). In a previous study with a time delay between capture and sacrifice, hepatic EROD activity in cotton rats collected from a site contaminated with petroleum hydrocarbons was induced less than 2 fold compared to rats captured at a matched reference site (Lochmiller et al 1999).

ENVIRONMENTAL RELEVANCE

Measuring changes in sensitive biochemical markers of exposure, such as CYP enzymes, glutathione and GST, over time possibly could be used to monitor effectiveness of land farming remediation (Qualls et al 1998). Such hepatic effects may be used to establish early cellular indicators of toxicity in wild rodents chronically exposed to a variety of toxicants at non-lethal levels. This work may in the future be used to extrapolate the relative threat to other organisms, including humans.

OBJECTIVE

The overall objective of this research project is to increase our understanding of terrestrial toxicant bioavailability and hepatic effects of chronic exposure to landfarmed petroleum wastes, and to determine the suitability of cotton rats as bioindicator organisms.

HYPOTHESES

H_O: There will be no significant differences found in hepatic detoxification enzymes surveyed between animals killed on the day of capture and those killed 48 hours after capture within one site.

H_A: Animals from landfarm sites killed on the day of capture will have more pronounced differences, whether induction or depletion, when compared to animals captured on reference sites.

H_O: Hepatic CYP IA1, IA2 and 2B will not differ when comparing cotton rats collected from landfarmed sites with those inhabiting reference sites.

H_A: Hepatic CYP IA1, IA2 and 2B dependent enzyme activities will be elevated in animals collected from landfarm sites when compared to those animals inhabiting matched reference sites with CYP1AI induced to a greater extent than either CYP IA2 or 2B.

H_O: Hepatic glutathione S-transferase levels will be similar in animals collected both from landfarmed sites and matched reference sites.

H_A: Hepatic glutathione S-transferase levels will be elevated in animals from landfarmed sites compared to animals from matched reference sites.

H_O: Hepatic GSH and total glutathione levels will be similar in animals from landfarm sites and from matched reference sites.

H_A: Hepatic GSH and total glutathione levels will be decreased in animals from landfarm sites compared to animals from matched reference sites.

H_O: Hepatic histology slides from reference and treatment animals will be similar.

H_A: Cellular differences will be detected in hepatic histology slides when comparing animals from landfarm sites to animals from matched reference sites.

TO TEST THESE HYPOTHESES:

1. Developed microplate fluorimetric procedures for determination of ethoxyresorufin O-dealkylation (EROD), methoxyresorufin O-dealkylation (MROD), and pentoxyresorufin O-dealkylation (PROD) as enzymatic markers for CYP IA1, CYPIA2, and CYP2B, respectively.
2. Evaluated total glutathione and oxidized vs. reduced glutathione and glutathione S-transferase levels as indicators of hepatic Phase II biotransformation enzyme induction.
3. Compared hepatic EROD, MROD, PROD, and GST activities and glutathione levels in cotton rats in situ (day of capture) with the enzyme activities found in rats brought back to lab and held for 48 hours before termination.
4. Compared the sensitivity and reliability of Western blotting for CYPIAI and CYPIA2 to EROD and MROD determinations.
5. Evaluated the histopathology of the liver of rats inhabiting landfarms.

MATERIALS AND METHODS

Male and female cotton rats from two unremediated and two remediated landfarm sites and four matched reference sites were sampled during summer, fall and winter. Each reference and landfarm site is named by its location. This study, like other previous studies, delayed terminations by 36-48 hours in order to conduct immunological tests on the rats (Elangbam et al 1989, Lochmiller et al 1999, Elangbam et al 1991). A comparison was done between liver enzyme activities in rats sacrificed following capture and those held up to 48 hours before termination. Ethoxyresorufin o-dealkylation (EROD), methoxyresorufin o-dealkylation (MROD), pentoxyresorufin o-dealkylation (PROD), Western blotting of cytochrome P4501A1 and cytochrome P450 1A2, glutathione s-transferase (GST), and glutathione assays were preformed and histology was evaluated. All data with each site analyzed using t-tests separating rats collected using season and sex. A split-plot design (SAS) was used to analyze the data over all seasons, treatments and sexes.

	Sept. 1998	Aug. 1999	Feb. 2000	Feb. 2000	Sept. - Oct. 2000
	Delayed Termination	Delayed Termination	Day of Capture Termination	Delayed Termination	Day of Capture Termination
Ponca City Landfarm and Reference	Female Male	Female Male	Female Male	Female Male	Female Male
Duncan Landfarm and Reference	Female Male	Female Male		Female Male	Female Male
Mounds East Landfarm and Reference		Female Male	Male	Female Male	Female Male
Mounds West Landfarm and Reference		Female Male	Male	Female Male	Female Male

Table 1: Dates, sites, sexes and times of termination for the rats collected. Rats from September 1998 were only used for histopathology. The August 1999, February 2000 and September - October 2000 termination are referred to in the rest of the text as the summer, winter and fall terminations, respectively.

STUDY SITES

Mounds, OK: Two study sites were located on approximately 53 hectares of land in Creek County, Oklahoma. From 1976 to 1980, this land was owned and used as a petroleum waste dump location by Consolidated Tank Cleaning. Over this time period more than 300,000 barrels of petroleum-based sludge from local oil companies were delivered to the site. Two unlined storage ponds l-4 m deep were constructed to hold the waste. This waste was then landfarmed. Landfarming continued until 1977, but the ponds continued to function as waste storage areas well after the operation was shut down (Kelly 1985). Kelly Inc. Companies submitted a clean-up plan to the Creek County courthouse on 3 July 1981. Recovery of the site to its precontaminated state was the primary goal of the plan. The upper 2 m of soil were highly contaminated with petroleum based sludge. Sludge along the bottoms of ponds was the primary area of contamination, but adjacent grasses also were highly contaminated (Kelly 1985). Metal drums were discovered at the bottom of one of the ponds and buried throughout the site. Drums contained 1,000 tons of sludge, which consisted of 28% oil, 50% water and 22% solid waste. Drum sludge was treated with biodegradation (Kelly 1985). Analysis of contaminant mobility was the first step of the remediation. New ponds were constructed as part of a retention system to handle surface runoff. Subsurface drainage was controlled by installation of impermeable clay trenches. Conditional landfarming was used to treat the contaminated soils and pond sludge on site. The conditions used were: oil in the soil could not exceed 8% by weight, no sludge could be applied if the soil

moisture exceeded 70%, and incorporation of sludge had to be done on the day of application. The bottoms of the original ponds were tilled and treated with microbes capable of degrading hydrocarbons. Then ponds were filled with dirt, tilled and graded. The soil after treatment was found to contain less than 1% oil. Contaminated soil was tilled and treated with microbial degradation (Kelly 1985). Upon completion land was seeded with Bermuda grass *(Cynodon dactylon)*, Chinese lespedeza *(Serica lespedeza),* and leguminous species, such as sweet clover (*Melilotus* spp.). Unauthorized grazing of cattle has taken place in the past, but steps were taken to eliminate this activity (Kelly 1983).

Ponca City, OK: The site is located on a recently abandoned land farm plot inside the still active Conoco refinery. Conoco's refinery is one of the largest in the state. Landfarming is the refinery's main method of petroleum waste disposal. There are active landfarming activities on the plots adjacent to the study site. Little information is available about the methods employed in landfarming on this site.

Duncan, OK: Two study sites are located on an inactive refinery five miles south of Duncan. The refinery covers 162 hectares and originally known as the Rock Island Oil and Refining Company, which produced aviation fuel during the 1920's. The refinery changed owners and expanded until 1980 when the Tosco Corporation purchased it from the Sun Petroleum Company. Automotive gasoline, diesel fuel, fuel oil, liquid propane gas, petroleum feedstock, and petroleum coke were produced at the refinery. The refinery's capacity was 55,000 barrels of oil per day. Operation ceased July 1983, and treatment and

storage of the refinery contents were handled under the Resource Conservation and Recovery Act from 1983-1986. In 1986 the refinery was sold to Alpha Oil. Tosco Co. and the Oklahoma Department of Environmental Quality began work, in 1995, to clean up contaminated sites on the refinery. A cut-off wall and extraction wells were installed to prevent further leaching into Claridy Creek. No recovery plan has been established (Coleman 1997). One of these sites, isolated on three sides by a stream and on another by a double set of railroad tracks, did not have enough rats on site to continue captures and terminations after 1998. Grazing has occurred on the refinery and signs of grazing still occur. Landowners surrounding the Duncan refinery also have used settling pond water from the refinery to irrigate their wheat fields (Wilson personal observation).

Reference sites: Each study site has a reference site that is as close in proximity and vegetation as possible. Reference sites include some unused field bordering and owned by Cotton Creek Golf Course in Mounds, Lake Warika in Duncan and Kaw Lake in Ponca City. At all reference sites, owners had agreed not to mow, burn or apply chemicals to the reference sites for the duration of the study.

RAT CAPTURE AND HANDLING

Rats (n=6 females and 6 males per site) were collected in summer (July), fall (October) and winter (February) from two unremediated, two remediated landfarm sites and four reference sites by use of Sherman live traps (Sherman Traps Inc, Tallahassee Fl). During winter, traps were supplied with cotton for bedding to prevent hypothermia. Animals weighing over 100 g were

preferentially taken with the weight limit being lowered in 10 g increments until sample sizes were satisfied. Prior to termination rats were brought to the laboratory and held for 48 hours in individual polycarbonate cages with wire tops containing corn cob as bedding and fed Purina 5001 Laboratory Rodent Chow (Purina Mills, St. Louis MO) and water ad libitum. Each rat was assigned a number so that all animals in a group were not processed as a unit. On the morning of termination, rats were anesthetized with Metofane (Pittman-Moore, Mundelein IL), had blood drawn from retro-orbital sinus plexus and then were killed by cervical dislocation. After length, mass and sex had been determined, rats had ice cold cell media injected into their abdominal cavity. The abdomen was massaged then the media extracted by syringe. The spleen was removed aseptically thru a small slit. Then the abdomen was cut completely open and the liver removed. The intact gall bladder was clamped and carefully removed. The liver was weighed to the nearest 0.01g then divided, with half going into ice cold Tris-KCl buffer (0.5M Tris,1.15% KCl, pH 7.5) and the other half refrigerated for metal analyses.

TIME OF SACRIFICE

To test whether delay in collecting livers had an effect on induction of biotransformation enzymes, after 24 rats had been collected from a matched set of landfarm and reference sites during the winter 2000 termination, additional rats (up to 24 more) were taken. Livers were dissected and processed within 2-6 hours of capture and in the same manner as livers from rats terminated 48 hours after capture. A comparison was made between enzyme activities in these

animals and those brought back to the lab and held for 48 hrs before killing. Insufficient female animals were captured from Duncan, Mounds West and Mounds East to allow a day of capture termination in the winter of 2000. Insufficient male rats of were captured at Duncan to allow for a day of capture winter termination. During fall 2000 all cotton rats were terminated on the day of capture.

MICROSOME AND CYTOSOL PREPARATION

Hepatic microsomes (for CYP enzymes) and cytosol (for GST and glutathione) were prepared using differential centrifugation (Omura and Sato 1964). Liver was placed in individual glass beakers filled with ice-cold Tris-KCl buffer, minced with scissors, and then homogenized using a Wheaton glass and teflon homogenizer with a 30 sec rest on ice between each pass. Homogenates were transferred into individual plastic centrifuge tubes and centrifuged at 10,000xg at 4°C for 20 min in a Sorvall RC5C centrifuge with a Sorvall SM24 rotor. Supernatants were collected and centrifuged at 100,000xg at 4°C using Beckman Ti30 rotor in a Beckman LS-70M Ultracentrifuge or Beckman Optima LE-80K Ultracentrifuge. The resulting supernatant or cytosol was decanted into cryovials (Arrowhead Scientific, Lenexa KS) and frozen at -85°C. Pellets were washed with 0.25M sucrose and then resuspended in a 0.25M sucrose solution. The resulting microsomal preparations were aliquoted into cryovials and frozen. In order to preform the glutathione assays, a forth of the livers from the fall 2000 capture was deproteinated with metaphosphoric acid. The metaphosphoric acid was subsituted for the Tris buffer. The processing procedure was identical to the

above, except only cytosol was kept for glutathione determinations. All samples were frozen at -85°C until used. Microsomal and cytosolic protein concentrations were determined using a DC protein assay kit (BioRad, Hercules, CA) that is a variation of the Lowry procedure (Lowry et al 1951), using bovine serum albumin as a standard. Protein readings were performed in 96-well microplates (Arrowhead Scientific, Lenexa KS) with a SpectraMax 340 spectrophotometer and analyzed by SoftMax Pro 2.2.1 software. During all assays including protein determination, samples were number coded and run in groups by location with both landfarm and reference animals. Determination of which rats were treatment or reference was done after each assay was finished.

EROD, MROD, PROD

Dealkylation of resorufin ethers is highly specific for the major CYP isoezymes and has been used as a biochemical markers of CYP isoenzyme induction (Burke and Mayer 1974, Lubet et al 1985, Nerurkar et al 1993). Activities of CYP1A1, CYP1A2 and CYP2B were measured spectrofluorometrically using EROD, MROD and PROD activities, respectively (Burke and Mayer 1974, Lubet et al 1985). Briefly, microsome samples were diluted to a protein concentration of 6mg/ml with 0.25M sucrose. Standards were sequential dilutions of resorufin (Sigma, St Louis, MO). Ethoxyresorufin, methoxyresorufin or pentoxresorufin (Sigma, St. Louis, MO) in 0.1M Hepes buffer containing 5mM $MgCl_2$ was pipetted into 96-well microplates (Arrowhead Scientific, Lenexa KS). Beta-NADPH (Boehringer Mannheim, Indianapolis, IN) was dissolved in ice-cold Hepes buffer. All components were protected from

light. After incubation at 37°C, a reading of the resorufin formation was taken with an ICN Titertek Fluoskan II spectrofluorometer set to an emission wavelength of 590nm and excitation wavelength of 544nm. Incubation times for EROD, MROD and PROD were 10, 40 and 100 min, respectively. Readings were analyzed by DeltaSoft II 4.0 software (Biometallics Inc, Princeton, NJ). Each reading was corrected for protein concentration and incubation time between addition of NADPH and reading. Enzyme activities were expressed as nmol resorufin/min/mg microsomal protein.

WESTERN BLOTTING

Expression of CYP1A1 and CYP1A2 proteins was determined using Western blotting (Towbin et al 1979). Two lots of polyclonal goat anti-rabbit P450-1A1 and -1A2 (Oxford, Oxford MI) had differing ideal conditions for dilution and protein concentration and are given separately. Hepatic microsomal proteins, diluted 1:25 or 1:5, were separated under denaturing conditions using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Hoefer Model SE600-15-1.5) consisting of 10% stacking gels and 12.5% polyacrylamide separating gels. They were transferred to a 0.45μm nitrocellulose membrane for 15 h at 30V using Hoefer transfer unit (TE 42). The membranes were dried for approximately 10 min to fix protein. They were then blocked with 5% skim milk (BioRad, Hercules CA) in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for one h. The membrane was then incubated with a 1:1000 concentration of poly-clonal goat anti-rabbit P450 1A1 and 1A2 antibody in 1% skim milk TBS-T for one h. After five, ten and fifteen minute washings with TBS-T, an hour incubation

with anti-goat secondary (Santa Cruz, Santa Cruz CA) at a 1:2000 concentration in 1% skim milk TBS-T was used to label the primary with horseradish peroxidase. The proteins were visualized with chemiluminescence (SuperSignal, Pierce Chemical Co, Rockford, IL) and semi-quanitated by densitometry using a ScanJet 5300C scanner (Hewlett-Packard Co.) and Scion Image software (Beta 4.0.2).

GLUTATHIONE S-TRANSFERASE

Hepatic cytosolic glutathione s-transferase activity was determined spectrophotometrically using a microplate reader enzyme assay measuring the formation of the conjugate of glutathione (GSH) and 1-chloro,2,4-dinitrobenzene (CDNB) at 340nm (Habig and Jakoby 1981). Briefly, cytosol samples were diluted 1:10 with 0.1M phosphate buffer pH 6.5. Samples were then pipetted into a 96-well microplate (Arrowhead Scientific, Lenexa, KS) that contained phosphate buffer. Blank wells were left for reference. An assay mixture of CDNB, glutathione (Sigma, St Louis, MO) and phosphate buffer was then pipetted into wells and the plate was read immediately using a SpectraMax 340 spectophotometer (Molecular Devices Corp.). The resulting data from 5 min of scanning at 15 sec intervals at 37°C were analyzed using SoftMax Pro 2.2.1 software. The CDNB absorbency changes when it is conjugated to GSH by glutathione S-transferase. GST readings were corrected for protein content. Finite nonenzymatic catalysis was minimized with low substrate concentration and low pH. CDNB has been recognized as a general substrate for all glutathione transferases, making separation of different isoezymes unnecessary

for total glutathione S-transferase determination (Mannervik and Danielson 1988).

GLUTATHIONE

Total cytosolic glutathione and glutathione disulfide (GSSG) were determined spectrophotometrically using a commercial microplate enzymatic-recycling assay kit made by Cayman Chemical Company (Ann Arbor MI). Total glutathione measurement started with deproteination of the cytosol samples after homogenization with metaphosphoric acid and triethanolamine. Wells were assigned on a microplate. The standards were prepared by adding specific amounts of GSSG standard to MES Buffer to create concentrations from 0 to 8.0 M GSSG, which were 0 to 16.0 M at the conclusion of the assay. These standards and the cytosol samples were pipetted into the microplate. The assay mixture containing MES Buffer (0.4M 2-(N-morpholino)ethanesulphonic acid, 0.1M phosphate, 2mM EDTA pH 6), cofactor mixture (NADP and glucose-6-phosphate), glutathione reductase, glucose-6-phosphate dehydrogenase and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) was then added to each well. Any GSSG present was reduced to GSH by glutathione reductase. The sulfhydryl group of GSH reacted with DTNB and produced a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The GSTNB mixed disulfide, which also was produced, was reduced by glutathione reductase to recycle the GSH and produce more TNB. The plate was then incubated in the dark. A SpectraMax 340 microplate reader was used to measure absorbance at 405 nm at 5 min intervals for 30 min. Analysis was done by SoftMax Pro 2.2.1 software. The rate of TNB production

was directly proportional to the recycling reaction, which is directly proportional to the concentration of total GSH. GSSG levels alone were determined by derivatizing GSH with 2-vinylpyridine after deproteinization. The 2- vinylpyridine was added to the standards, because it has some inhibiting effect on color development. Other than these changes the GSSG levels were determined in the same manner as total glutathione.

HISTOPATHOLOGY

I also compared liver histopathology in rats from reference and petrochemical contaminated sites using basic histological techniques (hematoxylin and eosin - H&E staining) to determine if there has been any damage caused by the contaminants or the electrophilic intermediates formed during Phase I detoxification. Liver samples from summer 1998 were preserved by fixing for 24 h in Carson's modified formalin, paraffin-embedded and sectioned at 5μm using routine histological procedures. Slides were prepared, assigned code numbers and stained by the Oklahoma Animal Disease Diagnostic Laboratory, Oklahoma State University. Twenty-four individual slices of liver, from 24 rats, were analyzed for each location (12 rats collected from landfarm sites and 12 rats collected from the matched reference sites). Analysis of 5 fields of vision within each liver slice was done on slides from Duncan and Ponca City to determine general cell size, presence of cells exhibiting cloudy-swelling, presence of pyknotic nuclei, acellularity, and immune cell invasion. Beyond tissue necrosis, or malignancy, liver hypertrophy and proliferation of the smooth

endoplasmic reticulum are possible accompaniments to CYP induction (Ioannides and Parke 1990, Philips et al 1987, Rattner et al 1993).

STATISTICS

The statistical program, Instat, was used to perform *t*-tests on all data with each site analyzed separately during each season, and males and females analyzed separately. Asterisks indicate significant differences. A split-plot design (SAS) was used to analyze the data over all seasons, treatments and sexes. For the SAS program, the remediated landfarm sites of Mounds West and East were classified as remediated and the unremediated landfarm sites of Ponca City and Duncan were classified as treatment sites. Letters were used to indicated significant differences within the split plot graphs a & b are significantly different as are c & d and e & f, etc. An α of 0.05 was assigned.

RESULTS

EROD and CYP1A1

All females collected from landfarm sites showed no difference in EROD activity among reference animals (Fig 1). EROD activity was significantly elevated ($p=0.0003$) in male rats collected in summer 1999 from the Mounds West landfarm site compared to males from its reference site and males from the Duncan landfarm approached significance ($p=0.0618$). Males from the landfarm sites in Ponca City and Mounds East showed no difference in EROD activity compared to animals from the reference sites. Although animals from landfarm sites compared to reference were not significantly different , CYP1A1 levels for both females and males showed similar trends as EROD activities, except for the rats from Duncan (Figure 1).

In winter 2000, female cotton rats from the landfarm sites in Ponca City and Mounds East exhibited significantly elevated EROD activities ($p=0.0231$ and $p=0.0292$, respectively; Figure 2) when compared to females from the reference sites. As only one reference female was caught in Duncan no statistical test for significance could be performed. Mounds West females from the landfarm site showed no difference in EROD activity compared to animals from the reference site. Winter 2000 male cotton rats from each landfarm site showed no difference in enzyme activity from males collected from the corresponding reference sites. The differences in the CYP1A1 levels in female rats from Ponca City, Duncan and Mounds West in winter 2000 were in general agreement with the EROD activities. The CYP1A1 levels of females from the Mounds East landfarm site did

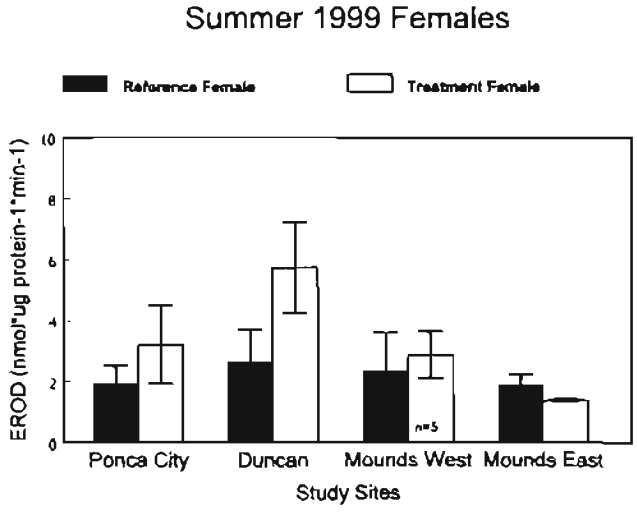
not show elevation of EROD activities when compared to the reference females from the corresponding reference site. No significant differences were found in the CYP1A1 levels of the female rats collected from any of the landfarm sites compared to the rats collected at matched reference sites. CYP1A1 protein levels of male cotton rats from Ponca City, Mounds West and Mounds East were in general agreement with the EROD activities with no elevations detected. Male cotton rats from the landfarm site at Duncan exhibited a significant elevation ($p=0.0281$) of CYP1A1 levels whereas they showed no elevation in EROD activity when compared to the males from the corresponding reference site (Figure 2).

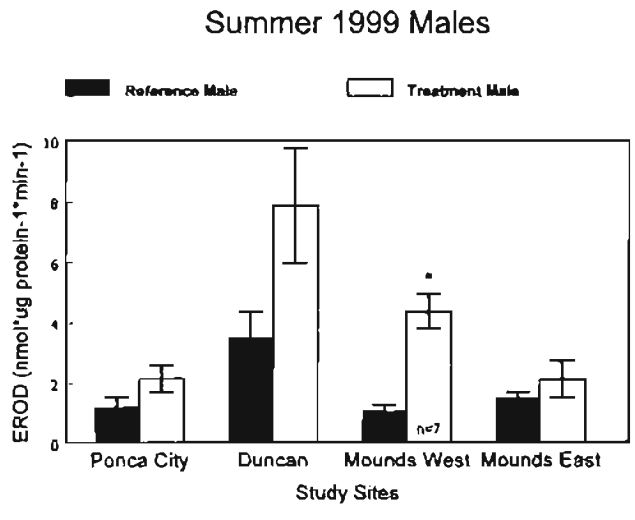
Ponca City females, from the landfarm site, sacrificed on the day of capture exhibited a significant elevation ($p=0.0063$) in EROD activity when compared to females from the corresponding reference site (Figure 3). Males from the landfarm site in Ponca City also had a significant elevation ($p=0.0175$) of EROD activity when compared to males from the matched reference site. No differences in EROD activity were found in males collected from landfarm sites in Mounds East and Mounds West when compared to males from the corresponding reference site. Unlike the EROD activity, the CYP1A1 levels showed no differences for the Ponca City females or males collected from the landfarm site compared to the reference site. A significant elevation of CYP1A1 levels ($p=0.0024$) was found in Mounds West males collected from the landfarm site compared to males from the reference site (Figure 3).

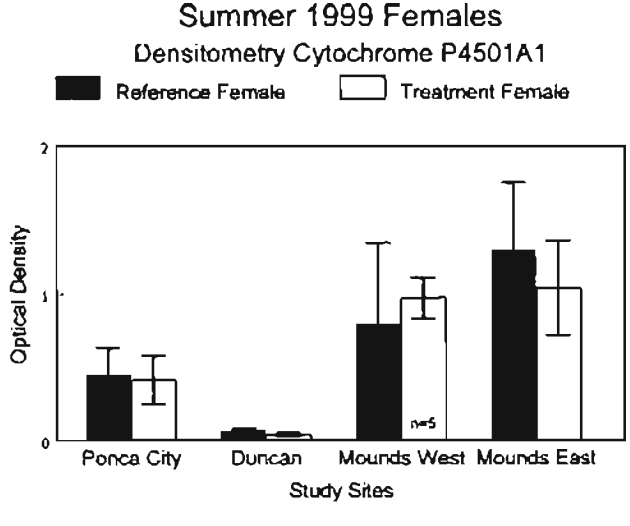
Cotton rats collected in fall 2000 were terminated on the day of capture. Ponca City females and males had elevated EROD activities ($p=0.008$ and 0.0039, respectively) (Fig 4). No differences were found in the EROD activities of female or male cotton rats of Duncan, Mounds West and Mounds East. No differences were found in the CYP1A1 levels at any of the sites (Figure 4).

Using split plot analysis, all seasons, site histories, and both sexes were analyzed for patterns in EROD activity (Figure 5). A history-season-sex interaction was found ($p=0.0322$). In the winter females from treatment sites, EROD activity was found to be significantly greater ($p<.0001$) than the activities found in females from reference and remediated sites. In the fall, females from the treatment sites were found to have significantly greater EROD activity ($p=0.0283$) than females from reference sites. Treatment females in the winter were found to have significantly higher activity ($p=0.0004$, 0.0003, respectively) than treatment females in the summer or fall. Within fall males, males from remediated sites had significantly elevated EROD activity ($p=0.0316$) when compared to reference animals. In the summer, males from the treatment sites had significantly greater EROD activity ($p=0.011$) when compared to the reference males. Reference males from the winter collection had a significantly greater EROD activity ($p=0.0056$, $p=0.0304$ respectively) than summer or fall males. When comparing females and males in the winter, reference females had a significantly greater EROD activity ($p=0.005$) than reference males, but treatment females had a significantly lower enzyme activity ($p=0.003$) than treatment males.

Figure 1. Mean (± SEM) O-dealkylation of ethoxyresorufin and optical density of cytochrome P4501A1 immunoreactive bands of female and male cotton rats caught in summer 1999. Asterisks indicate a significant elevation of the treatment enzyme activity over the reference enzyme activity within one site ($p<0.05$). n=6, except where noted in the graphs.


Summer 1999 Females
Reference Female
Treatment Female
EROD (nmol*ug protein-1*min-1)
0
2
4
6
8
10
n=5
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Summer 1999 Males
Reference Male
Treatment Male
EROD (nmol*ug protein-1*min-1)
0
2
4
6
8
10
n=7
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Summer 1999 Females
Densitometry Cytochrome P4501A1
Reference Female
Treatment Female
Optical Density
0
1
2
n=5
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

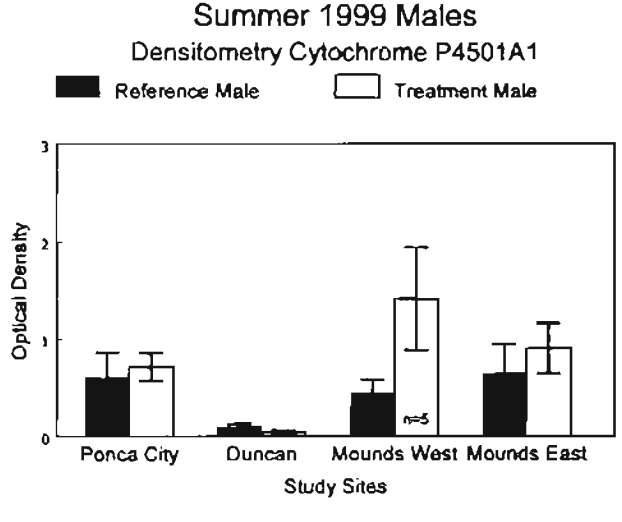
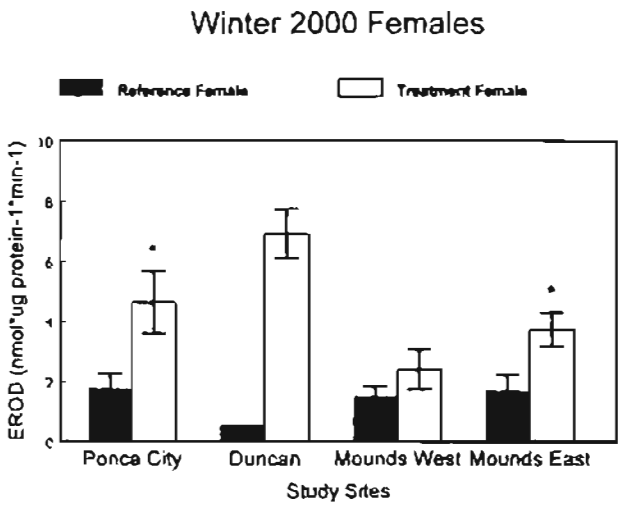
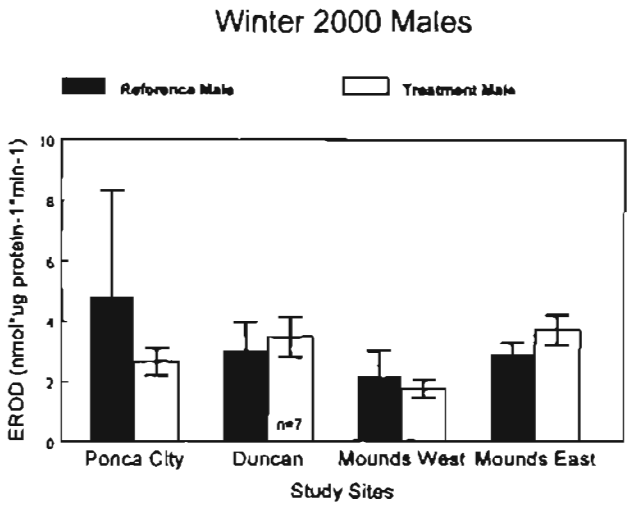
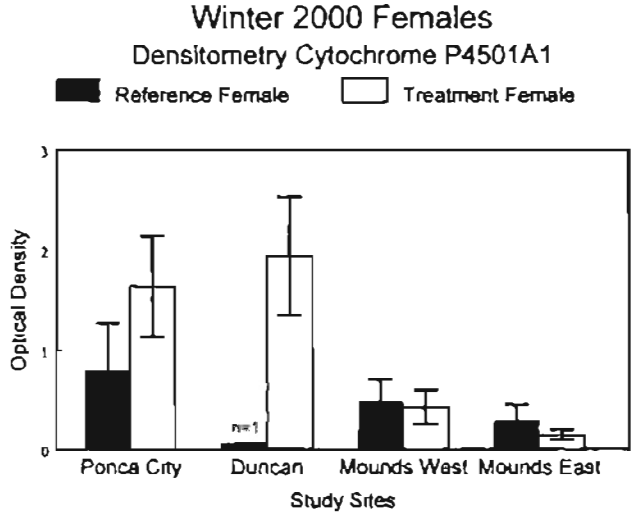

Summer 1999 Males
Densitometry Cytochrome P4501A1
Reference Male
Treatment Male
Optical Density
0
1
2
3
n=5
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

Figure 2. Mean (± SEM) O-dealkylation of ethoxyresorufin and optical density of cytochrome P4501A1 immunoreactive bands of female and male cotton rats caught in winter 2000. Asterisks indicate a significant elevation of the treatment enzyme activity over the reference enzyme activity within one site ($p<0.05$). n=6, except where noted in the graphs.


Winter 2000 Females
Reference Female
Treatment Female
EROD (nmol*ug protein-1*min-1)
0
2
4
6
8
10
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Winter 2000 Males
Reference Male
Treatment Male
EROD (nmol*ug protein-1*min-1)
0
2
4
6
8
10
n=7
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Winter 2000 Females
Densitometry Cytochrome P4501A1
Reference Female
Treatment Female
Optical Density
0
1
2
3
n=1
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

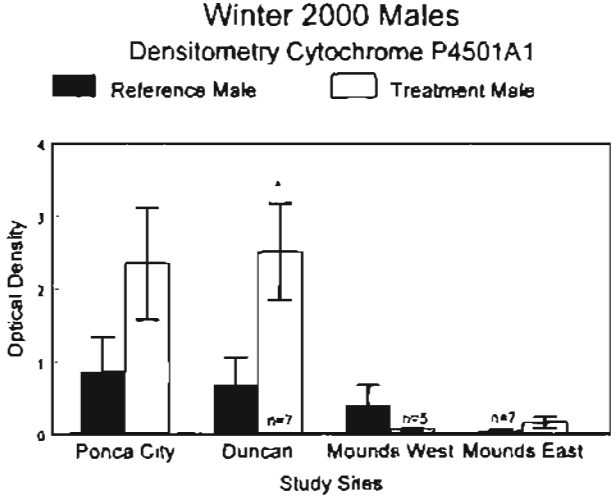
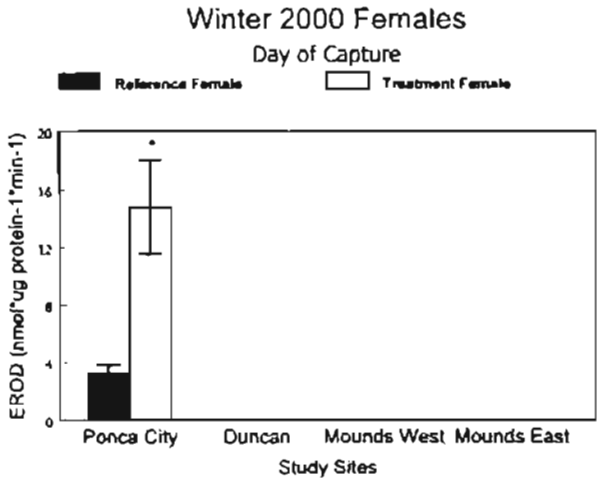
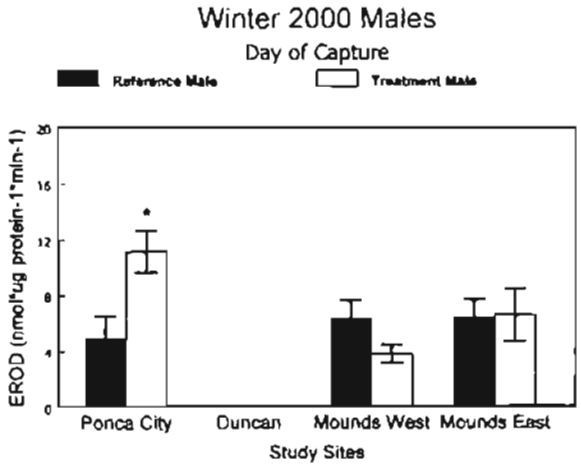
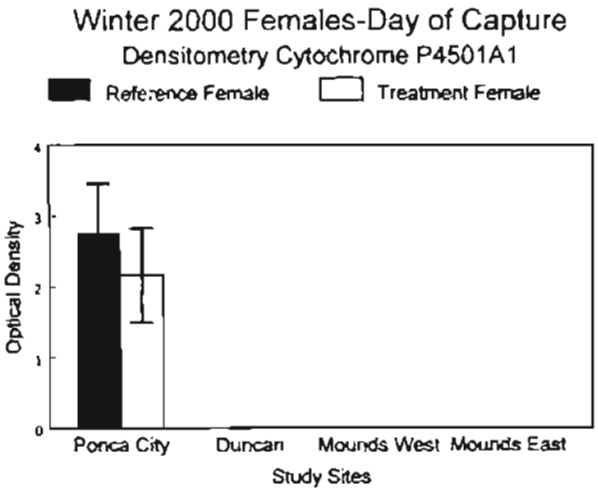

Winter 2000 Males
Densitometry Cytochrome P4501A1
Reference Male
Treatment Male
Optical Density
0
1
2
3
4
n=7
n=5
n=7
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

Figure 3. Mean (± SEM) O-dealkylation of ethoxyresorufin and optical density of cytochrome P4501A1 immunoreactive bands of female and male cotton rats caught in winter 2000 and sacrificed on the day of capture. Asterisks indicate a significant elevation of the treatment enzyme activity over the reference enzyme activity within one site ($p<0.05$). n=6, except where noted in the graphs.


Winter 2000 Females
Day of Capture
Reference Female
Treatment Female
EROD (nmol*ug protein-1*min-1)
0
4
8
12
16
20
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Winter 2000 Males
Day of Capture
Reference Male
Treatment Male
EROD (nmol*ug protein-1*min-1)
0
4
8
12
16
20
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Winter 2000 Females-Day of Capture
Densitometry Cytochrome P4501A1
Reference Female
Treatment Female
Optical Density
0
1
2
3
4
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

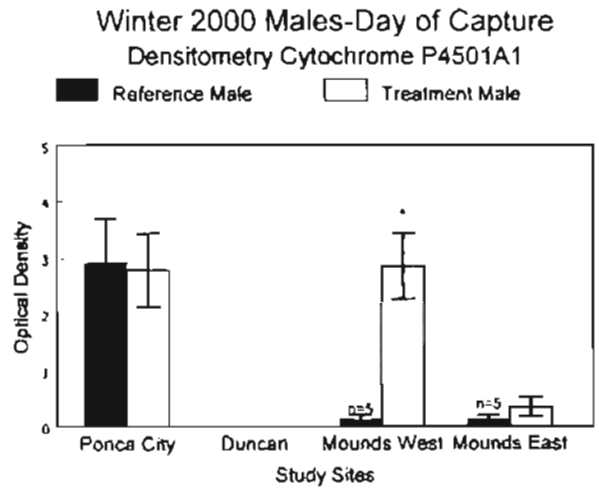
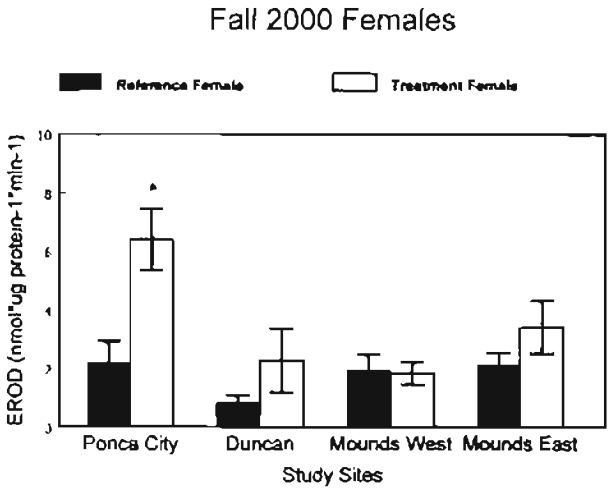
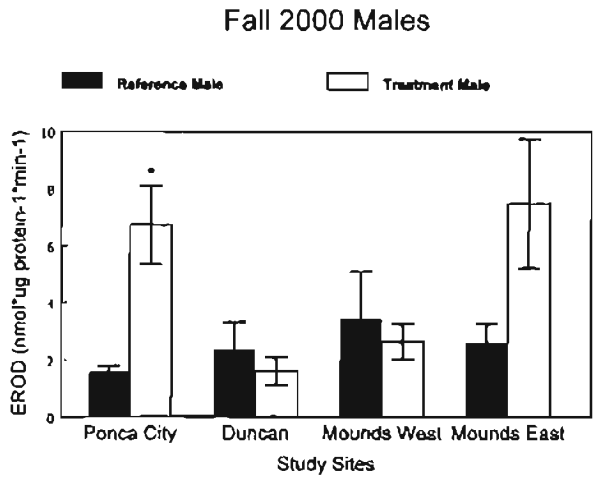
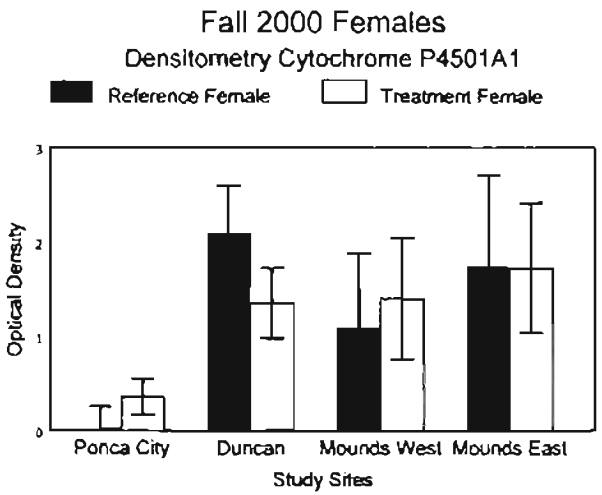

Winter 2000 Males-Day of Capture
Densitometry Cytochrome P4501A1
Reference Male
Treatment Male
Optical Density
0
1
2
3
4
5
n=5
n=5
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

Figure 4. Mean (± SEM) O-dealkylation of ethoxyresorufin and optical density of cytochrome P4501A1 immunoreactive bands of female and male cotton rats caught in fall 2000. Asterisks indicate a significant elevation of the treatment enzyme activity over the reference enzyme activity within one site ($p<0.05$). n=6, except where noted in the graphs.


Fall 2000 Females
Reference Female
Treatment Female
EROD (nmol*ug protein-1*min-1)
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Fall 2000 Males
Reference Male
Treatment Male
EROD (nmol*ug protein-1*min-1)
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Fall 2000 Females
Densitometry Cytochrome P4501A1
Reference Female
Treatment Female
Optical Density
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

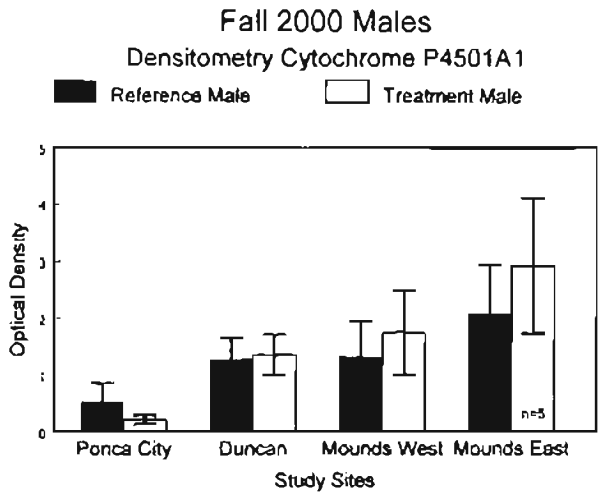
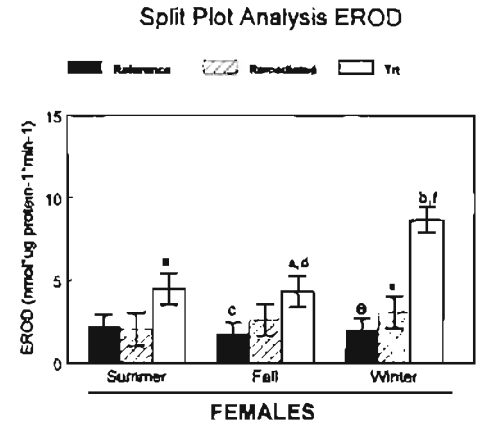
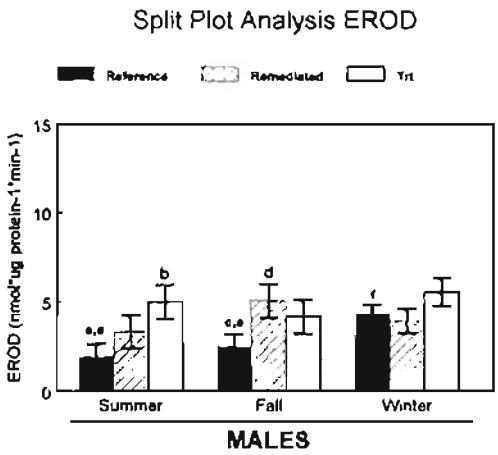
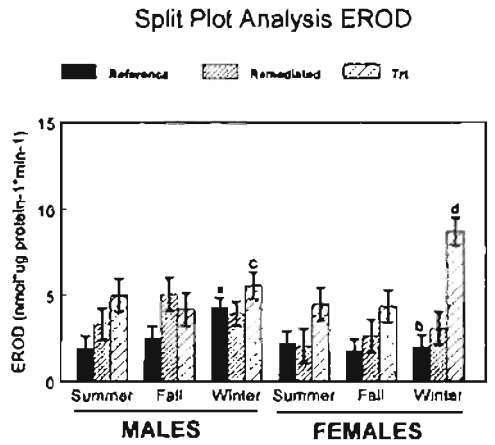

Fall 2000 Males
Densitometry Cytochrome P4501A1
Reference Male
Treatment Male
Optical Density
n=5
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

Figure 5. Split plot analysis. Mean (± SE) O-dealkylation of ethoxyresorufin of female and male cotton rats. Within each graph, ascending letters indicate significant differences ($p<0.05$). Letters a & b, c & d and e & f indicate significant difference.


Split Plot Analysis EROD
Reference
Remediated
Trt
EROD (nmol*ug protein-1*min-1)
15
10
5
0
Summer
Fall
Winter
FEMALES


Split Plot Analysis EROD
Reference
Remediated
Trt
EROD (nmol*ug protein-1*min-1)
15
10
5
0
Summer
Fall
Winter
MALES


Split Plot Analysis EROD
Reference
Remediated
Trt
EROD (nmol*ug protein-1*min-1)
15
10
5
0
Summer
Fall
Winter
Summer
Fall
Winter
MALES
FEMALES

Split plot analysis was used to determine if there were any patterns in EROD activity between winter 2000 animals sacrificed on the day of capture and those held up to 48 hours before termination (Figure 6). Male and female cotton rats collected from the landfarm site and matched reference site at Ponca City were analyzed together as the Ponca City sites were the only ones where sufficient female animals were collected. A history-time of termination interaction was found ($p=0.0019$). There was no difference found between animals collected from the reference site at Ponca City sacrificed at either time point (day of capture or 48 hours after capture). The animals collected from the treatment site at Ponca City had a significant decline ($p<0.0001$) in EROD activity between animals sacrificed on the day of capture and those sacrificed 48 hours later. An analysis was conducted on EROD activities of male rats collected from the landfarm site and matched reference site at Mounds East and West. A time of termination effect was found ($p<0.0001$) for EROD activities of male rats collected at both sites at Mounds East and West. Both the reference and landfarm sites at Mounds East and West had a significant decline in activity between the EROD activity from animals terminated on the day of capture and those sacrifice up to 48 hours later. An analysis was conducted on EROD activities of male rats collected from the landfarm site and matched reference site at Mounds East, Mounds West and Ponca City. A history-time of termination interaction was found ($p=0.0191$). For the reference sites, a significant decline ($p=0.0207$) in activity between the EROD activity from animals terminated on the day of capture and those sacrificed up to 48 hours later was found. A decline

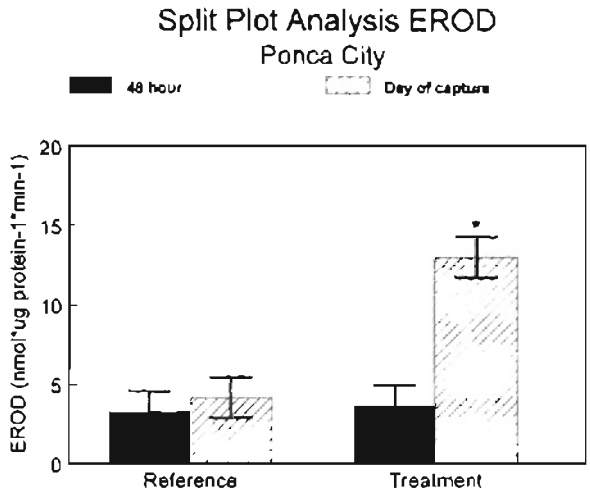
($p<0.0001$) was also found in the EROD activity between animals from the treatment site terminated on the day of capture and those sacrifice up to 48 hours later. No difference in activity due to time delay was found between the males collected from the remediated sites (Figure 6).

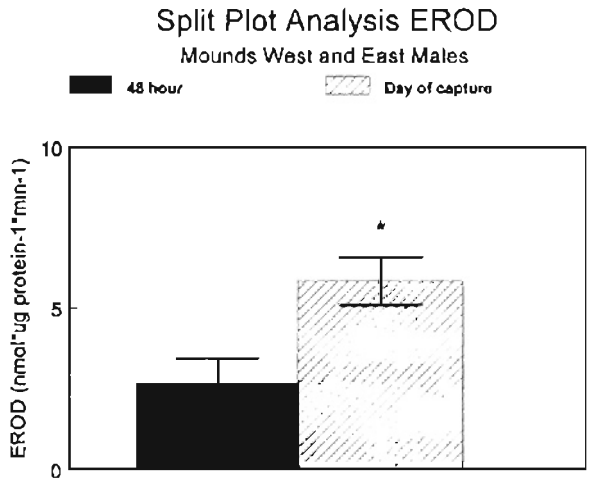
MROD and CYP1A2

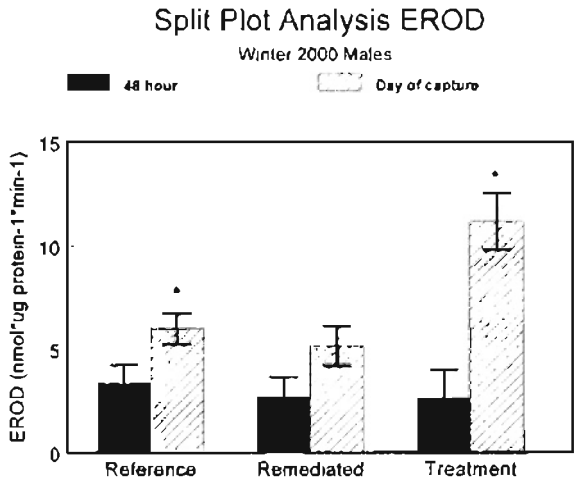
There were no significant differences between sites in MROD acitivity in the females collected in the summer of 1999 (Figure 7). Within the summer males, Ponca City and Mounds West animals from the landfarm sites had a significant elevation of MROD activity ($p=0.0431$, $p=0.0034$, respectively) when compared to males from corresponding reference sites. Males from the landfarm site in Duncan had a nonsignificant elevation of MROD activity ($p=0.0524$) when compared to animals from the corresponding reference site. No significant differences were found in the CYP1A2 levels for either males or females (Figure 7).

In the winter of 2000, a significant elevation of MROD activity ($p=0.0007$) was detected in females from the landfarm site in Ponca City when compared to animals from the matched reference site (Figure 8). A significantly lower MROD activity ($p=0.0379$) was found in the males from the landfarm site in Mounds West when compared to the males from the corresponding reference site. Significant elevation ($p=0.0047$, $p<0.0001$) was detected in CYP1A2 levels in both males and females from the landfarm site in Ponca City when compared to animals from the matched reference site. The CYP1A2 levels in males from the landfarm site was significantly greater ($p=0.0447$) than that of animals from the

Figure 6. Split plot analysis. Mean (± SE) O-dealkylation of ethoxyresorufin of winter 2000 rats sacrificed on the day of capture and those sacrificed 48 hours later (female and male cotton rats collected from the landfarm site and matched reference site in Ponca City, male cotton rats collected from the landfarm site and matched reference sites in Mounds West and Mounds East and male cotton rats collected from the landfarm site and matched reference sites in Mounds West, Mounds East and Ponca City). Within each graph, asterisks indicate significant differences ($p<0.05$).


Split Plot Analysis EROD
Ponca City
48 hour
Day of capture
EROD (nmol*ug protein-1*min-1)
20
15
10
5
0
Reference
Treatment


Split Plot Analysis EROD
Mounds West and East Males
48 hour
Day of capture
EROD (nmol*ug protein-1*min-1)
10
5
0

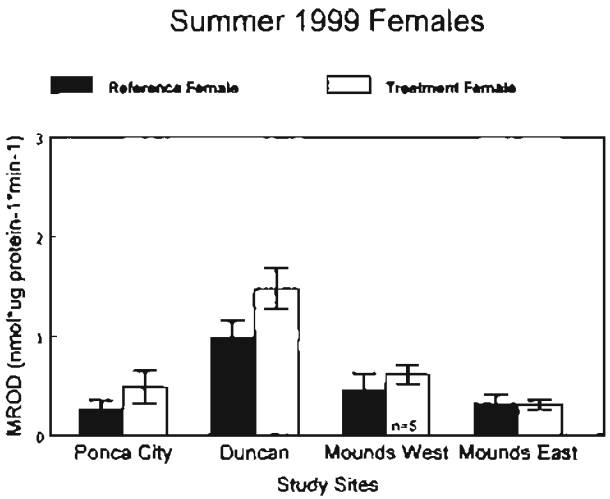

Split Plot Analysis EROD
Winter 2000 Males
48 hour
Day of capture
EROD (nmol*ug protein-1*min-1)
15
10
5
0
Reference
Remediated
Treatment

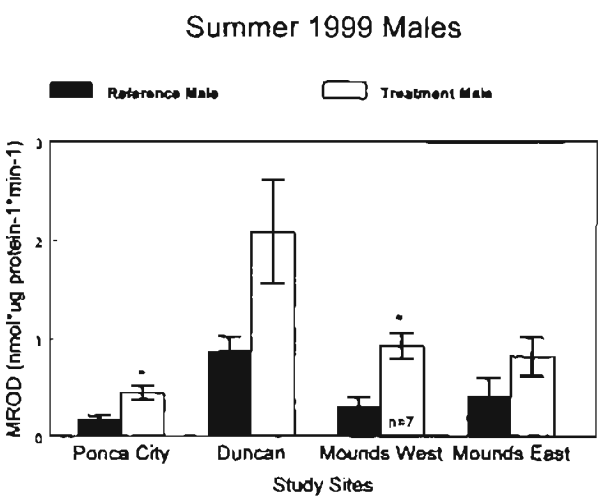
corresponding reference site in Mounds East. As only one reference female was caught in Duncan no statistical test could be performed. No other differences were found in the other sites (Figure 8).

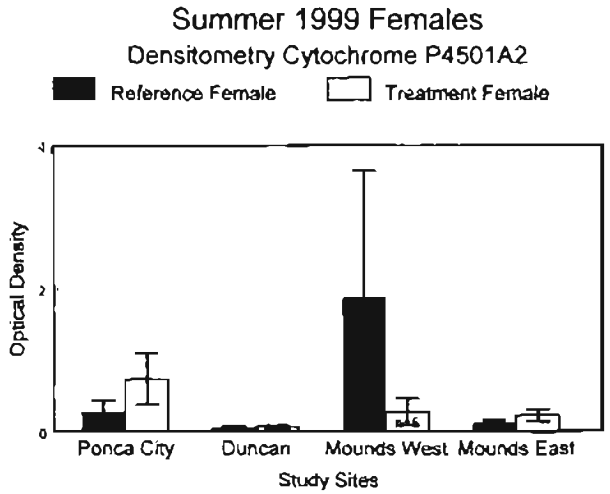
Insufficient animals were captured in Duncan to allow a day of capture termination in the winter of 2000 (Figure 9). In Mounds West and East inadequate numbers of females were caught to allow a day of capture termination. Ponca City females and males from the landfarm site had significantly elevated MROD activities ($p=0.0071$, $p=0.027$ respectively) and CYP1A2 levels ($p=0.0164$, $p=0.0111$) when compared to animals from the reference site. No differences were detected in MROD activity and CYP1A2 levels in males from Mounds West and East sacrificed on the day of capture in the winter of 2000 (Figure 9).

Within females caught in fall 2000, only animals caught in the landfarm site in Ponca City showed induction of MROD activity ($p=0.0003$) when compared to the females from the corresponding reference site (Figure 10). Males from the landfarm sites in both Ponca City and Mounds East showed an elevation of MROD activity ($p=0.002$, $p=0.0433$ respectively) when compared to the males from the matching reference sites. Duncan and Mounds West males showed no differences in MROD activity. Ponca City males collected from the landfarm sites were the only animals, male or female, that showed a significant induction of CYP1A2 levels ($p=0.0169$) when compared to animals from the corresponding reference sites (Figure 10).

Figure 7. Mean (± SEM) O-dealkylation of methoxyresorufin and optical density of cytochrome P4501A2 immunoreactive bands of female and male cotton rats caught in summer 1999. Asterisks indicate a significant elevation of the treatment enzyme activity over the reference enzyme activity within one site ($p<0.05$). n=6, except where noted in the graphs.


Summer 1999 Females
Reference Female
Treatment Female
MROD (nmol*ug protein-1*min-1)
0
1
2
3
n=5
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Summer 1999 Males
Reference Male
Treatment Male
MROD (nmol*ug protein-1*min-1)
0
1
2
3
n=7
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Summer 1999 Females
Densitometry Cytochrome P4501A2
Reference Female
Treatment Female
Optical Density
0
2
4
n=6
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

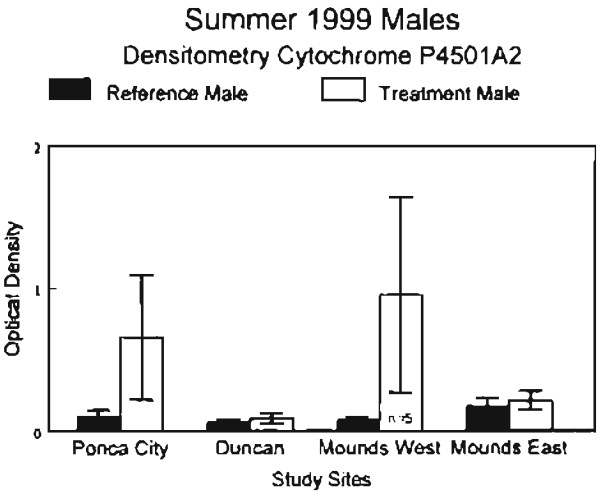
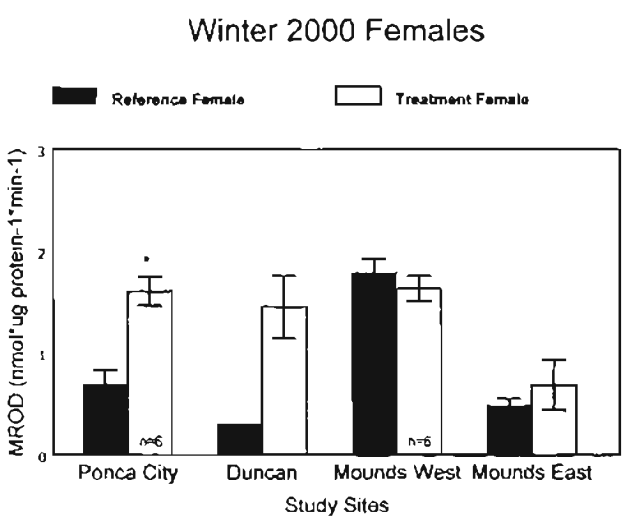
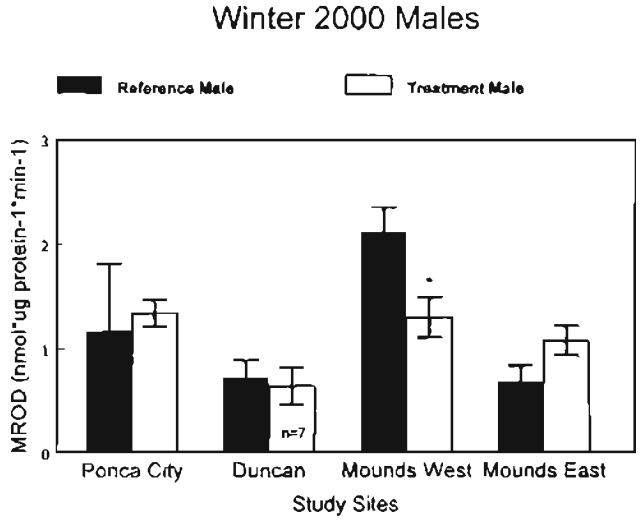
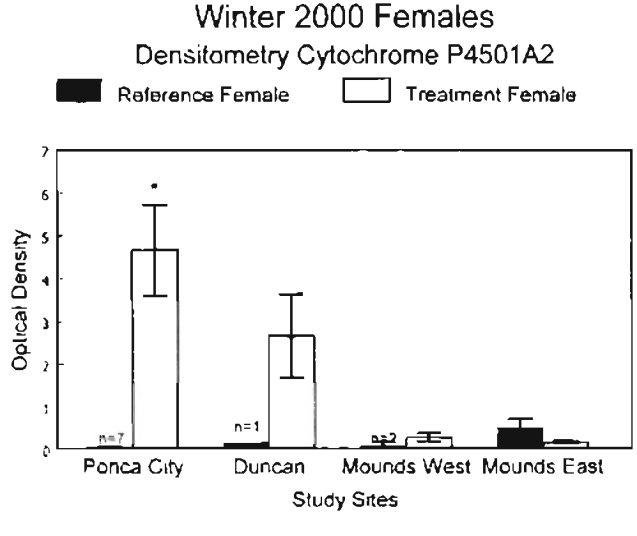

Summer 1999 Males
Densitometry Cytochrome P4501A2
Reference Male
Treatment Male
Optical Density
0
1
2
n=5
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

Figure 8. Mean (± SEM) O-dealkylation of methoxyresorufin and optical density of cytochrome P4501A2 immunoreactive bands of female and male cotton rats caught in winter 2000. Asterisks indicate a significant elevation of the treatment enzyme activity over the reference enzyme activity within one site ($p<0.05$). n=6, except where noted in the graphs.


Winter 2000 Females
Reference Female
Treatment Female
MROD (nmol*ug protein-1*min-1)
n=6
n=6
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Winter 2000 Males
Reference Male
Treatment Male
MROD (nmol*ug protein-1*min-1)
n=7
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Winter 2000 Females
Densitometry Cytochrome P4501A2
Reference Female
Treatment Female
Optical Density
n=7
n=1
n=2
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

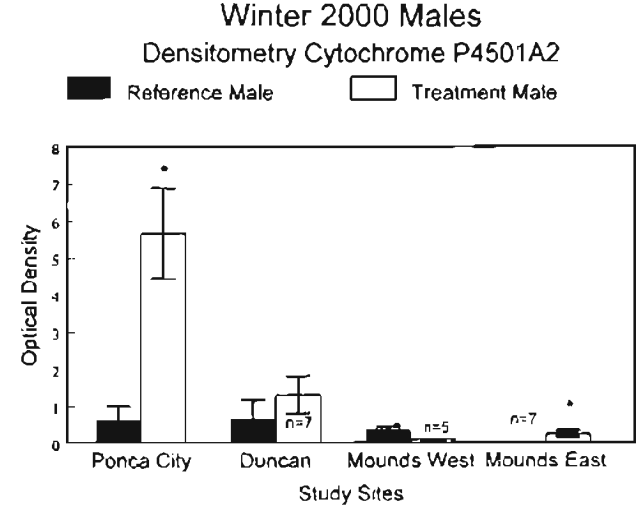
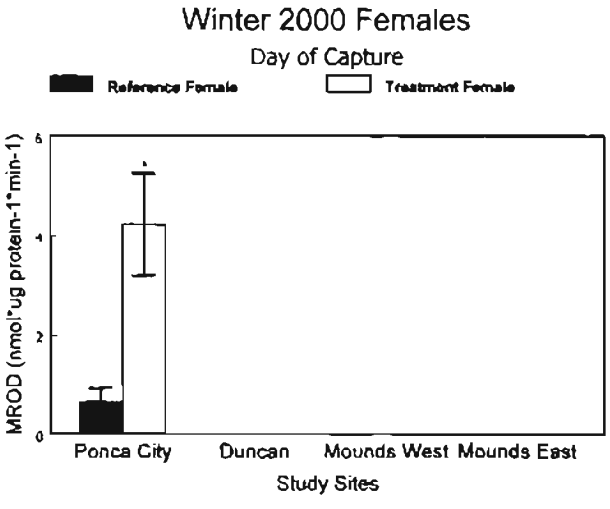
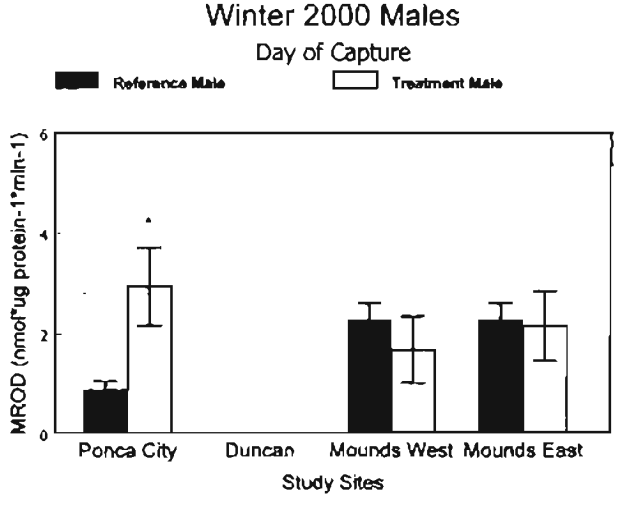
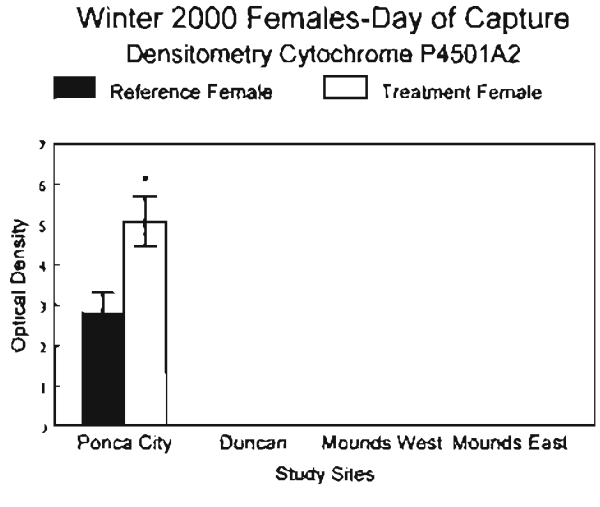

Winter 2000 Males
Densitometry Cytochrome P4501A2
Reference Male
Treatment Male
Optical Density
n=7
n=5
n=7
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

Figure 9. Mean (± SEM) O-dealkylation of methoxyresorufin and optical density of cytochrome P4501A2 immunoreactive bands of female and male cotton rats caught in winter 2000 and sacrificed on the day of capture. Asterisks indicate a significant elevation of the treatment enzyme activity over the reference enzyme activity within one site ($p<0.05$). n=6, except where noted in the graphs.


Winter 2000 Females
Day of Capture
Reference Female
Treatment Female
MROD (nmol*ug protein-1*min-1)
0
2
4
6
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Winter 2000 Males
Day of Capture
Reference Male
Treatment Male
MROD (nmol*ug protein-1*min-1)
0
2
4
6
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Winter 2000 Females-Day of Capture
Densitometry Cytochrome P4501A2
Reference Female
Treatment Female
Optical Density
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

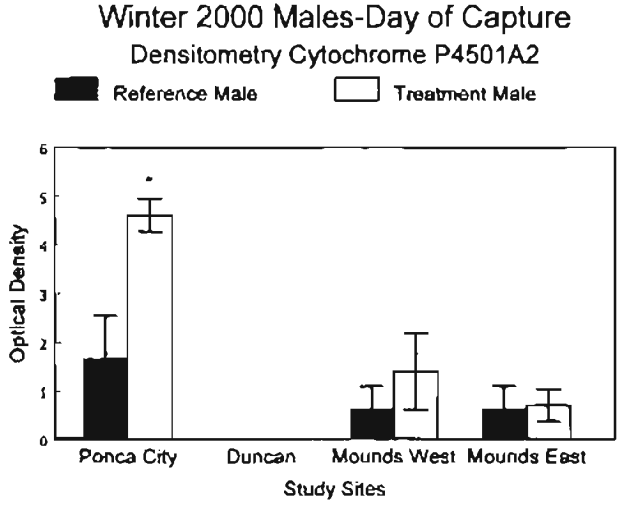
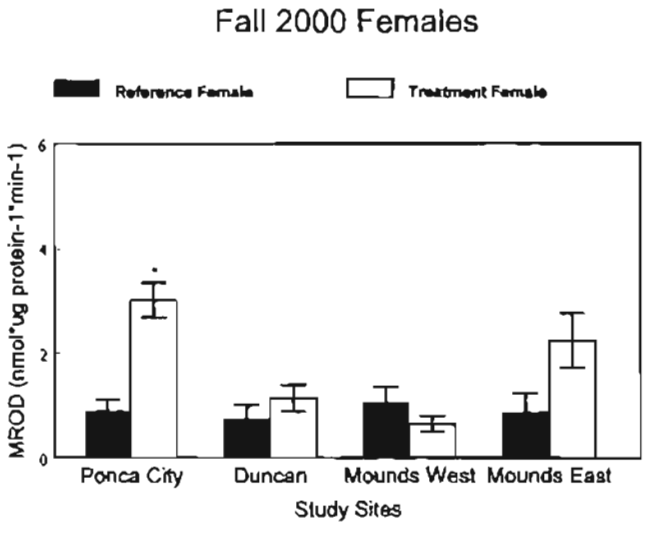
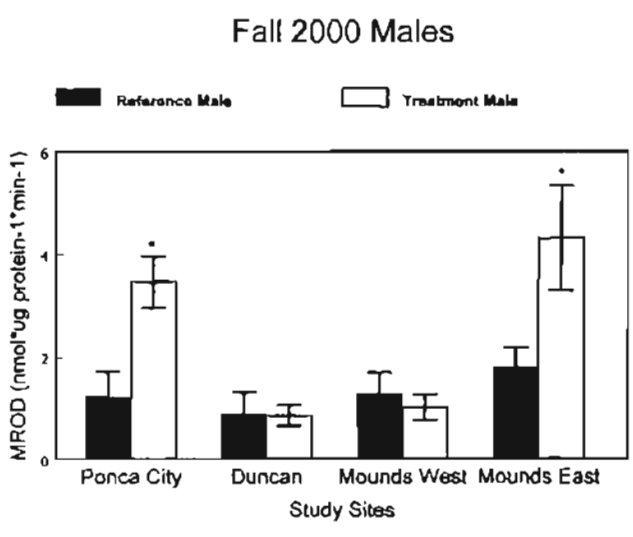
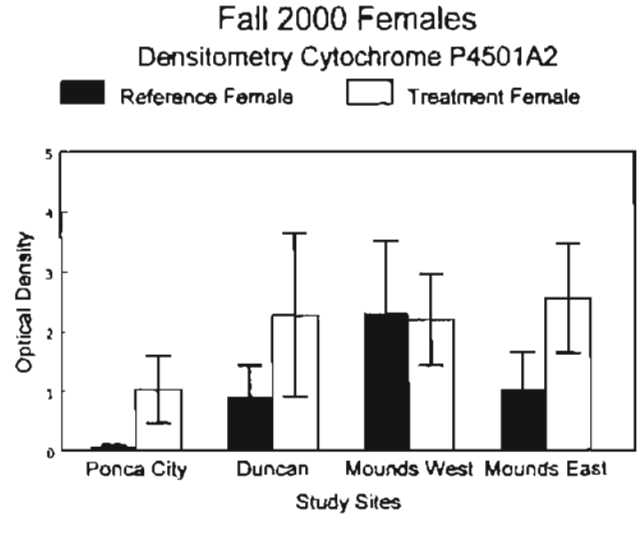
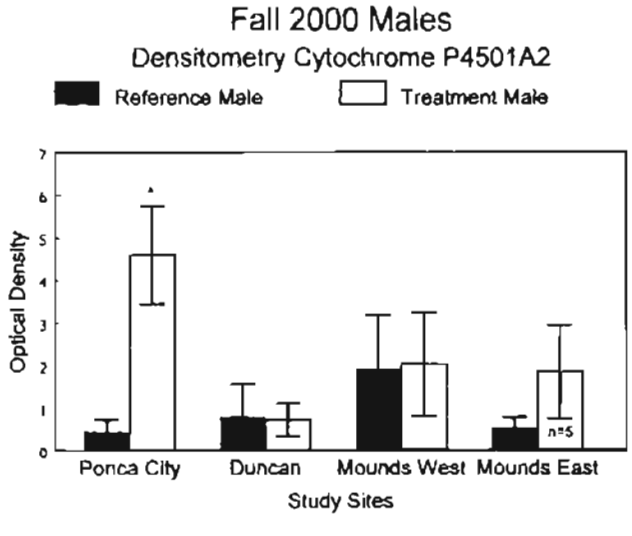

Winter 2000 Males-Day of Capture
Densitometry Cytochrome P4501A2
Reference Male
Treatment Male
Optical Density
0
1
2
3
4
5
6
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

Figure 10. Mean (± SEM) O-dealkylation of methoxyresorufin and optical density of cytochrome P4501A2 immunoreactive bands of female and male cotton rats caught in fall 2000. Asterisks indicate a significant elevation of the treatment enzyme activity over the reference enzyme activity within one site ($p<0.05$). n=6, except where noted in the graphs.


Fall 2000 Females
Reference Female
Treatment Female
MROD (nmol*ug protein-1*min-1)
0
2
4
6
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Fall 2000 Males
Reference Male
Treatment Male
MROD (nmol*ug protein-1*min-1)
0
2
4
6
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Fall 2000 Females
Densitometry Cytochrome P4501A2
Reference Female
Treatment Female
Optical Density
0
1
2
3
4
5
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Fall 2000 Males
Densitometry Cytochrome P4501A2
Reference Male
Treatment Male
Optical Density
0
1
2
3
4
5
6
7
n=5
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

A split plot analysis was used to analyze MROD activity across all seasons, site histories, and both sexes (Figure 11). An interaction was found between history and sex ($p=0.0504$). Male animals from remediated sites were found to have significantly higher MROD activity ($p=0.0054$) than females from the same sites. Female treatment rats were determined to have significant induction of MROD activity ($p=0.0232$) when compared to reference females (Figure 11).

Split plot analysis was used to determine if there were any patterns in MROD activity between winter 2000 animals sacrificed on the day of capture and those held up to 48 hours before termination (Figure 12). Male and female cotton rats collected from the landfarm site and matched reference site at Ponca City were analyzed together as the Ponca City sites were the only ones where sufficient female animals were collected. A history-time of termination interaction was found ($p=0.0032$). There was no difference found between animals collected from the reference site at Ponca City sacrificed at either time point (day of capture or 48 hours after capture). The animals collected from the treatment site at Ponca City exhibited a significant decline ($p=0.0002$) in MROD activity between animals sacrificed on the day of capture and those sacrificed 48 hours later. An analysis was conducted on MROD activities of male rats collected from the landfarm site and matched reference site at Mounds East and West. A time of termination effect was found ($p=0.0017$). Both the reference and landfarm sites at Mounds East and West had a significant decline in activity between the MROD activity from animals terminated on the day of capture and those

sacrificed up to 48 hours later. An analysis was conducted on MROD activities of male rats collected from the landfarm site and matched reference site at Mounds East, Mounds West and Ponca City. A time of termination effect was found ($p=0.0008$). The MROD activity declined between the males terminated on the day of capture and those sacrificed up to 48 hours later (Figure 12).

PROD

Of all cotton rats capture in summer 1999, only females collected from the landfarm site in Duncan had elevated PROD activity ($p=0.0299$) when compared to females from its reference site (Figure 13). In winter 2000 female rats from the landfarm site at Ponca City showed induction of PROD activity ($p=0.0039$) when compared to reference animals. Winter 2000 males collected from the landfarm site in Mounds West had significantly lower PROD activity ($p=0.0389$) when compared to reference males. No other differences were found in PROD activities in animals collected in the winter of 2000 (Figure 13).

Induction of PROD activity ($p=0.0468$) was found in females from the landfarm site in Ponca City when compared to animals from the reference site (Figure 14). No differences were found in the males sacrificed on the day of capture in the winter of 2000 collection. In fall 2000, males and females captured at the landfarm site at Ponca City showed an induction of PROD activity ($p=0.0075$, $p=0.0031$ respectively) when compared to the reference site animals. No other differences were detected in the fall of 2000 animals (Figure 14).

Split plot analysis was used to compare PROD activities across all seasons, site histories, and both sexes (Figure 15). An interaction was found

Figure 11. Split plot analysis. Mean (± SE) O-dealkylation of methoxyresorufin of female and male cotton rats. Within each graph, ascending letters indicate significant differences ($p<0.05$). Letters a & b and c & d indicate significant difference.

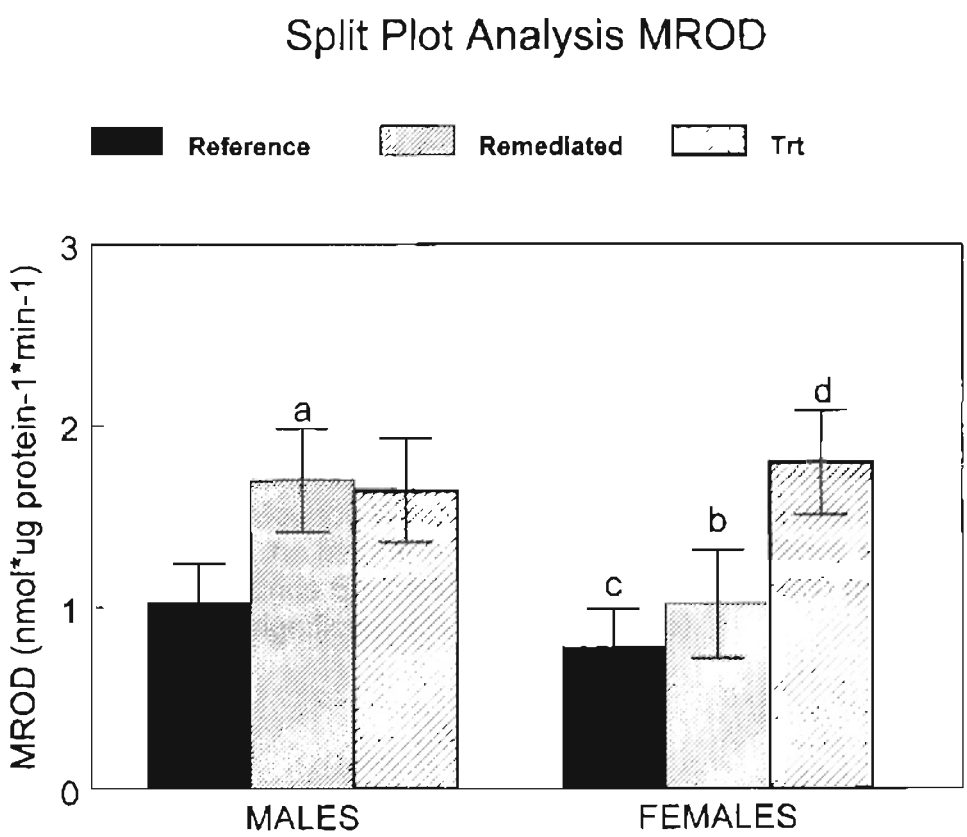
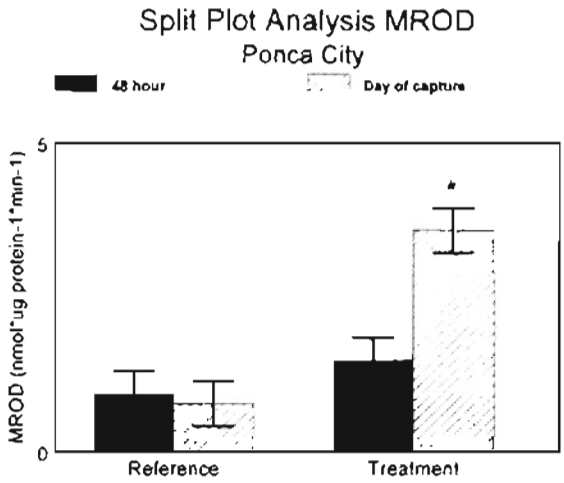
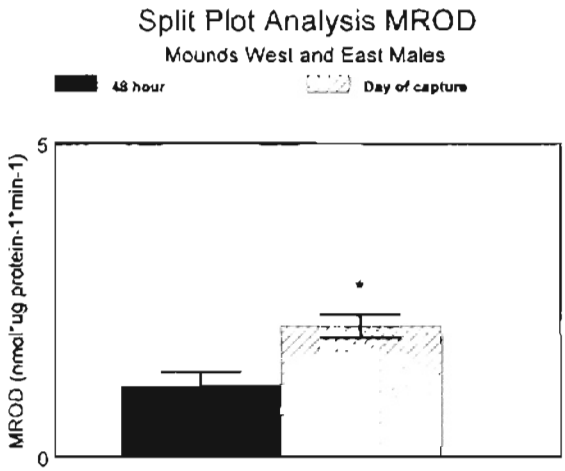

Split Plot Analysis MROD
Reference
Remediated
Trt
MROD (nmol*ug protein-1*min-1)
3
2
1
0
a
b
c
d
MALES
FEMALES

Figure 12. Split plot analysis. Mean (± SE) O-dealkylation of methoxyresorufin of winter 2000 rats sacrificed on the day of capture and 48 hours later (female and male cotton rats collected from the landfarm site and matched reference site in Ponca City, male cotton rats collected from the landfarm site and matched reference sites in Mounds West and Mounds East and male cotton rats collected from the landfarm site and matched reference sites in Mounds West, Mounds East and Ponca City). Within each graph, asterisks indicate significant differences ($p<0.05$).


Split Plot Analysis MROD
Ponca City
48 hour
Day of capture
MROD (nmol*ug protein-1*min-1)
5
0
*
Reference
Treatment


Split Plot Analysis MROD
Mounds West and East Males
48 hour
Day of capture
MROD (nmol*ug protein-1*min-1)
5
0
*

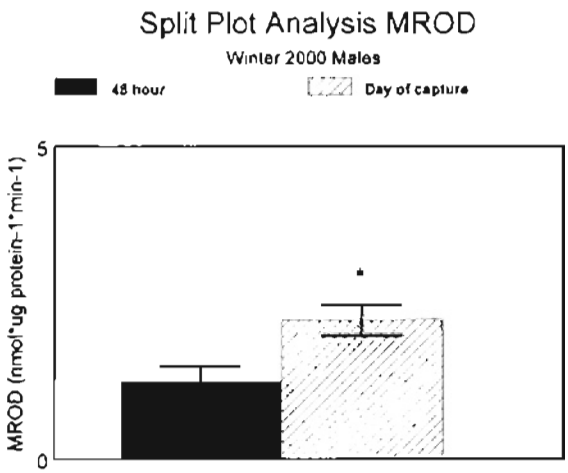
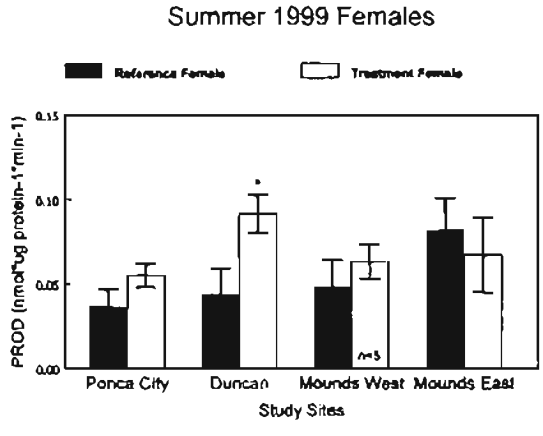
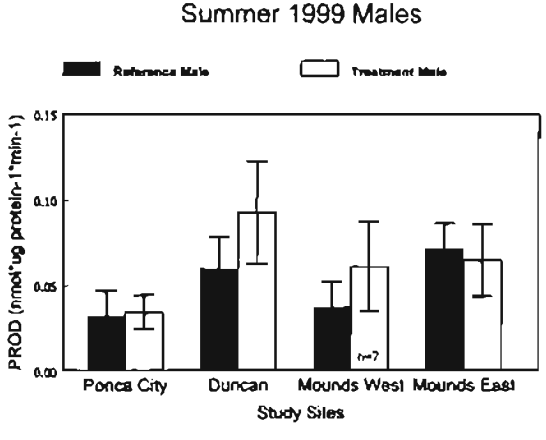
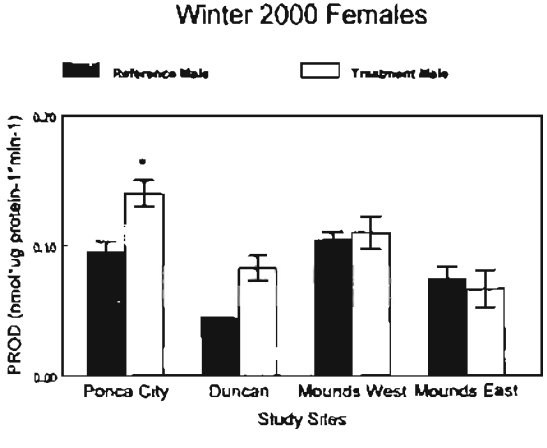

Split Plot Analysis MROD
Winter 2000 Males
48 hour
Day of capture
MROD (nmol*ug protein-1*min-1)
5
0
*

Figure 13. Mean (± SEM) O-dealkylation of pentoxyresorufin of female and male cotton rats caught in summer 1999 and winter of 2000. Asterisks indicate a significant difference in the treatment enzyme activity when compared to over the reference enzyme activity within one site ($p<0.05$). n=6, except where noted in the graphs.


Summer 1999 Females
Reference Female
Treatment Female
PROD (nmol*ug protein-1*min-1)
0.15
0.10
0.05
0.00
n=5
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Summer 1999 Males
Reference Male
Treatment Male
PROD (nmol*ug protein-1*min-1)
0.15
0.10
0.05
0.00
n=7
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Winter 2000 Females
Reference Male
Treatment Male
PROD (nmol*ug protein-1*min-1)
0.20
0.15
0.00
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

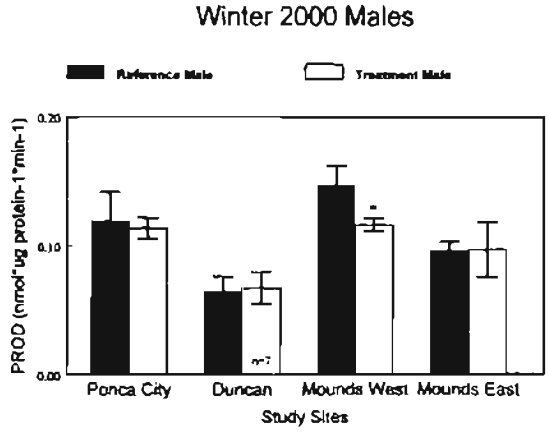
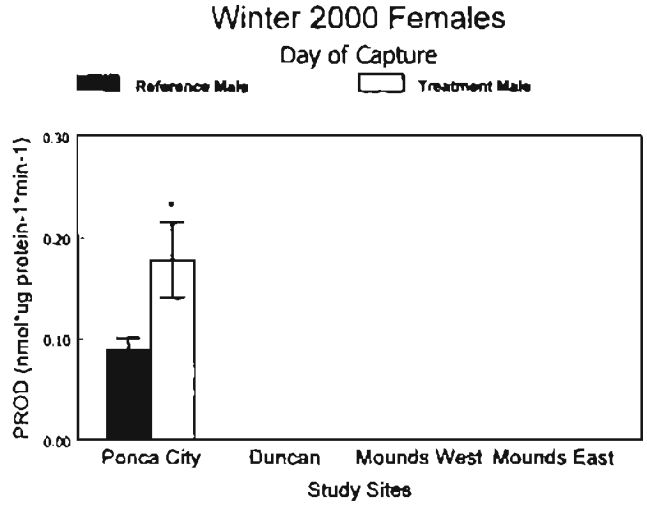
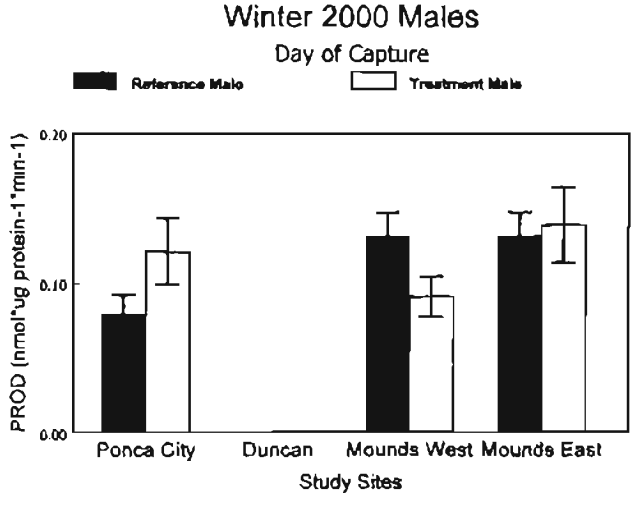
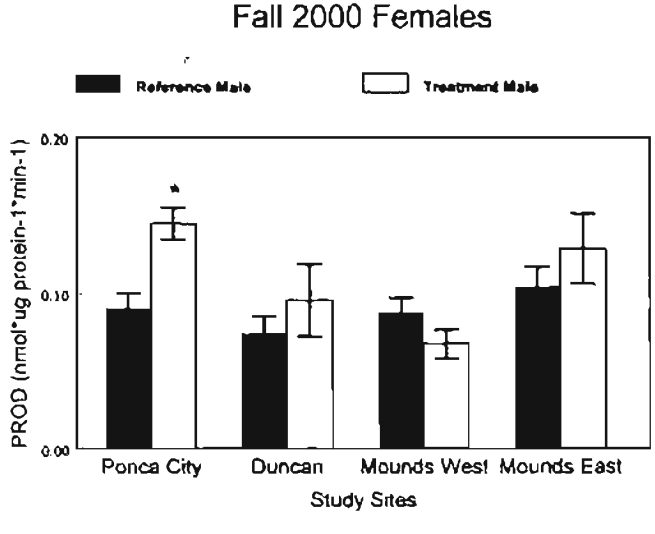
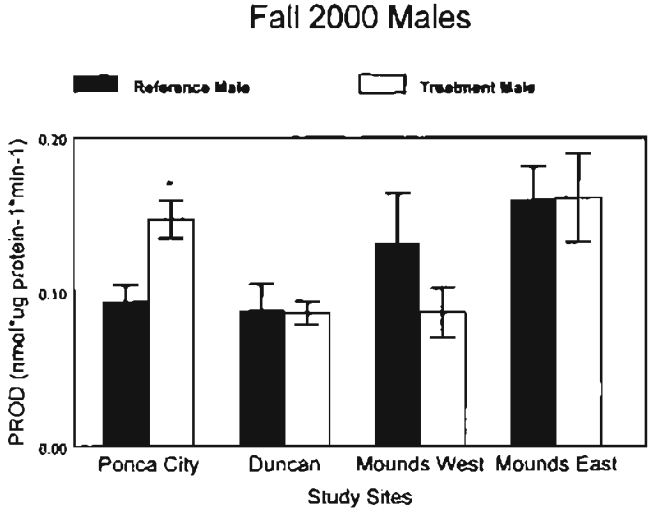

Winter 2000 Males
Reference Male
Treatment Male
PROD (nmol*ug protein-1*min-1)
0.20
0.10
0.00
n=7
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

Figure 14. Mean (± SEM) O-dealkylation of pentoxyresorufin of female and male cotton rats caught in winter 2000 and fall 2000 that were sacrificed on the day of capture. Asterisks indicate a significant elevation of the treatment enzyme activity over the reference enzyme activity within one site ($p<0.05$). n=6, except where noted in the graphs.


Winter 2000 Females
Day of Capture
Reference Male
Treatment Male
PROD (nmol*ug protein-1*min-1)
0.30
0.20
0.10
0.00
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Winter 2000 Males
Day of Capture
Reference Male
Treatment Male
PROD (nmol*ug protein-1*min-1)
0.20
0.10
0.00
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Fall 2000 Females
Reference Male
Treatment Male
PROD (nmol*ug protein-1*min-1)
0.20
0.10
0.00
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Fall 2000 Males
Reference Male
Treatment Male
PROD (nmol*ug protein-1*min-1)
0.20
0.10
0.00
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

between site history and sex (p=0.0317). Landfarmed sites in Ponca City and Duncan were classified as treatment sites. The landfarm sites in Mounds West and East were classified as remediated. No differences were detected in the males. Treatment females were found to have higher PROD activities (p=0.017, p=0.0313 respectively) when compared to reference and remediated females.

Split plot analysis was used to determine if there were any patterns in PROD activity between winter 2000 animals sacrificed on the day of capture and those held up to 48 hours before termination. Male and female cotton rats collected from the landfarm site and matched reference site at Ponca City were analyzed together as the Ponca City sites were the only ones where sufficient female animals were collected. An analysis was done of PROD activities of male rats collected from the landfarm site and matched reference site at Mounds East and West. An analysis was also done of PROD activities of male rats collected from the landfarm site and matched reference site at Mounds East, Mounds West and Ponca City. No differences in PROD activity were observed from the time of termination in any of these analyses.

Histology

No differences were detected in acellularity, picnotic nuclei, cloudy swelling, immune cell clumps or cell size in males and females captured from either Ponca City or Duncan in the Summer of 1998 (Figure 16).

Figure 15. Split plot analysis. Mean (± SE) O-dealkylation of pentoxyresorufin of female and male cotton rats. Within each graph, ascending letters indicate significant differences ($p<0.05$). Letters a & b indicate significant difference.

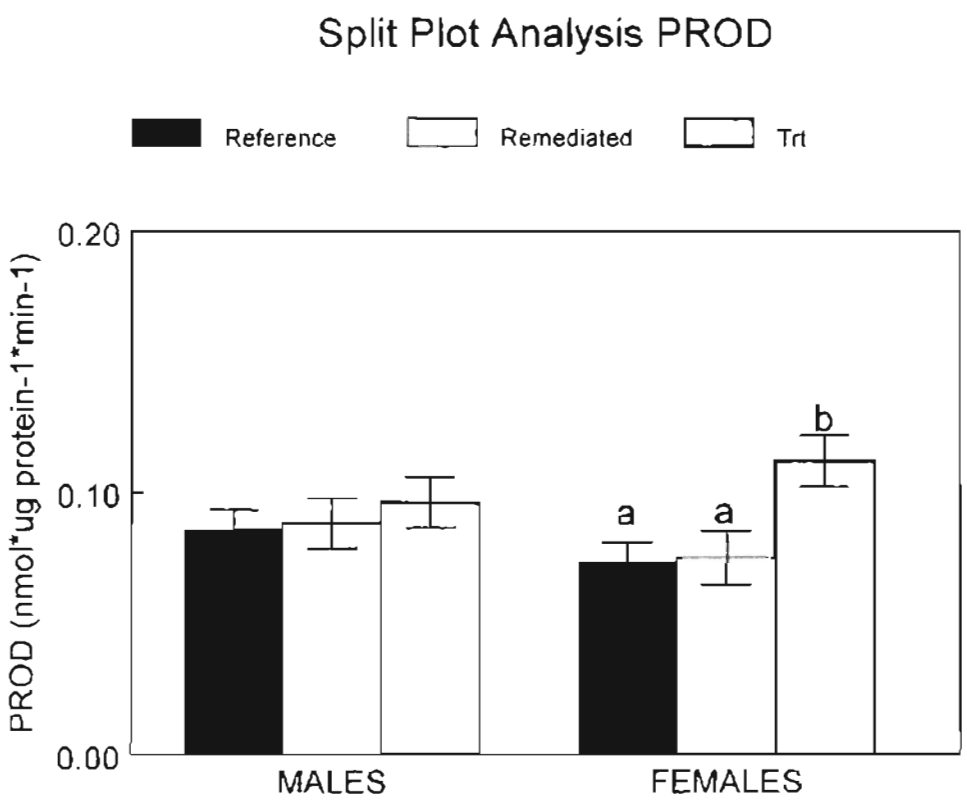
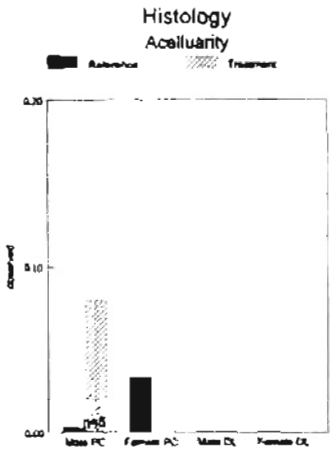
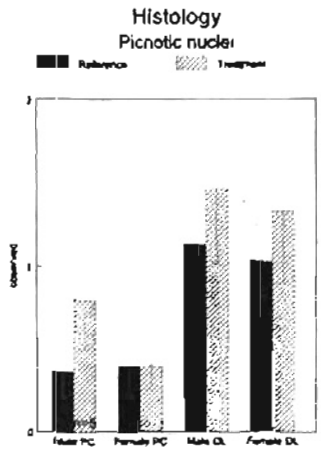
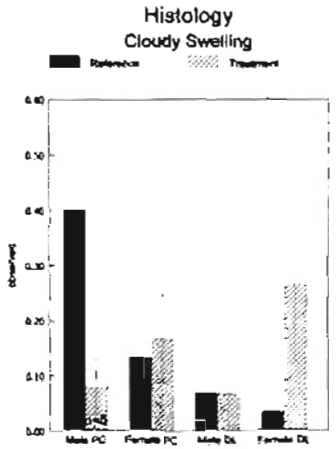
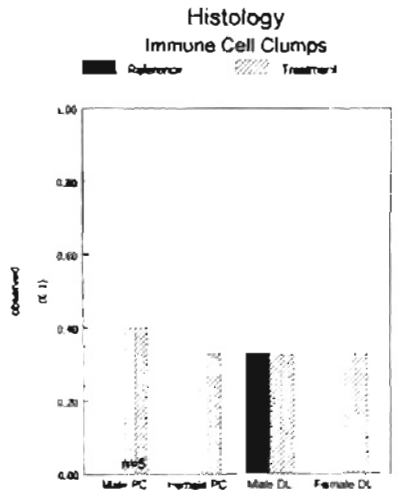

Split Plot Analysis PROD
Reference
Remediated
Trt
PROD (nmol*ug protein-1*min-1)
0.20
0.10
0.00
a
a
b
MALES
FEMALES

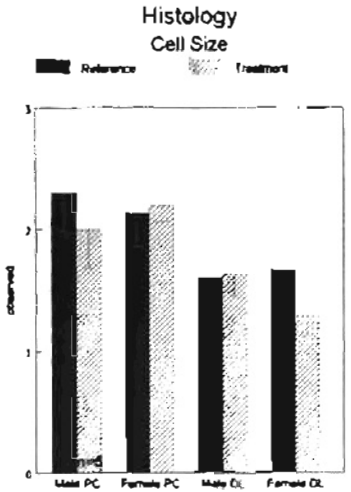
Figure 16. Mean (± SEM) observations of histological features of the livers of female and male cotton rats caught in summer 1998. n=6, except where noted in the graphs.


Histology
Acellularity
Reference
Treatment
observed
0.00
0.10
Male PC
Female PC
Male DL
Female DL


Histology
Picnotic nuclei
Reference
Treatment
observed
0
1
Male PC
Female PC
Male DL
Female DL


Histology
Cloudy Swelling
Reference
Treatment
observed
0.00
0.10
0.20
0.30
0.40
0.50
Male PC
Female PC
Male DL
Female DL


Histology
Immune Cell Clumps
Reference
Treatment
observed
0.00
0.20
0.40
0.60
0.80
1.00
Male PC
Female PC
Male DL
Female DL


Histology
Cell Size
Reference
Treatment
observed
0
1
2
3
Male PC
Female PC
Male DL
Female DL

Glutathione S-Transferase

No differences were detected in the glutathione s-transferase activity in females or males in the summer of 1999 or winter of 2000 collections terminated 48 hours after capture (Figure 17).

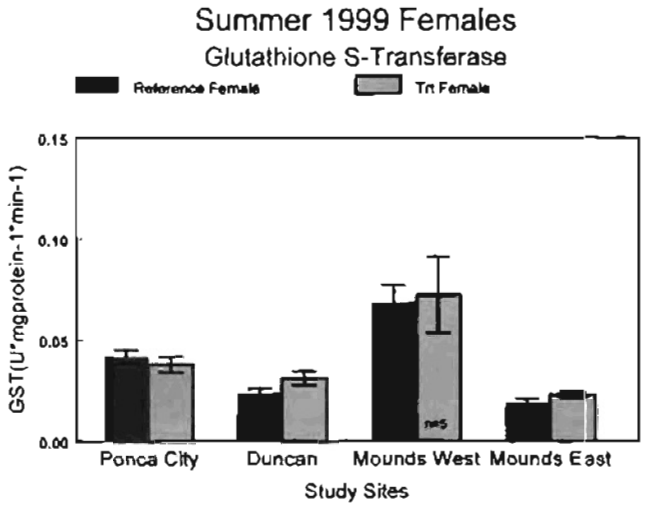
In sites where sufficient numbers of animals were collected, no differences in glutathione s-transferase activity were detected in winter 2000 rats sacrificed on the day of capture (Figure 18). In the fall 2000 collection, females collected from the landfarm site in Ponca City had greater glutathione s-transferase activity ($p=0.0043$) when compared to reference animals. Males from the landfarm site in Mounds West had a detectable decline in glutathione s-transferase activity ($p=0.013$) compared to reference animals in the fall. No other differences were detected in the fall of 2000 glutathione s-transferase activity (Figure 18).

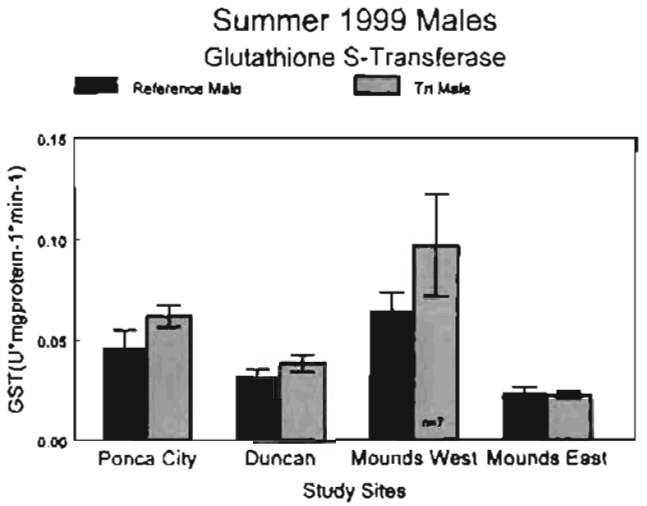
Split plot analysis of glutathione s-transferase activity across all seasons, site histories, and both sexes was performed (Figure 19). Season and sex ($p=0.0135$) were found to be interactive factors, as were history and season ($p=0.027$). In the summer of 1999, animals from remediated landfarm sites (Mounds West and East) were found to have higher glutathione s-transferase activity ($p=0.0037$, $p=0.0315$ respectively) when compared to animals from reference and treatment sites. Animals collected from the remediated sites had higher glutathione s-transferase activity ($p<0.0001$ and $p=0.0002$ respectively) in the summer and winter than in the fall. The reference and treatment sites had rats with higher enzyme activity ($p=0.0019$, $p= 0.0219$ respectively) in the winter than summer. The difference for higher activity ($p<0.0001$, $p=0.0006$

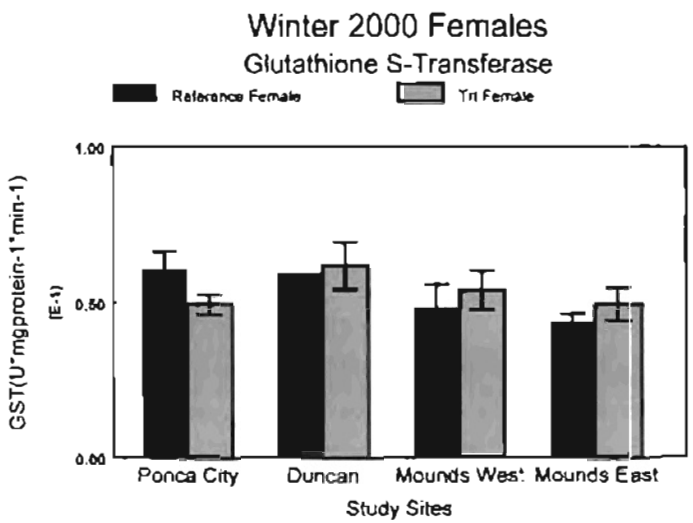
respectively) in the reference and treatment animals in the winter was also present when comparing to the fall. The GST activity levels of the rats collected from the remediated site were higher ($p<0.0001$, $p=0.0002$ respectively) in the summer and winter when compared to the animals in the fall. Female rats in the summer and winter had higher enzyme activity ($p=0.0252$, $p<0.0001$) than females in the fall. This relationship of summer and winter animals having higher activity ($p<0.0001$) than fall animals was also true for the males. In the summer, male rats were found to have higher glutathione s-transferase activity ($p=0.0066$) than the females (Figure 19).

Split plot analysis was used to determine if there were any patterns in glutathione s-transferase activity between winter 2000 animals sacrificed on the day of capture and those held up to 48 hours before termination. Male and female cotton rats collected from the landfarm site and matched reference site at Ponca City were analyzed together as the Ponca City sites were the only ones where sufficient female animals were collected. An analysis was done of glutathione s-transferase activities of male rats collected from the landfarm site and matched reference site at Mounds East and West. An analysis was also done of glutathione s-transferase activities of male rats collected from the landfarm site and matched reference site at Mounds East, Mounds West and Ponca City. No differences in glutathione s-transferase activity were found to result from the time of termination in any of these analyses.

Figure 17. Mean (± SEM) glutathione s-transferase activity of female and male cotton rats caught in summer 1999 and winter 2000. n=6, except where noted in the graphs.


Summer 1999 Females
Glutathione S-Transferase
Reference Female
Trt Female
GST(U*mgprotein-1*min-1)
0.15
0.10
0.05
0.00
n=5
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Summer 1999 Males
Glutathione S-Transferase
Reference Male
Trt Male
GST(U*mgprotein-1*min-1)
0.15
0.10
0.05
0.00
n=7
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Winter 2000 Females
Glutathione S-Transferase
Reference Female
Trt Female
GST(U*mgprotein-1*min-1)
(E-1)
1.00
0.50
0.00
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

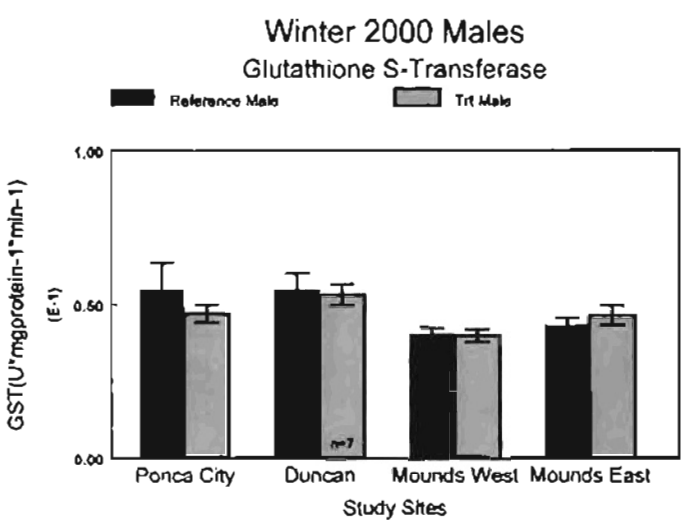
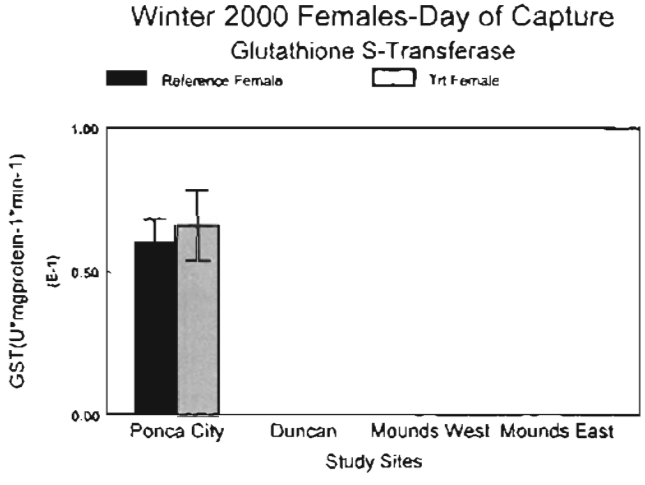
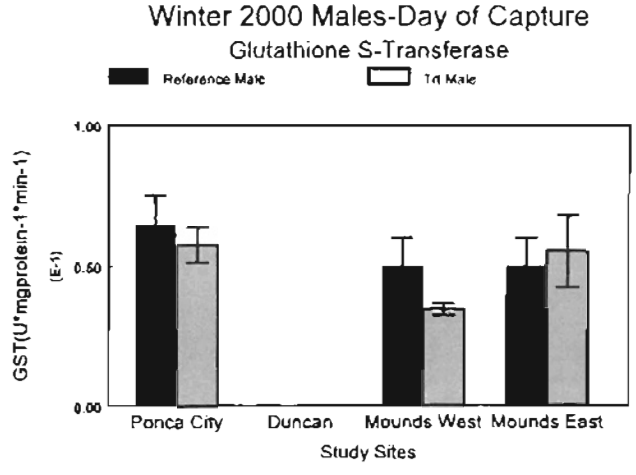
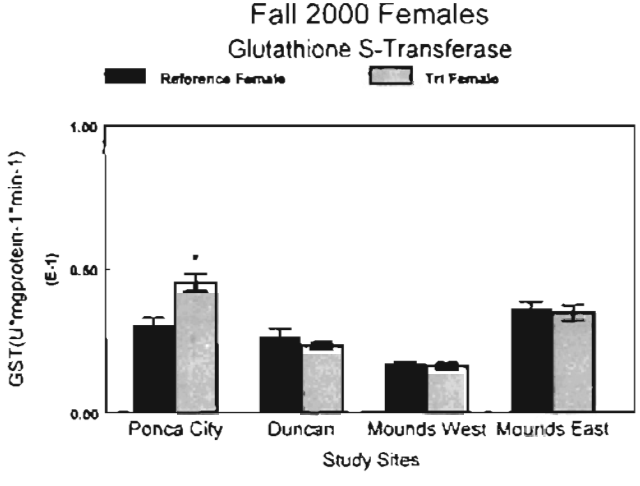

Winter 2000 Males
Glutathione S-Transferase
Reference Male
Trt Male
GST(U*mgprotein-1*min-1)
(E-1)
1.00
0.50
0.00
n=7
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

Figure 18. Mean (± SEM) glutathione s-transferase activity of female and male cotton rats caught in winter 2000 and fall 2000 that were sacrificed on the day of capture. Asterisks indicate a significant difference in the treatment enzyme activity compared to the reference enzyme activity within one site ($p<0.05$). n=6, except where noted in the graphs.


Winter 2000 Females-Day of Capture
Glutathione S-Transferase
Reference Female
Tri Female
GST(U*mgprotein-1*min-1)
(E-1)
1.00
0.50
0.00
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Winter 2000 Males-Day of Capture
Glutathione S-Transferase
Reference Male
Tri Male
GST(U*mgprotein-1*min-1)
(E-1)
1.00
0.50
0.00
Ponca City
Duncan
Mounds West
Mounds East
Study Sites


Fall 2000 Females
Glutathione S-Transferase
Reference Female
Tri Female
GST(U*mgprotein-1*min-1)
(E-1)
1.00
0.50
0.00
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

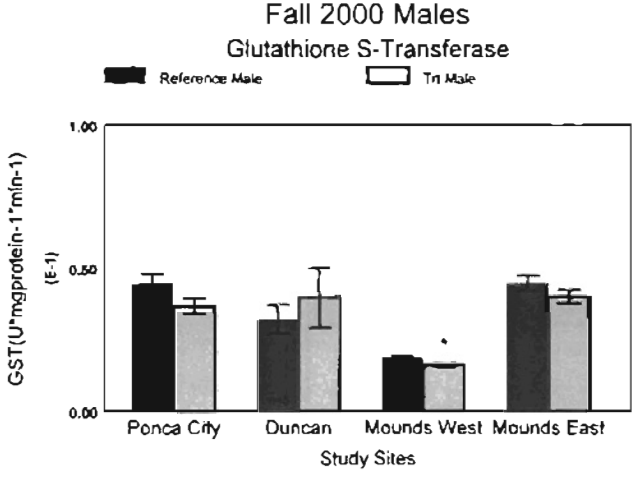
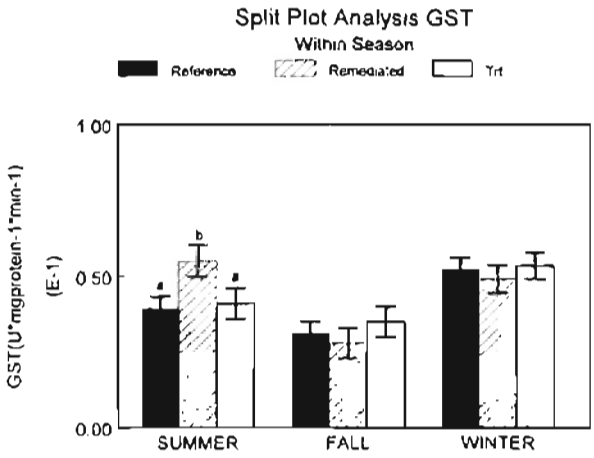
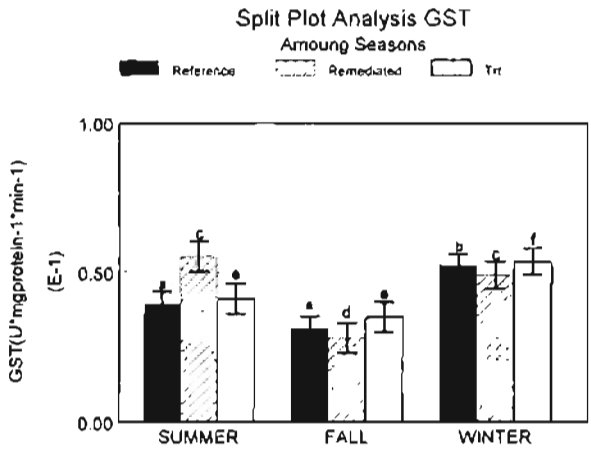
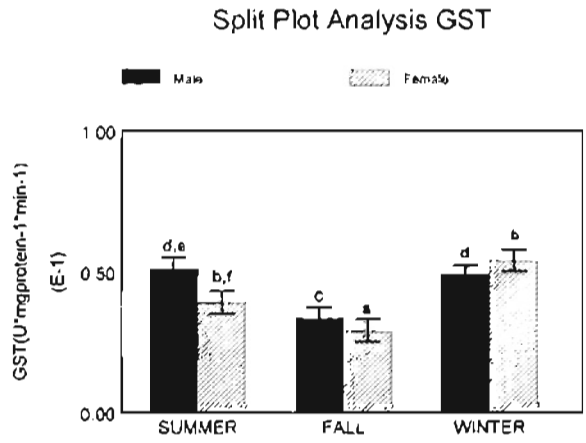

Fall 2000 Males
Glutathione S-Transferase
Reference Male
Tri Male
GST(U*mgprotein-1*min-1)
(E-1)
1.00
0.50
0.00
Ponca City
Duncan
Mounds West
Mounds East
Study Sites

Figure 19. Split plot analysis. Mean (± SE) glutathione s-transferase activity of female and male cotton rats. Within each graph, ascending letters indicate significant differences ($p<0.05$). Letters a & b, c & d and e & f indicate significant difference.


Split Plot Analysis GST
Within Season
Reference
Remediated
Trt
GST(U*mgprotein-1*min-1)
(E-1)
1.00
0.50
0.00
a
b
a
SUMMER
FALL
WINTER

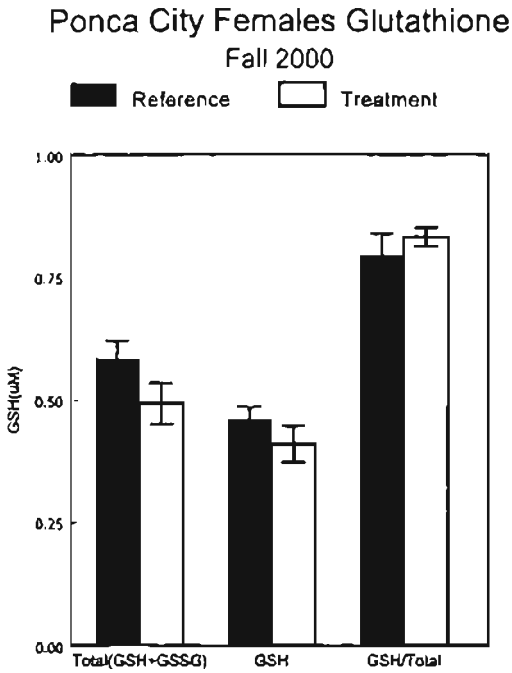

Split Plot Analysis GST
Amoung Seasons
Reference
Remediated
Trt
GST(U*mgprotein-1*min-1)
(E-1)
1.00
0.50
0.00
a
c
a
a
d
a
b
c
f
SUMMER
FALL
WINTER


Split Plot Analysis GST
Male
Female
GST(U*mgprotein-1*min-1)
(E-1)
1.00
0.50
0.00
d,e
b,f
c
a
d
b
SUMMER
FALL
WINTER

Glutathione

No differences were observed in total glutathione levels, glutathione (GSH) levels or GSH to total glutathione ratios of either males or females from Ponca City in the fall of 2000 (Figure 20). Split plot analysis of Ponca City glutathione levels in the fall of 2000 across histories, and both sexes was done using SAS (Figure 21). No interactions were found for the ratio of GSH to total glutathione. An interaction of history and sex as factors was determined for total glutathione ($p=0.015$). Treatment males had more total glutathione ($p=0.0233$) than reference males. There was a nonsignificant difference for males collected from unremediated landfarm sites (treatment sites) to have more total glutatione ($p=0.051$) than females from the same sites. An interaction of history and sex ($p=0.0251$) was also found for GSH levels. Again treatment males had more GSH ($p=0.0172$) than reference males. For GSH, however, the relationship of male treatment animals having more GSH ($p=0.0355$) than females was significant (Figure 21).

Figure 20. Mean (± SEM) glutathione levels in female and male cotton rats from the reference and treatment sites at Ponca City captured in fall 2000. n=6.


Ponca City Females Glutathione
Fall 2000
Reference
Treatment
1.00
0.75
0.50
0.25
0.00
GSH(uM)
Total(GSH+GSSG)
GSH
GSH/Total

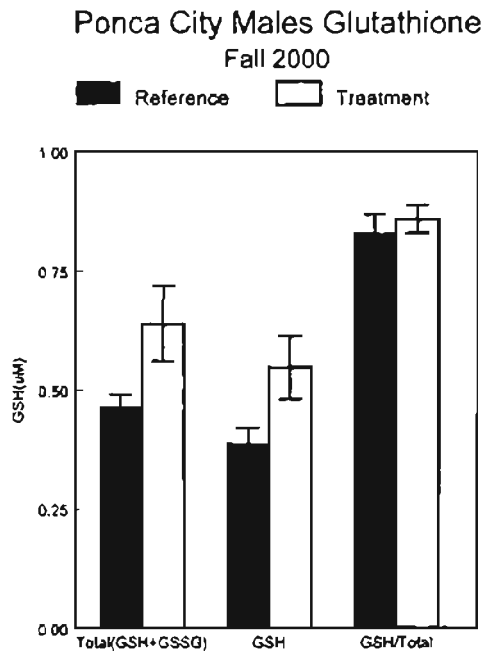
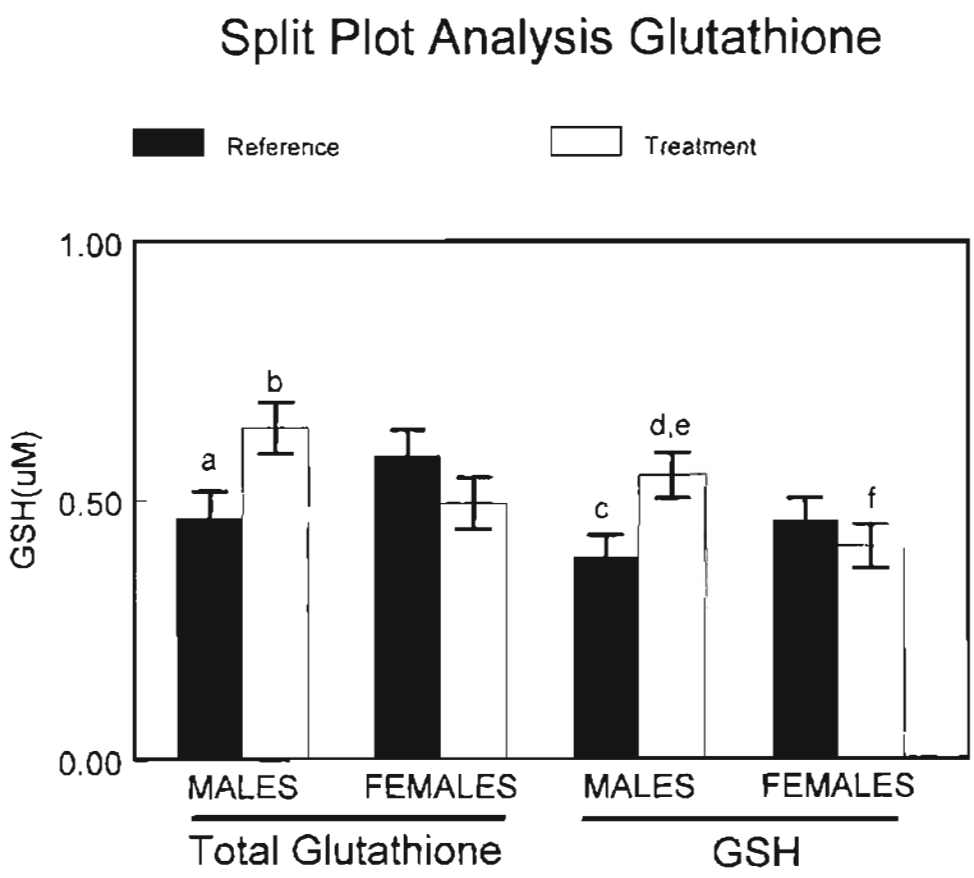

Ponca City Males Glutathione
Fall 2000
Reference
Treatment
1.00
0.75
0.50
0.25
0.00
GSH(uM)
Total(GSH+GSSG)
GSH
GSH/Total

Figure 21. Split plot analysis. Mean (± SE) of glutathione levels of female and male cotton rats from the reference and treatment sites at Ponca City captured in fall 2000. Within each graph, ascending letters indicate significant differences ($p<0.05$). Letters a & b, c & d and e & f indicate significant difference.


Split Plot Analysis Glutathione
Reference
Treatment
1.00
0.50
0.00
GSH(uM)
a
b
c
d,e
f
MALES
FEMALES
MALES
FEMALES
Total Glutathione
GSH

Coefficients of Variability

Assay	Variability	Coefficient of Variability	N
EROD	Within A Plate	4.17	6
EROD	Between Plates	7.46	3
MROD	Within A Plate	5.598	8
MROD	Between Plates	1.89	2
PROD	Within A Plate	0.67	3
PROD	Between Plates	3.19	4
Densitometry - CYP1A1	Between gels	67.87	11
Densitometry - CYP1A2	Between gels	40.16	11
GST	Within A Plate	4.10	3
GST	Between Plates	5.07	11

Table 2: Coefficients of Variability of Assays in this study.

DISCUSSION

Cotton rats and other native rodents are being considered for monitoring of soil bound contaminants (Elangbam et al 1989a; Flickinger and Nichols 1990; Fouchecourt et al 1999; Lochmiller et al 1999). Petroleum hydrocarbons are vital to today's economy and there is concern about disposal of the resulting wastes. Certain contaminants such as PAHs have a high bioavailability in mammals. Monitoring of rodent liver enzymes and other hepatic parameters evaluates the bioavailability and possible impact of the contaminants as well as effects of those contaminants on wild animals exposed to environmental stressors. The objective of this study was to increase understanding of terrestrial toxicant bioavailability and hepatic effects of chronic exposure to landfarmed petroleum wastes, and to determine the suitability of cotton rats as bioindicator organisms.

TIME OF SACRIFICE

Previous studies have examined CYP isozyme alterations in cotton rats exposed to petrochemical wastes using hepatic EROD, MROD, and PROD activities as biochemical markers of exposure to CYP1A and CYP2B inducing compounds. Incorporated a time delay between capture and sacrifice of rats of approximately 48 hours (Elangbam et al 1989a, b, 1991a, b; Lochmiller et al 1999; Kim et al 2001b). This could be problematic from a biomonitoring perspective as certain CYP isozymes, particularly CYP1A, are known to rapidly down regulate once exposure to contaminants is removed. The half lives of CYP isozymes vary from 8 to 35 hours (Guengerich and Liebler 1985). Studies in old world rats (*Rattus norvegicus*) reported limited biological half lives of PAHs (e.g.

half life of benzo(a)pyrene was 5-10 minutes) (Eisler 1987). In gulls dosed with a low level of crude oil, EROD activities decreased significantly after 24 hours and were back to control levels by 72 hours (Peakall et al 1989). Therefore, a major objective of this thesis was to compare hepatic EROD, MROD and PROD activities in cotton rats held for 48 hours following capture with enzyme activities determined as soon as possible following capture.

To achieve this objective, three separate split plot analyses were done. Split plot analysis allows for elucidation of interactions and patterns involving EROD or MROD, the time of sacrifice and the site or rat characteristics, such as treatment or sex. Within the animals collected from the unremediated landfarm site at Ponca City both males and females had significantly higher EROD and MROD activity (3.5, 2-fold respectively) on the day of capture sacrifice compared to a later sacrifice. No difference in activity with change in time of sacrifice was found in the males and females from the reference site at Ponca City. At two remediated landfarm sites (Mounds West and East) and their matched reference sites, male cotton rats had significantly higher EROD and MROD activity (2, less than 2-fold respectively) when sacrificed on the day of capture when compared to those sacrificed up to 48 hours later. When the males from all three landfarm sites (Ponca City, Mounds West and Mounds East) were analyzed along with their matched reference sites a different pattern of changes due to time of sacrifice appeared. Again males from the Ponca City unremediated landfarm site had a higher EROD activity (4-fold) when sacrificed on the day of capture when compared to those sacrificed up to 48 hours later. The reference males from all

three sites had a higher EROD activity (2-fold) when sacrificed on the day of capture when compared to those rats from the same site terminated up to 48 hours later. In contrast to the analysis including only the males from Mounds West and East remediated landfarm and matched reference sites, the analysis including males from the landfarm sites and matched reference sites at Ponca City, Mounds West and Mounds East revealed a pattern of no difference in EROD activity resulting from the time of sacrifice within the males from Mounds West and Mounds East remediated landfarm sites. MROD activity was also different in the analysis including the males from the three sites (Ponca City, Mounds West and Mounds East). Males from the unremediated landfarm site (Ponca City), remediated landfarm sites (Mounds West and Mounds East) and reference sites all had higher MROD activity (less than 2-fold) when sacrificed on the day of capture when compared to the males from the same sites sacrificed up to 48 hours later. In a previous study with a time delay between capture and sacrifice, EROD has been found to been induced less than 2 fold in cotton rats captured at a refinery compared to those captured at a matched reference site (Lochmiller et al 1999).

The differences found between the separate analyses of the unremediated (Ponca City) and remediated landfarm (Mounds West and East) sites may have resulted from degradation of Ah receptor agonists that induce CYP1A at the remediated landfarm sites. The lack of differentiation in the EROD and MROD activities between the reference and remediated landfarm sites at Mounds West and East may be due to historic contamination of the reference sites. Nearby to

Mounds West and East reference sites was one of the biggest oil finds in American history (Glenn Pool strike) (Franks 1984). This strike commenced North of Mounds West and East expanded rapidly south (Franks 1984). Due to inadequate storage capacity, earthen pits were flooded with oil (Franks 1984). In addition to the seepage from these pits, the wooden storage tanks and pipelines when they were built were known to leak (Franks 1984). The wooden storage tanks were sometimes toppled by the wind, which is the reason they were eventually replaced by steel tanks (Franks 1984). During this time, it was not uncommon to observe Polecat Creek, which ran thru Glenn Pool, thickly covered with crude oil (Franks 1984).

In summary, hepatic EROD and MROD activities in this study were found to significantly decline within 48 hours after capture of rats (Figs 6,12). If there is a delay between capture and sacrifice of cotton rats, biological monitoring of sites using rodent hepatic EROD and MROD activities may well give an erroneously low picture of contaminant exposure. Although it limits the use of the animals for certain immunological tests, the results of this study indicate that when using EROD and MROD activities as biochemical markers of exposure to AhR agonists, animals should be sacrificed as quickly as possible after capture to get an accurate idea of exposure.

In general the CYPIIB family of enzymes have longer half-lives when compared to CYPIA enzymes (Ioannides and Parke 1990). This is in accordance with the findings of this study. The same three split plot analyses were performed for PROD activities as were done for EROD and MROD. No

differences were found between PROD activities from animals sacrificed on the day of capture and those sacrificed up to 48 hours after capture. Thus when monitoring for compounds that induce CYP2B enzymes keeping the time between capture and sacrifice minimal is less vital.

CYTOCHROME P450

Within the order Rodentia interspecific variability has been found among cytochrome P450-dependent enzymes both in inducibility and substrate specificity (Astrom et al 1986, Hincks and Brindley 1986, Novak and Qualls 1989, Qualls et al 1998). Classical inducers of CYP2B and CYP1A include phenobarbital and 3-methylcholanthrene, respectively. Substrate specificity was thought to contribute to differences found in EROD, MROD and PROD activity between phenobarbital induced Sprague-Dawley (Old World) rats and phenobarbital induced cotton rats (Novak and Qualls 1989). Cotton rats and Sprague-Dawley rats displayed similar reactions to 3-methylcholanthrene with elevation of EROD, MROD and PROD activity (Novak and Qualls 1989). Phenobarbital administration resulted in elevated EROD activity with no change in MROD or PROD activity in Sprague-Dawley rats, but in cotton rats it resulted in elevated MROD and PROD activity with no change in EROD activity (Novak and Qualls 1989). Use of indigenous rodents increases the ecological relevance when dealing with contaminated sites as compared to use of lab strain rodents with little genetic variability.

Cotton rats dosed with injections of classical inducers of CYP2B and CYP1A reported total cytochrome P450 levels increased 1.5 to 2-fold in both

sexes (Elangbam et al 1989b). Another study using the same inducers found significant induction in EROD, MROD and PROD activity ([10, 5], [6, 8.5], and [4.5, 2]-fold [females and males] for EROD, MROD and PROD respectively), in cotton rats treated with 3-methylcholanthrene (Novak and Qualls 1989). In males, treatment with phenobarbital induced EROD (less than 2-fold), but not MROD or PROD, with no significant effect found in the females (Novak and Qualls 1989). Aroclor 1254 dosing induced EROD and MROD (4 to 8-fold) and PROD (2 to 3-fold) in cotton rats (Henneman et al 1994). In a study comparing a Superfund toxic waste dump and its matched reference site, male cotton rats collected from the Superfund site were found to have elevated EROD, MROD and PROD activities (8, 4, and 3-fold respectively) (Elangbam et al 1991a). Thus, cotton rats appear to respond to classical CYP1A inducers similarly to laboratory strains of rats.

Roos et al (1996) found that at PAH contaminated sites (4 former coking plants and a former gas plant), most of the 2- to 4-ring PAHs were degraded by microbial action, but about one-third of 5- and 6- ring PAHs remained in the soil and were bioavailable to rodents. Roos et al (1996) exposed Sprague-Dawley rats to a diet containing soil from the 5 PAH contaminated sites and found that the extent of EROD elevation in the liver had a linear correlation with the amount of 5 & 6- ring PAHs in the soil and not with the total PAHs or PAH/Total organic carbon ratio. The Roos et al (1996) study would indicate that on PAH contaminated sites monitoring of EROD activities in rodents gives a good indication of the 5- and 6-ring PAHs that are bioavailable. This correlates with

another study in which lab mice were injected with an artificial reconstituted mixture of PAHs manufactured to resemble gas plant residue or fractions of this mixture containing 2-ring, 3-ring or ≥4-ring PAHs (Chaloupka et al 1995). With these PAH mixtures it was determined that PAHs with 4 or more rings induced hepatic EROD and CYP1A1 mRNA levels (Chaloupka et al 1995). Induction of hepatic MROD and CYP1A2 mRNA levels was found with PAHs containing 4 or more rings, but a large portion of the induction was attributed to 3-ring PAHs (Chaloupka et al 1995). Low induction was found for 2-ring PAHs for either EROD and CYP1A1 mRNA levels or MROD and CYP1A2 mRNA levels (Chaloupka et al 1995). Considering previous studies, evaluation of EROD and MROD induction in native rodents on sites contaminated with PAHs would elucidate the bioavailability of PAHs with 3 or more rings.

The differences found between this study and others done on petrochemical waste sites may be due to variation in contaminants and contaminant location. Various polyaromatic hydrocarbons have been detected on the sites in this study. The enzyme induction found in the animals from these sites is likely due to these contaminants. Not detectable in the soil from the reference sites, several 3-, 4-, and 5-ring PAHs (acenapthene, anthracene, acenapthylene and benzo(k)fluoranthene) were detected on both the remediated and unremediated landfarm sites with the detected levels of 3,4,5-ring PAHs being higher on unremediated landfarm sites (Schroder unpublished). Soil levels of phenanthrene, benzo(a)anthracene, chrysene, fluoranthrene, pyrene, benzo(a)pyrene, benzo(b)fluoranthrene, dibenzo(a,h)anthracene and

indeno(1,2,3)CD-pyrene (3-, 4-, 5-, 6-ring PAHs) were elevated on the unremediated landfarm sites when compared to the detectable levels found on the reference and remediated landfarm sites (Schroder unpublished). Napthalene and benzo(g,h,i)perylene (2, 6-ring PAHs) levels found in the soil had a declining pattern, with the unremediated landfarm sites having more than the remediated landfarm sites which had more than the reference sites. The presence of the PAHs at the reference sites is not surprising as they are ubiquitous in the environment, although results may be skewed because of the possible contamination at the two Mounds reference sites as mentioned previously (Eisler 1987). Exposure of cotton rats to these 4,5,6-ring PAHs are the most likely contaminants responsible for the enzyme alterations observed in this study.

In this study, low level (2 to 4 fold) induction of hepatic EROD activity was determined in cotton rats collected from three of the landfarm sites (Ponca City, Mounds West and Mounds East) when compared to rats from matched reference sites. The animals from one of the unremediated landfarm sites (Duncan) had no elevation of EROD when compared to the animals of its matched reference site. The lack of EROD induction observed at Duncan may be due to lack of biological availability or may be due to poor habitat on the site causing rodents to avoid areas with high concentrations of contaminants. The Duncan landfarm site has large areas of bare ground, which does not suit cotton rat habitat preferences (Cameron and Spencer 1981).

In general, induced MROD activities found in this study were slightly lower than EROD. MROD has been found to be induced less than 2 fold in cotton rats captured at an abandoned refinery compared to those captured at a matched reference site (Lochmiller et al 1999). In the present study, low levels (2 to 7 fold) of MROD induction were found in animals from one of the unremediated landfarm sites (Ponca City) when compared to rats from the matched reference site.

Consistent, but low induction of PROD activity (less than 2 to 2-fold) was observed in the present study in female rats collected from one of the unremediated landfarm sites (Ponca City) when compared to females from the matched reference site. Males from the same unremediated landfarm site had elevated PROD levels (less than 2 fold) compared to the males from the matched reference site only in the fall. Females from the other unremediated landfarm site (Duncan) when compared to the matched reference site demonstrated an elevation of PROD activity (2-fold) only in the summer. Of the animals from the two remediated landfarm sites only males in the winter from the one site showed a difference in PROD activity compared to the animals from the matched reference site. Interestingly this difference was a decline in PROD activity. This selective inhibition may have been due to metal contamination on the site. Both the unremediated and remediated landfarm site soils had elevated levels of As, Ba, Ni and F compared to the reference sites (Schroder unpublished). It has been found that certain metal ions can inhibit CYP enzyme activities (Testa and Jenner 1981, Lewis 1996). There is some evidence that certain metals inhibit

CYP isoforms preferentially (i.e. cadmium salts inhibit CYP2E, but not CYP3A) (Lewis 1996). Another study reported a less than 2- to 10-fold increase of PROD activity in Sprague Dawley rats fed various soils from sites contaminated with PAHs (Roos et al 1996). PROD activities in Sprague-Dawley rats exposed to PAH-contaminated soil were correlated with 5- & 6- ring PAHs (Roos et al 1996). Elevated PROD activity was assumed to be due to induction of CYP1A1 isozymes (Roos et al 1996). While CYP isozymes have specificity towards different substrates , considerable substrate overlap can exist (Guengerich and Liebler 1985). Constitutive CYP2B content has been found to be higher in males (presumably Old World rats) (Ioannides and Parke 1990). However, no differences in constitutive PROD activity were found between males and females in this study. In addition, no seasonal patterns were found in the PROD activities of the rats collected from any of the sites. Immunoblotting in conjunction EROD and PROD activity in male Wistar rats found CYP1A to be responsible for EROD and in part PROD activity and CYPIIB was responsible for PROD (Nakajima et al 1990). In this study, PROD may have been enhanced by CYP1A activity as was suspected in the Roos et al (1996) study.

CYP1A WESTERN BLOTTING

CYP1A1 and CYP1A2 were differentiated using a polyclonal antibody that recognized both isozymes. After SDS and heat treatment, the two isozymes separated on polyacrylamide gels into a distinct doublet of immunoreactive bands because of differences in their size and conformation, with CYP1A2 traveling slightly slower than CYP1A1. Determination of isozyme identity was

performed using information from the company that produced the antibody and confirmed by similar findings in another study (Letcher et al 1996). Determination of CYP1A1 and CYP1A2 protein served two purposes in this thesis. First, it provided confirmation that CYP1A1 and CYP1A2 proteins were actually present in hepatic microsomes, supporting the use of EROD and MROD as catalytic markers of CYP1A1 and CYP1A2, respectively, in cotton rats. Second, an objective of this thesis was to compare CYP1A Western blotting as an alternative method to measuring EROD and MROD.

Immunoblotting of hepatic CYP1A1 in the present study determined a 3-10 fold increase, but only in males captured in the winter from one unremediated landfarm site (Duncan) and one remediated landfarm site (Mounds West) compared to males collected from matched reference sites. Immunoblotting of CYP1A2 determined an increase of less than 2 to 30-fold following the pattern of MROD induction, but only in animals captured during the winter and fall from one of the unremediated landfarm sites (Ponca City and Duncan) compared to the animals from the matched reference site.

No significant correlation was found between EROD and CYP1A1 immunoreactive bands or MROD and CYP1A2 immunoreactive bands (data not shown). High variability in the Western blotting determinations may have contributed to the lack of correlation. There was a distinct visual correlation between hepatic MROD induction and increased CYP1A2 protein levels in cotton rats collected from the unremediated landfarm site and matched reference site at Ponca City in the winter and fall. Such a correlation was not evident when

examining the relationship between EROD induction and CYP1A1 protein levels in cotton rats collected from the same sites. This may be due to increased CYP1A2 protein resulting in increased MROD and EROD activity due to overlap in substrate specificities. Another possibility is that the variability found in Western blotting of CYP1A1 bands, which was greater in CYP1A1 than CYP1A2 bands, was high enough to mask any changes.

GLUTATHIONE S-TRANSFERASE

No consistent patterns of increased GST activity were observed in cotton rats collected from any of the contaminated sites in this study. No induction of GST activity was found in a laboratory study pretreating cotton rats with microsomal enzyme inducers (phenobarbital, 3-methylcholanthrene or pregnenolone 16α-carbonitrile) (Watkins 1991). A study of male cotton rats inhabiting various hazardous waste sites found a slight decrease in GST activity (less than 2-fold) in cotton rats on only one site compared to its matched reference site (Rattner et al 1993). Animals from two other sites in the Rattner et al (1993) study had no change in GST activity when compared to their matched reference sites. Sex related differences in GST have been found in mice, apparently under the control of testosterone (Mannervik and Danielson 1988). In cotton rats, constitutive GST activities were found to be higher in males than females (Watkins 1991). Males collected from both reference and treatment sites in summer of 1999 did have a higher level of GST activity than females collected from the same sites in the same season, but this was the only season that differences due to sex were found. Both males and females showed a

seasonal pattern of higher GST activity in the summer and winter than in the fall. Animals collected from the unremediated landfarm and reference sites had higher GST activity in the winter when compared to the fall or summer. This contrasts with another study of cotton rats inhabiting various hazardous waste sites and matched reference sites that found higher GST activity in the winter than in the summer (Rattner et al 1993). Animals from the remediated landfarm sites (Mounds West and East) exhibited this pattern of higher activity in the winter and summer when compared to the fall. The only pattern found between sites was for animals from the remediated landfarm sites to have higher GST activity than animals from the reference and unremediated landfarm sites, but only in the summer. With the low levels of EROD, MROD and PROD induction, large changes in GST activity were not expected.

Certain metal compounds (cadmium iodide, cadmium chloride, copper chloride and lead acetate) have been found to inhibit GST (Mannervik and Danielson 1988). This may have confounded the findings in this study as these sites are contaminated with metals as mentioned previously.

GLUTATHIONE

Males collected from the unremediated landfarm sites (Ponca City and Duncan) were found to have higher GSH levels than females from the same sites. Males collected from the unremediated landfarm sites (Ponca City and Duncan) were found to have higher total glutathione and GSH levels than males collected from the reference sites. This may have been due to metal exposure, PAHs or a combination of both.

Alterations of glutathione status generally follow the pattern of decreasing GSH and increasing GSSG, deviating from the nonchallenged status of approximately 99.5% GSH and 0.05% GSSG (Redegeld and Galaris 1990, Smith and Mitchell et al 1989). Some cases of pathological or chemical insult have resulted in higher than normal GSH concentrations (Kosower and Kosower 1989). GSH levels are influenced by nutritional status and stress, such as fasting, refeeding after fasting and dietary protein levels (Taniguchi et al 1989). Glutathione turnover is faster in males than females in mice (Taniguchi et al 1989). However, no difference was found in total glutathione between males and females in this study.

Recently phytochelatins, enzymes that detoxify heavy metals (particularly cadmium), have been discovered in the nematode (*Caenorhabditis elegans*) (Vatamaniuk et al 2001). Phytochelatins are synthesized from glutathione, thus furthering the importance of glutathione in the detoxification of heavy metals (Vatamaniuk et al 2001). Perhaps after validation of phytochelatin assays in mammals, they too will be monitored in animals on metal contaminated sites in the future.

As the subcellular pools of GSH (microsomal and cytosolic) appear to respond independently, further study of glutathione status should look at the mitochondrial pool as it has shown to be sensitive to administration of oxidants (Romero and Galaris 1990). Important for experimental design is the fact that hepatic GSH levels have been found to undergo circadian rhythms with a peak in the morning and lower levels in the afternoon (Taniguchi et al 1989).

HISTOLOGY

No patterns were found in any of the histological markers examined at any of the sites in the present study. These histology findings are consistent with the pattern of low EROD and MROD induction and lack of GST and glutathione changes. Difficulties were encountered because of loss of cell contents in some of the slides. A laboratory study exposing cotton rats to Aroclor 1254 found hypertrophy, fat accumulation and areas of cellular necrosis in liver (Henneman et al 1994). Proliferation of smooth endoplasmic reticulum is not always visible with a light microscope, and sometimes it is necessary to use an electron microscope (Cullen and Ruebner 1991). Total cytochrome P450 increase has been correlated with proliferation of the smooth endoplasmic reticulum in cotton rats collected from a site contaminated with polychlorinated biphenyls, so much so that mitochondria were displaced (Elangbam et al 1991b). Proliferation of the smooth endoplasmic reticulum is expected with large induction of CYP enzymes as this is where the enzymes are located in the cell.

SEASON AND SEX EFFECTS

Season and the sex of the animals were found to have a significant effect on EROD activities and CYP1A1 content. Females collected from the unremediated landfarm sites (Ponca City and Duncan) had higher EROD activities in the winter compared to males. The only elevations in CYP1A1 content above females were detected in males collected from one unremediated

and one remediated landfarm site (Duncan and Mounds West, respectively) in the winter. This agrees with patterns found under laboratory conditions with sex, reproductive status, and biological rhythms reported as factors that influence the cytochrome P450 system (Rattner et al 1989). Sex was found to have a significant effect on MROD activity, with males collected from remediated landfarm sites (Mounds West and East) having higher MROD activity than females collected from the same sites. Unlike the lack of seasonal pattern within MROD activity, season was found to have an effect on CYP1A2 levels, with induction found in the winter and fall, but not the summer.

A seasonal pattern in EROD and MROD activity has been previously reported in cotton rats collected on petrochemical contaminated sites, with higher activity found in the summer, but not in the winter (Lochmiller et al 1999). This is in contrast to the pattern of higher EROD levels detected in the winter when compared to the summer and the lack of seasonal pattern in MROD in this study. Higher total cytochrome P450 content in the winter compared to the summer was found in another study involving cotton rats inhabiting three hazardous waste sites (Rattner et al 1993). Seasonal effects in CYP activity or content have been found in previous studies, but the season of greatest change was not consistent between studies (Rattner et al 1989, 1993, Lochmiller et al 1999, Kim et al 2001a). Possible reasons for this seasonal effect include shifting dietary habits by season, seasonal stress (i.e. reproduction), seasonal activity and rapid population turnover. Cotton rats are known to shift their dietary habits and even

activity (larger rats were less active in the winter) by season (Cameron and Spencer 1981, Kincaid and Cameron 1982, Eifler and Slade 1998).

The constitutive CYPIA content has generally been found to be higher in females (presumably in Old World rats) (Ioannides and Parke 1990). Higher inducibility of total cytochrome P450 levels in females has been found in several different rodents (Astrom et al 1986). A laboratory study examining EROD, MROD and PROD induction following exposure to Aroclor 1254 found female cotton rats to be less sensitive than males (Henneman et al 1994). In cotton rats exposed to 3-methylcholanthrene (a classic inducer of CYPIA), females were found to have a higher EROD, MROD and PROD activity than males (Novak and Qualls 1989, Ioannides and Parke 1990). Phenobarbital-induced male cotton rats were found to have slightly higher EROD, MROD and PROD activities than females (Novak and Qualls 1989). In the present study, a pattern of higher EROD activity in females collected in winter was found in the unremediated landfarm sites (Ponca City and Duncan) and reference sites, but not in female rats collected in winter from the remediated landfarm sites (Mounds West and East). This pattern was not observed in MROD activity; in fact males from the remediated landfarm sites were found to have higher MROD activity than females from the same sites. Previous studies using cotton rats reported induction of EROD, MROD and PROD activities and total cytochrome P-450 content in males, but nonsignificant elevation in females (Elangbam et al 1989a, 1991a). This was attributed to high individual variability, particularly in females (Elangbam et al 1991a).

It has been suggested that high individual differences among female cotton rats diminishes their usefulness in monitoring toxicants in the environment (Elangbam et al 1991a). This idea was not supported in this study, since induction of hepatic EROD, MROD and PROD activities were evident in both males and females. Additionally, a problem often encountered in studies using animals captured in the field is obtaining sufficient numbers of animals. Low cotton rat population levels have been encountered in the field, thus limiting statistical evaluation (Elangbam et al 1991a, Rattner et al 1993). Limiting a study to one sex of animal would make collections more labor intensive, more expensive (more traps needed) or not possible in areas with low population densities caused by either natural factors (i.e. habitat or food source) or contaminants. Additionally, predatory factors such as fire ants can lower the number of live animals collected (Rattner et al 1993). Rattner et al (1993) encountered a site that had a combination of low population density and high mortality caused by fire ants resulting in the capture of only four male rats.

REMEDIATED AND UNREMEDIATED LANDFARMS

Refiners in the United States, Canada and Europe have employed landfarms in the disposal of petroleum waste (American Petroleum Institute 1984). In the United States, the US Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) or Superfund act of 1980 is the legislative action dealing with sites such as the ones in this study.

Adding to the genetic variability found when performing field studies with wild organisms is the variability in site contamination and treatment. Different

refinery processes produce different grades of petroleum and result in the generation of different wastes (American Petroleum Institute 1984). Certain hydrocarbons and heavy metals (i.e. Cr, Pb, Cd, As and Ni), if present in the waste, can have an inhibitory effect on microbial activity (Huesemann 1994). Soil characteristics such as pH, temperature, moisture and natural microbes present influence biodegradation of hydrocarbons (American Petroleum Institute 1984, Huesemann 1994). High organic carbon content has been found to be a critical parameter for successful microbial degradation of PAHs with minimal microbial toxicity (Roos et al 1996). Anaerobic bacteria have been found that can dechlorinate polychlorinated biphenyls, producing mixtures that have a lower EROD induction potential in rat cell cultures (Mousa et al 1998). Studies assessing the bioavailability of contaminants to mammals are important when considering future uses for landfarmed land. Considering whether petroleum waste is bioavailable to mammals is also important because of the concern about children playing on or near contaminated sites and inhaling fine particles or ingesting small quantities of soil while at play (Roos et al 1996).

Higher enzyme activity in animals collected from unremediated landfarm sites compared those collected from remediated landfarm sites were only evident in females (EROD in the winter and PROD). Additionally, higher enzyme activity (EROD in fall males and GST in the summer) in the animals collected from remediated landfarm sites compared to those collected from reference sites were not consistent across sex or season. This indicates that the animals from remediated landfarm sites probably range in between animals from unremediated

landfarm or reference sites in exposure to contaminants. This is consistent with the PAH concentrations on the sites as mentioned previously. Based on these data, this would indicate that remediation is effective to a certain degree. Alternatively, the history of the remediated landfarm sites leaves the possibility that exposure is inconsistent resulting from patchiness of contaminants in the environment.

RATS & BIOMONITORING

Wider variation is expected when looking at wild species because of genetic and environmental factors (Walker 1980). It has been suggested that terrestrial carnivores may have less metabolizing capacity in general when compared to herbivores or omnivores (Walker 1980). This leaves the possibility of greater threat to carnivores than to herbivores or omnivores, but the population densities of carnivores are often very low prohibiting statistical analysis especially when considering the genetic variation that is encountered in wild populations. Although bioavailability of PAHs to microorganisms decreases with increasing total organic carbon values, this relationship does not necessarily apply to mammals (Roos et al 1996). No correlation was found in a study of PAH contaminated soil between bacterial toxicity and mammalian bioavailability (Roos et al 1996). This lack of correlation between microorganism and mammal bioavailability limits the use of microorganisms as surrogate organisms for mammals.

RECOMMENDATIONS ON ENDPOINTS FOR BIOMONITORING

CYP enzymes and GST have been detected in the intestine of laboratory rodents (Marcus et al 1978, Sarasquete and Segner 2000). Monitoring CYP enzymes and GST levels in the intestine may lead to a more complete picture of exposure, particularly very low exposure to toxicants. Although the intestine is not a major organ for detoxification, it would be one of the first organs exposed in cases of ingestion of toxicants. Pulmonary tissue of wild and laboratory Old World rats has also been found to contain inducible total CYP, EROD activities and CYP1A levels (less than 2 to 12-fold) that can be induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin or polychlorinated biphenyl contaminated fine soil particles (Beebe et al 1990, Nessel et al 1992, Fouchecourt and Riviere 1995 and 1996, Fouchecourt et al 1998). CYP enzymes found in the pulmonary tissue were found to be more sensitive at nonmaximal induction doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin (Nessel et al 1992). Exposure to polychlorinated biphenyl, petrochemical waste or polychlorinated biphenyl and petrochemical waste contaminated soils found Old World rat EROD more inducible in the lung with induction of 3 to 7-fold for the liver and 9 to 28-fold for the lung (Fouchecourt and Riviere 1995 and 1996, Fouchecourt et al 1998).

Therefore, future studies should consider monitoring CYP activities with EROD and MROD. Both preferentially monitor CYP1A isoform activities. High variability and large time investment leads to the recommendation of preferential use of EROD and MROD. However, Western blotting should be conducted in each study using animals with an uncharacterized CYP system to confirm the presence of specific isozymes. Monitoring of PROD or other assays with a

preference for CYP isoforms other than CYP1A allows for economical monitoring for other contaminants on sites with unclear contamination histories. Monitoring of total glutathione and GSH gives an idea of the state of phase II hepatic detoxification. Monitoring of glutathione s-tranferase activity did not add information to this study and although the assay is of great ease, the components of the assay are conspicuously toxic.

Light microscopy of histological slides was also not informative. Evaluation of hepatic sections with an electron microscope may provide a better indication of cellular toxicity. After validation of phytochelatin assays in mammals, monitoring phytochelatin levels in animals on metal contaminated sites may yield future insight into metal detoxification. The seasonal effects found in this and other studies, as mentioned previously, illustrate the importance of monitoring among different seasons. As mentioned previously, the time between capture and sacrifice should be minimized. Further study of glutathione status should look at the mitochondrial pool as well as the cytosolic pool, keeping the approximate time of day at sacrifice consistent.

Important to keep in mind is that monitoring of biomarkers produces correlation information rather than cause and effect relationships (Peakall 1992b). The correlation between CYP1A activity and PAHs and the findings of this study would indicate that using the EROD and MROD activities of native rodents is a accurate and cost effective method to monitor ≥3-ring PAH bioavailability on contaminated sites (Chaloupka et al 1995, Roos et al 1996).

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APPENDIX A: Microsome and Cytosol Preparation

Procedure

1. All glassware should be at 4°C in ice buckets. Start refrigeration of centrifuge at 4°C.
2. Check pH of 0.05M Tris 1.15% KCl buffer. Readjust to 7.5 if necessary. Keep on ice.
3. Prepare one 50ml beaker, filled with 20 ml of Tris/KCl buffer and keep them on ice.
4. Weigh each rat, then sacrifice it by cervical dislocation. Rapidly take out the liver. Rinse the liver with Tris/KCl buffer at 4°C.
5. Weigh each liver and place it in 50 ml beaker with Tris/KCl at 4°C.
6. Cut livers into pieces with scissors and transfer to large homogenizer. Homogenize 5 times at speed 5 (reverse), keeping the tube on ice. Rest one minute on ice. Homogenize 5 times at speed 5 (reverse) and transfer into plastic centrifuge tube, again on ice.
7. Fill each tube to the shoulder with Tris/KCl buffer. Balance tubes that will oppose each other in the rotor. Centrifuge 20 minutes at 10,000xg at 4°C.
8. Decant supernatant into ultracentrifuge tubes. Discard pellet.
9. Fill each tube to the shoulder with Tris/KCl buffer. Balance tubes that will oppose each other in the rotor (CAREFULLY!). Centrifuge 60 minutes at 100,000xg at 4°C.
10. Prepare cyrovials for cytosol and microsome storage.
11. Aliquot supernatant into cytosol tubes. Freeze at -80°C.
12. Wash the pellet 3 times with 1ml 0.25M sucrose. Resuspend each pellet with 1ml of 0.25M sucrose and loosen pellet with glass rod. Transfer to a small homogenizer.
13. Homogenize 3 times at speed 3 at 4°C, keeping the tube on ice.
14. Aliquot into cyrovials labeled microsomes. 0.25ml/ vial into 5 vials. Freeze immediately at -80°C.

Stock solution recipes

0.05M Tris 1.15% KCl pH 7.5 4°C (Tris/KCl buffer)

	1 liter	2 liters
KCl	11.5g	23.0g
Tris	6.05g	12.11g

In a beaker add KCl, Tris and most of the volume of distilled water. Adjust pH to 7.5 with HCl and then make up the volume with remaining distilled water. Store at 4°C. Readjust pH to 7.5 before using. Stable one month at 4°C.

0.25M Sucrose

	100ml	500ml	1 liter
Sucrose	.56g	42.79g	85.57g

Add sucrose and full volume of distilled water to beaker. Stir until dissolved and then filter through paper. Stable for about a week at 4°C.

APPENDIX B: DC Protein Assay (BIORAD, Hercules CA)

Procedure

1. Prepare 1 in 50 dilution (5 μg of microsome in 250 μl distilled H_2O) of microsome samples and 1 in 25 dilution (10 μg of cytosol in 250 μl distilled H_2O) of cytosol samples in distilled H_2O of samples to be assayed.
2. Prepare 1 in 50 dilution of standards each day. A bovine serum albumin (BSA) protein standard, of concentration 1.47 μg/μl, is diluted in distilled H_2O.

   Prepare standard curve

   1. **0.1 μg/μl** protein: 6.8 μl protein standard + 93.2 μl di H_2O
   2. **0.2 μg/μl** protein: 13.6 μl protein standard + 86.4 μl di H_2O
   3. **0.4 μg/μl** protein: 27.2 μl protein standard + 72.8 μl di H_2O
   4. **0.6 μg/μl** protein: 40.8 μl protein standard + 59.2 μl di H_2O
   5. **0.8 μg/μl** protein: 54.4 μl protein standard + 45.6 μl di H_2O
   6. **1.0 μg/μl** protein: 68.0 μl protein standard + 32.0 μl di H_2O
   7. **1.47 μg/μl** protein: 100 μl protein standard

   If using a BSA protein standard of a different concentration use dilution calculations to adjust the above protein amounts.

   Initial concentration/Desired concentration = Dilution Factor
   Desired Final volume/Dilution Factor = Volume of Sample
   Desired Final volume-Volume of Sample = Volume of Solvent
3. Pipette 5μl of standards and samples into clean, dry microplate.
4. Add 25 μl of kit reagent A to each well using a multichannel pipettor.
5. Add 200 μl of kit reagent B to each well using a multichannel pipettor.
6. Mix for 15 minutes protected from light.
7. Read using microplate reader at 750 nm absorbance.
8. Correct readings for 1 in 50 dilution.

APPENDIX C: EROD & MROD & PROD Assay

Temp. 37°C for rats/20°C for fish. Light subdued
Amounts are determined for one 96 well microplate

Procedure

1. Make Resorufin dilution standards (Protect from light)
   Prepare standard curve dilutions of Resorufin as follows:
   1. **0.0005 mM** Resorufin: 6ul **0.01mM** stock + 114ul DMSO
   2. **0.001 mM** Resorufin: 12ul **0.01mM** stock + 108ul DMSO
   3. **0.0025 mM** Resorufin: 30ul **0.01mM** stock + 90ul DMSO
   4. **0.005 mM** Resorufin: 6ul **0.1mM** stock + 114ul DMSO
   5. **0.01 mM** Resorufin: 120ul
   6. **0.02 mM** Resorufin: 24ul **0.1mM** stock + 96ul DMSO
   7. **0.06 mM** Resorufin: 72ul **0.1mM** stock + 48ul DMSO
   8. **0.1mM** Resorufin: 120ul
2. Calculate dilutions to obtain 6mg protein/ml for each sample.
3. Defrost microsomes on ice.
4. Turn on spectrofluorometer(Biochemistry 349)(Needs 45 minutes to warm up)
5. Assign sample #s to wells of the plate(96 wells per plate) (Microsome samples done in duplicate and Standards done in triplicate).
6. Take an aliquot of Hepes buffer containing 5mM $MgCl_2$ (30mL per plate) from fridge and allow equilibrate to room temp.
7. Remove bottle of Beta-NADPH from fridge, and set at room temp. for 1/2 hr before opening bottle to weigh out Beta-NADPH.
8. Obtain bucket of ice
9. Label eppendorf tubes with sample numbers
10. Mix 136uL of 1mM Eth-, Meth-, Pent- oxyresorufin stock in 25mL of Hepes Buffer containing 5mM $MgCl_2$. Shield from light
11. To each well add 230ul of Eth-, Meth-, Pent- oxyresorufin/Buffer solution with multichannel pipettor and cover plate with plate sealer and wrap in aluminum foil.
12. Dilute microsomes to 6mg of protein per ml with 0.25M Sucrose (final volume 100ul). 950ul of Control microsome.
13. Place diluted microsomes on ice
14. Mark lids and return stock microsomes to -70°C. Freeze
15. Make up 1mL (for each plate) 6.25 mM Beta-NADPH in cold Hepes Buffer. Cover with aluminum foil and keep on ice. Place Beta-NADPH in amber 2ml vial.
16. Take plate(s), timer, pipette tips, 10uL pipettor, gloves, microsomes and solutions (6.25 mM Beta-NADPH & Resorufin dilution) to Biochemistry

17. Set spectrofluorometer temp to 37°C-Preset #2

18. Access "Qualls" folder from main menu of Macintosh

19. Access Carlson folder from Qualls and then template-Ruth 1

20. Under Filter Select Menu -Set emission wavelength to 590nm and excitation wavelength to 544nm=Preset #3&3 on Spectrofluorometer

21. For each sample-well add the following sequentially:

For the Standard Blank:
10ul 6mg/ml control microsome in 0.25M sucrose(stir to mix with pipette tip)
For the Standards:
10ul 6mg/ml control microsome in 0.25M sucrose
10ul of specified concentration of Resorufin (stir to mix with pipette tip)
For the Unknown Blanks:
10uL of Microsomes (stir to mix with pipette tip)
For the Unknowns:
10uL of Microsomes(warm 2 minutes)
Add 10ul of 6.25mM NADPH (stir to mix with pipette tip)

22. PROD-Set timer for 100 minutes-place plate in incubator (rat microsomes)

MROD-Set timer for 40 minutes-place plate in incubator (rat microsomes)

EROD-Set timer for 10 minutes -place plate in incubator (rat microsomes)

23. After timer goes off take final reading

Standard Curve Procedure

Add sequentially:
230ul of Eth-, Meth-, Pent- oxyresorufin/Buffer Solution
10ul 6mg/ml control microsome in 0.25M sucrose
10ul of specified concentration of Resorufin is added

Resorufin Stock Solutions:

[Initial] Resorufin mM	[Final] Resorufin mM
0.0005	0.0025
0.001	0.005
0.0025	0.0125
0.005	0.025
0.01	0.05
0.02	0.10
0.06	0.3
0.1	0.5
0.16	0.8

Dilution Calculations

Initial concentration/Desired concentration = Dilution Factor

Desired Final volume/Dilution Factor = Volume of Sample
Desired Final volume-Volume of Sample = Volume of Solvent

Example Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	STD 2	STD 5	STD 8		U1	U5	U9	U13	U17	U21	+ C
B	Blank	STD 3	STD 5	STD 8		U1	U5	U9	U13	U17	U21	+ C
C	Blank	STD 3	STD 6	STD 8		U2	U6	U10	U14	U18	U22	
D	STD 1	STD 3	STD 6	STD 9		U2	U6	U10	U14	U18	U22	
E	STD 1	STD 4	STD 6	STD 9		U3	U7	U11	U15	U19	U23	
F	STD 1	STD 4	STD 7	STD 9		U3	U7	U11	U15	U19	U23	
G	STD 2	STD 4	STD 7			U4	U8	U12	U16	U20	U24	
H	STD 2	STD 5	STD 7			U4	U8	U12	U16	U20	U24	

Stock solution recipes

6.25mM Beta-NADPH Tetrasodium salt

Beta-NADPH	0.0055 g
HEPES - $MgCl_2$ Buffer (cold)	1 ml

Prepare fresh before each plate is run. MAKE LAST. Keep on ice at all times. Reduce exposure to light. Let NADPH equilibrate to room temp before weighing.

0.25M Sucrose

Sucrose	8.56g
Dist. water	100 ml
Filter through paper	
(Stable a couple of weeks max.)	

0.1M Hepes Buffer containing 5mM $MgCl_2$ pH 7.8

1)$MgCl_2$ * $6H_2O$ (MW 203.3)	Quantity	Final Conc.
$MgCl_2$ * $6H_2O$	10.165g	100mM
Dist. H_2O	500ml	
Store in fridge		

2)HEPES		
HEPES	11.915g	100mM
$MgCl_2$ 0.1M	25ml	5mM
Dist. H_2O	425ml	
Adjust to pH 7.8		
Adjust volume to 500ml with Dist. H_2O		
Store in fridge		

1 mM Ethoxyresorufin (MW 239.0)

Ethoxyresorufin	1.207 mg
DMSO	5 ml

Note: Ethoxyresorufin comes in 1mg vials & is very sticky. Warm to room temp and add 5ml of DMSO into ethoxyresorufin vial. Vortex well. Protect from light. May take up 24 or greater hours for all of the sample to go into solution.

1 mM Methoxyresorufin (MW 239.0)

Methoxyresorufin	1.207 mg
DMSO	5 ml

Note: Methoxyresorufin comes in 1mg vials & is very sticky. Warm to room temp and add 5ml of DMSO into methoxyresorufin vial. Vortex well. Protect from light. May take up 24 or greater hours for all of the sample to go into solution.

1 mM Pentoxyresorufin (MW 239.0)

Pentoxyresorufin	1.207 mg
DMSO	5 ml

Note: Pentoxyresorufin comes in 1mg vials & is very sticky. Warm to room temp and add 5ml of DMSO into pentoxyresorufin vial. Vortex well. Protect from light. May take up 24 or greater hours for all of the sample to go into solution.

Resorufin (MW=235.18g)

Store at room temp. Protect from light

Stock solutions.

A) **10.0 mM** in DMSO

Resorufin	11.759mg
DMSO	5ml

B) **1.0 mM** in DMSO

10.0mM stock	0.5 ml
DMSO	4.5ml

C) **0.1 mM** in DMSO

1.0 mM stock	0.5 ml
DMSO	4.5 ml

D) **0.01mM** in DMSO

0.1 mM stock	0.5 ml
DMSO	4.5 ml

APPENDIX D: Western Blotting

Separating Gel Mix - 12.5% acrylamide concentration

Stock Solution	One Gel	Two Gels
Lower Stock (pH 8.8, 1.5M Tris HCl, 0.4% SDS)	8ml	16ml
Acrylamide/Bis Stock (30/0.8%)	13.36ml	23.72ml
distilled water	10.1ml	20.18ml
10% Ammonium Persulfate	160ul	320ul
TEMED	14ul	28ul

Stacking Gel Mix

Stock Solution	One Gel	Two Gels
Upper Stock (pH 6.8, 0.5M Tris HCl, 0.4% SDS)	2.5ml	5.0ml
Acrylamide/Bis Stock (30/0.8%)	1.5ml	3.0ml
distilled water	6ml	12ml
10% Ammonium Persulfate	30ul	60ul
TEMED	10ul	20ul

Procedure

1. Clean the gel set-up with ethanol and make sure seals are lubricated.
2. Make up 10% Ammonium Persulfate solution.
   0.1g Ammonium Persulfate in 1ml distilled water
3. Combine separating gel mix components. Pour 28ml of mix for each gel.
4. Overlay gel with sec butanol
5. Allow 1/2 to 1 hour for polymerization.
6. Pour off sec butanol.
7. Rinse with dH_2O
8. Blot excess H_2O with filter paper.
9. Combine stacking gel components.
10. Pour stacking gel with combs in place.
11. Make sure there are no bubbles under the combs.
12. Polymerize for 1 hour.
13. Dilute samples with 2xSDS sample buffer with BME. Vortex diluted samples.
14. Remove comb.
15. Fill wells with dH_2O and then suction wells dry with vacuum apparatus fitted with a needle tip.
16. Make up 2L of 1xrunning buffer by dilution of 10xrunning buffer.
17. Heat diluted samples and kaleidoscope standard in a dry heat bath set at 90°C for 3-5 minutes.
18. Dry load gel.
19. Carefully top off wells by trickling running buffer along the spacers between wells.

20. Run gel at room temperature at constant Ma (check for bubbles at start) until dye line reaches the bottom of the glass plates.
    one gel: 32 Ma
    two gels: 70Ma
21. Remove gel from electrophoresis apparatus and from between glass plates. Remove stacking gel and discard. Cut upper left corner of the gel for orientation. Place in cold (4°C) transfer buffer.
22. Slide the 0.45uM nitrocellulose membrane under the gel.
23. Assemble transfer sandwich making sure to wet each component with transfer buffer. Assemble on the gray (+) side of the plastic sandwich.
    1)sponge
    2)filter paper
    3)0.45uM nitrocellulose membrane, which has the gel on top.
    4)gel
    5)filter paper - Roll glass tube over filter paper to remove air bubbles from between the gel and membrane.
    6)sponge
24. Orientate sandwich so that the gray (+) sides match up.
25. Fill transfer apparatus with transfer buffer.
26. Drop in stir bar.
27. Run transfer at 4°C (in fridge) at a constant voltage. Check for bubbles and moving stir bar at start.
    30V for 15 hours
28. After transfer, remove gel and filters.
29. Air dry nitrocellulose membrane for approximately 10 minutes. Dispose of gel.
30. Block membrane with room temperature TBS-T with 5% skim milk for 1 hour in a plastic container on a shaker.
31. Remove membrane. Drip off as much 5% solution as possible.
32. Incubate membrane in primary at appropriate dilution (1:1000) in room temperature TBS-T with 1% skim milk for at least 1 hour in a ziplock bag on a rolling platform.
33. Rinse membrane with TBS-T on a shaker in a plastic container.
    A) 5 minutes then dump and get new TBS-T
    B) 10 minutes then dump and get new TBS-T
    C) 15 minutes
34. Incubate membrane with secondary antibody linked to horseradish peroxide at appropriate dilution (1:2000) in 1% skim milk TBS-T for 1 hour at room temperature in a ziplock bag on a rolling platform.
35. Rinse membrane with TBS-T on shaker in a plastic container.
    A) 5 minutes then dump and get new TBS-T
    B) 10 minutes then dump and get new TBS-T
    C) 15 minutes
36. Develop membrane for 5 minutes in equal volumes of Reagent A and B from chemilumnescent substrate (5ml of each).
37. Wrap membranes in cling film and tape down to photograph cassette.

38. Take timer, film, gloves, developing reagents, drying rack and cassette to dark room.
39. Expose blue sensitive film to membranes in cassette for set times.
40. Run film through developer, water, and then fixer. Hang to dry.

Stock Solutions

Acrylamide Stock

	500ml	1L
30% Acrylamide	150g	300g
0.8% N,N-Methlyene-bis-acrylamide	4g	8g

Make up to volume of solution with dH_2O and dissolve with stirring for 1 hour. Filter solution through filter paper and store at 4°C. The solution is stable for 3-4 months.

Lower/Separating SDS-Page stock

	500ml
1.5M Tris base (pH 8.8)	90.825g
0.4% SDS	2g

Dissolve components in 450ml of dH_2O and adjust to pH 8.8. Make up remaining volume with dH_2O. Store at 4°C. Stable for months, possibly up to a year.

Upper/Stacking SDS-Page stock

	100ml
0.5M Tris base (pH 6.8)	6.06g
0.4% SDS	0.4g

Dissolve components in most of the dH_2O and adjust to pH 6.8. Make up remaining volume with dH_2O. Store at 4°C. Stable for months, possibly up to a year.

2x SDS Sample Buffer

	100ml
0.05M Tris base (pH 6.8)	0.605g
1% SDS	1g
30% Glycerine	30ml
0.01% Bromophenol Blue	0.01g

Dissolve Tris base, SDS, glycerine in most of the dH_2O, then adjust to pH 6.8. Add bromophenol blue and make up rest of volume with dH_2O. Stable at room temperature for 1 year. NOTE: Before using buffer for SDS-PAGE β-mercaptoethanol (20ul per 1ml of 2x sample buffer). Add the β-mercaptoethanol fresh on the day of sample dilution.

10x SDS-PAGE Running Buffer

	4L
247.8mM Tris Base	120g
1.918M Glycine	576g
1% SDS	40g

Dissolve all components in enough dH_2O to make up 4L. DO NOT ADJUST pH. Stable at room temperature indefinitely. To prepare 1X Running buffer, dilute 1:10 with dH_2O.

Transfer Buffer

	3L
25mM Tris base	9.084g
192mM Glycine	43.239g
20% Methanol	600ml
0.1% SDS	3g

Mix two batches of all components together and make up remaining volume with dH_2O. DO NOT ADJUST pH. Buffer can be used 3 times, but must be stored at 4°C. Buffer is stable for several months.

Tris-Buffered Saline-Tween 20 (TBS-T)

	4L
20mM Tris base (pH 7.5)	9.6912g
500mM NaCl	116.9g
0.05% Tween 20	2ml

Combine all components and most of the dH_20 volume. Use syringe for Tween 20 as it is very sticky. Adjust pH to 7.5 with HCl and then make up volume with remaining dH_20.

APPENDIX E: Glutathione s-Transferase Specific Activity Assay

Procedure

1. Make up Assay Mixture (enough for 1 plate)

phosphate buffer	14.66ml
glutathione (150mM)	0.20ml
CDNB (60mM)	0.50ml

2. Set up plate reader to stabilize light source and to warm up.
   Select:
   - Kinetics mode
   - Wavelength 340nm
   - Temperature 37°C
   - Scan time 5 minutes
   - Scan interval 15 seconds
3. Thaw and dilute cytosol samples in phosphate buffer (1:10 dilution). Keep samples on ice.
4. Using multichannel pipettor and disposable troughs, pipette 100ul of phosphate buffer into all wells of a microplate.
5. Add four repetitions in 10ul aliquots of each diluted cytosol sample to wells assigned to unknowns. Leave 8 wells with no unknowns added as blanks.
6. Using multichannel pipettor add 100ul of assay mixture to all wells.
7. Read plate immediately.
8. Correct readings for protein concentrations.

Stock Solutions

CDNB (1-chloro, 2,4-dinitrobenzene)

TOXIC- USE RESPIRATOR AND GLOVES.

60mM CDNB	0.121g
Ethanol	10ml

Solution stable for 2-4 days. Label solution as poison.

1 M Phosphate Buffer (pH 6.5)

Stock Solution #1: 0.2M sodium phosphate monobasic solution

NaH_2PO_4	24g
dH_2O	1L

Stock Solution #2: 0.2M sodium phosphate dibasic solution

Na_2HPO_4	28.4g
dH_2O	1L

Phosphate Buffer (0.1M, pH 6.5)

	200ml
Stock Solution#1	68.5ml
Stock Solution#2	31.5ml
dH_2O	100ml

Phosphate Buffer is stable for several weeks.

Glutathione

	150mM
Glutathione	0.461g
Phosphate Buffer	10ml

Solution is highly unstable. Mix fresh each day.

Example Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	U1	U3	U5	U7	U9	U11	U13	U15	U17	U19	U21
B	Blank	U1	U3	U5	U7	U9	U11	U13	U15	U17	U19	U21
C	Blank	U1	U3	U5	U7	U9	U11	U13	U15	U17	U19	U21
D	Blank	U1	U3	U5	U7	U9	U11	U13	U15	U17	U19	U21
E	Blank	U2	U4	U6	U8	U10	U12	U14	U16	U18	U20	U22
F	Blank	U2	U4	U6	U8	U10	U12	U14	U16	U18	U20	U22
G	Blank	U2	U4	U6	U8	U10	U12	U14	U16	U18	U20	U22
H	Blank	U2	U4	U6	U8	U10	U12	U14	U16	U18	U20	U22

APPENDIX F: Cayman Glutathione Assay Kit (Ann Arbor, MI)

Procedure

Liver Processing

1. All glassware should be at 4°C in ice buckets. Start refrigeration of centrifuge at 4°C.
2. Check pH of 20mM Bis Tris buffer. Readjust to 6.6 if necessary. Keep on ice.
3. Prepare one glass test tube, filled with 10 ml of Bis Tris buffer and keep them on ice.
4. Weigh each rat, then sacrifice it by cervical dislocation. Rapidly take out the liver. Rinse the liver with Bis Tris buffer at 4°C.
5. Weigh each liver and place it in tube with Bis Tris at 4°C.
6. Cut livers into pieces with scissors and transfer to large homogenizer with 5ml Bis Tris. Homogenize 5 times at speed 5 (reverse), keeping the tube on ice. Rest one minute on ice. Homogenize 5 times at speed 5 (reverse) and transfer into plastic centrifuge tube, again on ice.
7. Add 5ml MPA reagent, vortex, and let stand for 5 minutes at room temperature.
8. Centrifuge at 5,000 rpm in a microfuge for 5minutes.
9. Collect supernatant and transfer into 3 labeled cyrovials. Freeze immediately at -80°C.

Total Glutathione Assay

1. Equilibrate all reagents to room temperature.
2. Reconstitute kit reagents:
   - Dilute MES Buffer 2X with equal volume of distilled H_2O
   - Reconstitute cofactor mixture with 0.5 ml of distilled H_2O and mix well.
   - Dilute enzyme mixture with 2 ml of MES buffer 1X and mix well.
3. To thawed samples add 50 µl of TEAM reagent per ml of sample (5 µl TEAM reagent in 100 µl sample) to be used in this assay and vortex.
4. Dilute samples (from step 1) 75:1 with MES Buffer 1X (10 µl sample in 740 µl MES Buffer).
5. In separate tubes, prepare standards.
   A. **0 µM** GSSG: 0 µl GSSG standard + 500 µl MES buffer 1X.
   B. **0.25 µM** GSSG: 5 µl GSSG standard + 495 µl MES buffer 1X.
   C. **0.5 µM** GSSG: 10 µl GSSG standard + 490 µl MES buffer 1X.
   D. **1.0 µM** GSSG: 20 µl GSSG standard + 480 µl MES buffer 1X.
   E. **2.0 µM** GSSG: 40 µl GSSG standard + 460 µl MES buffer 1X.
   F. **4.0 µM** GSSG: 80 µl GSSG standard + 420 µl MES buffer 1X.
   G. **6.0 µM** GSSG: 120 µl GSSG standard + 380 µl MES buffer 1X.
   H. **8.0 µM** GSSG: 160 µl GSSG standard + 340 µl MES buffer 1X.
6. Add 50 µl of standard (A-H) to each designated well.
7. Add 50 µl of sample to each unknown well.

8. Cover plate with provided plate cover.
9. Reconstitute DTNB vial with 0.5 ml of distilled H_2O and mix well (Reconstituted DTNB must be used within 10 minutes).
10. Prepare Assay Cocktail

Assay Cocktail	
MES Buffer 1X	11.25 ml
reconstituted cofactor mixture	0.45 ml
reconstituted enzyme mixture	2.1 ml
distilled H_2O	2.3 ml
reconstituted DTNB	0.45 ml

11. Remove plate cover and add 150 µl of Assay Cocktail to each well using a multichannel pipettor.
12. Replace cover and incubate the plate in the dark on an orbital shaker.
13. Measure the absorbance at 405 nm using a microplate reader at 5 minute intervals for 30 minutes (6 readings total).

Glutathione Disulfide (GSSG) Assay

1. Equilibrate all reagents to room temperature.
2. Reconstitute kit reagents:
   - Dilute MES Buffer 2X with equal volume of distilled H_2O.
   - Reconstitute cofactor mixture with 0.5 ml of distilled H_2O and mix well.
   - Dilute enzyme mixture with 2 ml of MES buffer 1X and mix well.
3. In separate tubes, prepare standards.
   A. **0 µM** GSSG: 0 µl GSSG standard + 500 µl MES buffer 1X.
   B. **0.25 µM** GSSG: 5 µl GSSG standard + 495 µl MES buffer 1X.
   C. **0.5 µM** GSSG: 10 µl GSSG standard + 490 µl MES buffer 1X.
   D. **1.0 µM** GSSG: 20 µl GSSG standard + 480 µl MES buffer 1X.
   E. **2.0 µM** GSSG: 40 µl GSSG standard + 460 µl MES buffer 1X.
   F. **4.0 µM** GSSG: 80 µl GSSG standard + 420 µl MES buffer 1X.
   G. **6.0 µM** GSSG: 120 µl GSSG standard + 380 µl MES buffer 1X.
   H. **8.0 µM** GSSG: 160 µl GSSG standard + 340 µl MES buffer 1X.
4. To thawed samples add 50 µl of TEAM reagent per ml of sample (5 µl TEAM reagent in 100 µl sample) to be used in this assay and vortex.
5. Dilute samples (from step 1) 4:1 with MES Buffer 1X (10 µl sample in 390 µl MES Buffer).
6. Add 40 µl of 1M 2-vinylpyridine to each sample.
7. Add 5 µl of 1M 2-vinylpyridine to each standard.
8. Vortex the samples and standards and then incubate at room temperature for 60 minutes.
9. Add 50 µl of standard (A-H) to each designated well.
10. Add 50 µl of sample to each unknown well.
11. Cover plate with provided plate cover.
12. Reconstitute DTNB vial with 0.5 ml of distilled H_2O and mix well (Reconstituted DTNB must be used within 10 minutes).

13. Prepare Assay Cocktail

Assay Cocktail	
MES Buffer 1X	11.25 ml
reconstituted cofactor mixture	0.45 ml
reconstituted enzyme mixture	2.1 ml
distilled H_2O	2.3 ml
reconstituted DTNB	0.45 ml

14. Remove plate cover and add 150 µl of Assay Cocktail to each well using a multichannel pipettor.
15. Replace cover and incubate the plate in the dark on an orbital shaker.
16. Measure the absorbance at 405 nm using a microplate reader at 5 minute intervals for 30 minutes (6 readings total).

Glutathione (GSH) Concentration

Subtract the GSSG concentration from the total glutathione concentration for each sample.

Stock Solutions

Bis Tris Buffer pH 6.6 at 4°C

	20mM
Bis Tris	4.184g
distilled H_2O	1L

2-vinylpyridine

	1M
2-vinylpyridine	108 µl
ethanol	892 µl

MPA reagent

Metaphosphoric Acid	10g
distilled H_2O	100ml

TEAM reagent

Triethanolamine	531µl
distilled H_2O	469 µl

Example Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std A	Std A	Std A	U1	U1	U1	U9	U9	U9	U17	U17	U17
B	Std B	Std B	Std B	U2	U2	U2	U10	U10	U10	U18	U18	U18
C	Std C	Std C	Std C	U3	U3	U3	U11	U11	U11	U19	U19	U19
D	Std D	Std D	Std D	U4	U4	U4	U12	U12	U12	U20	U20	U20
E	Std E	Std E	Std E	U5	U5	U5	U13	U13	U13	U21	U21	U21
F	Std F	Std F	Std F	U6	U6	U6	U14	U14	U14	U22	U22	U22
G	Std G	Std G	Std G	U7	U7	U7	U15	U15	U15	U23	U23	U23
H	Std H	Std H	Std H	U8	U8	U8	U16	U16	U16	U24	U24	U24

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