ECOTOXICOLOGICAL RISKS ASSOCIATED WITH LAND TREATMENT OF PETROCHEMICAL WASTES IN OKLAHOMA

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Submitted to the faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May, 2002

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WITH LAND TREATMENT OF

PETROCHEMICAL WASTES

IN OKLAHOMA

Dissertation Approved:

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PREFACE

Environmental contamination has become a widespread and persistent problem throughout the world. Landfarming, as a method of treating petrochemical waste, uses microbial degradation to reduce soil contaminant levels. However, landfarming is of limited value for inorganic contaminants and high molecular weight hydrocarbons. The chronic effect of residual contamination on wildlife is unknown, especially among terrestrial mammals. Rodents inhabiting abandoned landfarms are a good model for studying these effects because they are plentiful, in close contact with contaminated soils, and have a limited home range. I used cotton rats (*Sigmodon hispidus*) as a model species and investigated the effects of residual petrochemical waste on the immune system, hematology, organ morphology, reproduction, and population dynamics of these rodents. In addition, I observed rodent assemblage structure for changes in composition on abandoned landfarms. This dissertation includes four manuscripts formatted for submission to *Journal of Toxicology and Environmental Health* (Chapter II), *Ecology* (Chapter III), *Journal of Mammalogy* (Chapter IV), and *Bulletin of Environmental Contamination and Toxicology* (Chapter V). The manuscripts are complete as written and need no supporting material.

ACKNOWLEDGEMENTS

This study was funded by a grant from the United States Environmental Protection Agency (EPA). Travel funding was provided by the Department of Zoology, Oklahoma State University and the Graduate Committee, Oklahoma State University. The U. S. Government is authorized to reproduce and distribute reprints for government purposes not withstanding any copyright notation herein.

I would like to thank Dr. Robert Lochmiller for teaching me the various immune techniques used during this project. Although he did not live to see the conclusion of my dissertation I think he would be happy with the result. After the death of Dr. Lochmiller, Drs. Eric Hellgren and David Janz were there to take me on as their graduate student and have treated me as one of their own even though my project did not exactly match their own research interests.

I would also like to thank Dan Rafferty for being the first person to welcome me to the lab and for his support and help in the lab and field. Steve Ditchkoff was also a welcome friend that provided the unwritten rules of the lab and introduced me around the department when I first arrived at OSU.

I would like to thank the people that helped me in the field and laboratory. This study could not have been done without the trapping efforts of Eric Webb and Dan Rafferty, who took the time to trap rats for two years. Terry Coffey was a welcome addition to my field study and made the time spent weighing rats and mice much

smoother. Keri Snethen was both helpful and entertaining during terminations. Finally, I would like to thank Dr. Claypool and Dr. Payton for their help with statistics.

Unfortunately, during my doctoral program I experienced a number of tragedies that might have led me into an unrecoverable darkness if it were not for one thing, the banjo. I would like to thank John Hickman and Byron Berline for providing a place of support that acted as an anchor in my life. It has been very important to me to go to Byron's bluegrass concerts every two weeks and to take banjo lessons from John, one of the best pickers in the U. S. In addition, Jeff Mohr and Jay Clark proved to be good picking partners and good friends.

I would not be on the path I am traveling if not for the influence of Robin Oliver. Her enthusiasm for biology has made me realize that no better life can be had than one that pursues an understanding of how the Universe functions. Throughout my studies I have always counted on the support and advice of Robin and her husband Sheldon. They have been there to inspire me and provide an understanding ear. Even to the last, Sheldon gave me support and comfort and made me feel like the world was at my feet. The advice and support that Robin and Sheldon gave will always be with me, and when I need to hear encouragement from Robin and Shel all I have to do is listen to the wind blow through the pines.

There have been a number of factors involved in my choice to be a biologist. My parents took me camping throughout my childhood, Robin Oliver's influence during high school, and of course the numerous wilderness documentaries I was exposed to during my childhood. The most important of those are the True Life Adventures series presented by Walt Disney. I would like to thank James Algar for his vision of nature and

for providing a fun and interesting (although not biologically sound at times) view of nature that was instrumental in getting me hooked on nature.

I would also like to thank Jennifer L. Parsons for her help and support during the dark times in my life. She has been a bright spot to focus on when I needed encouragement the most. I look forward to exploring life with her. Finally, I would like to thank my Mom and Dad for their continued support in my choice of professions. They have always been there when I needed them and have provided substantial financial and emotional support.

For Sheldon:

With whom biology was not only a career, but a way of life.

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CHAPTER I

OIL IN OKLAHOMA: HISTORY AND CONSEQUENCES OF PETROCHEMICAL WASTE

HISTORY

The first oil strike in Oklahoma was made in 1859, and produced approximately 10 bbls/day (Franks et al. 1981). However, the strike ran dry within 2 yrs and no further petroleum exploration was made in Oklahoma until the end of the Civil War. Primitive conditions prevented the movement of large equipment into Oklahoma, and legal problems with drilling on Indian Territory made oil production prohibitively expensive. It was not until 1897 that renewed oil drilling was seen in Oklahoma when the "Nellie Johnstone No. 1" well was drilled in what is now Bartlesville, OK (Franks et al. 1981). This was the first commercially viable well, producing 50 bbls/day of crude over the next 50 yrs.

Oil prospectors began drilling in the land occupied by what is now Tulsa, OK, in 1901. The strike was so rich and shallow that it rivaled the fields in Texas. Within a month of the first strike, over 1,000 oilmen moved to Tulsa and land leases quadrupled (Franks et al. 1981). Over the next five years, additional pools of oil were discovered by wildcatters in Sapulpa, Cushing, Drumright, Muskogee, and Glen Pool, OK. The strike at Glen Pool proved to be the richest in the state of Oklahoma and produced over 20,000,000 bbls of oil in 1908 and continued producing almost 2,000,000 bbls annually until 1935 (Franks et al. 1981). Consequently, the development of Tulsa was

dramatically affected by the rich oil deposits found there. Before 1901, Tulsa had a population of less than 2,000 people; however, by 1930 it had grown to 140,000.

Production of oil in Oklahoma continued through the 1940s and 1950s until the price of oil dropped by the development of oil wells and refineries in the Middle East. Production of oil in Oklahoma declined during the 1970s and stopped in all but the most productive areas during the 1980s. Refineries were abandoned and the petrochemical waste associated with those sites was often left in storage tanks. In addition, historically, crude oil and waste were often dumped on land set aside for storage of these products. The sight of large lakes of crude oil was not uncommon during the early years of Oklahoma's oil boom (Franks et al. 1981). Oklahoma's oil history has led to the production of numerous sites throughout Oklahoma that have some history of petrochemical contamination.

Advances in chemistry during the early 1900s led to widespread synthesis and use of chemicals. Pesticides, insecticides, medicines, and petroleum products all saw wide-spread development and use during the 1930-50s. Concern for the health and welfare of United States citizens led to the establishment of the National Institutes of Health in 1930 and the enactment of legislation developed to protect consumers and citizens from the toxic effect of chemicals in food and the environment (Klassen 1996). In the 1960s, analytical techniques were developed that were sensitive enough to detect small quantities of contaminants and enabled scientists to link health effects to minute quantities of contaminants. In response to the concern for environmental contaminants the United States formed the Environmental Protection Agency (EPA). The EPA was

responsible for monitoring risks to the public and regulating clean-up of contaminated sites.

The discovery of a contaminated site at Love Canal, NY led to the development of the Toxic Substances Control Act (TSCA) and the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), or the "Superfund Bill" (Peterle 1991, Klassen 1996). These two pieces of legislation allowed the EPA to monitor the release and effects of contaminants from their creation to disposal. The TSCA has four major objectives: 1) gather information on chemicals; 2) require testing of chemicals for possible risks; 3) screen new chemicals for risks; and 4) control chemicals to prevent risks (Peterle 1991). CERCLA established a fund to provide money for the clean-up of sites contaminated with hazardous waste.

The disposal of petroleum products and waste in land treatment facilities is strictly regulated under the Resource Conservation and Recovery Act (RCRA). Standards were developed under this legislation to protect human health and the environment for every step in the generation and disposal of petroleum waste (Klassen 1996). In 1984, the Hazardous and Solid Waste Amendment was added to RCRA to reduce direct land disposal of hazardous waste by refineries (Bryant and Moores 1991).

The latest round of legislation concerning the disposal of hazardous waste elevated protection to include wildlife. The National Environmental Policy Act (NEPA) was enacted to prevent damage to the biosphere (Peterle 1991). NEPA mandated the use of environmental assessment statements in all activities that may affect wildlife or habitats. Additional protection is provided to plants and animals in danger of extinction through the Endangered Species Preservation Act.

LANDFARMING

The combination of increased government regulation, advances in technology, and loss of available land has led to the need for safer, more efficient waste disposal solutions. One such solution has been the development of landfarming for municipal and petrochemical waste treatment (Line et al. 1996). It is a relatively low-cost solution to treating waste and requires little effort or equipment. Indeed, landfarming costs less than half than treating waste with chemical precipitation or demineralization (Green and Moore 1985). The most cost-prohibitive aspect of a landfarm is the large area required to treat suitable quantities of waste. During landfarming, waste oil is applied directly to a prepared bed of soil and is inoculated with microbes that consume petroleum. The soil-waste mixture is fertilized and turned over to promote maximal microbe growth and metabolism. However, most research has focused on improving the efficiency of landfarming rather than on the effects of residual contamination on wildlife.

It is estimated that refineries produce 0.32 tons of waste per thousand barrels of crude oil processed (Bryant and Moores 1991). Petroleum waste is a complex and highly concentrated mixture of hydrocarbons, metals, and caustics, and is generated in every process involved in petroleum refining (Bryant and Moores 1991). During the refining process, many chemical and physical methods are used to remove impurities from the petroleum product. Separation units remove impurities from the usable fraction of crude oil through the use of flotation or filter separation. The use of hydrofluoric acid as a catalyst for alkylation of hydrocarbons is responsible for the high fluoride content of refinery waste, and sulfides present in wastewater promote the precipitation of metals found in oil (Bryant and Moores 1991). In addition, caustics, such as NaOH, are used to

extract acidic compounds from the petroleum product and are responsible for adding phenolics, sulfides, and cyanides to waste (Bryant and Moores 1991).

Polyaromatic hydrocarbons (PAHs) are naturally occurring substances found in soils in concentrations of 1-10 μg/kg (Jones et al. 1989), and are also a major component of petrochemical waste, increasing soil PAH levels above naturally occurring levels. Low-molecular-weight PAHs are degraded by microbes, but larger PAHs are resistant to degradation (Zeng et al. 2000). Wilson and Jones (1993) report that no degradation was observed PAHs containing structures with 5 or more aromatic rings. The persistence, and carcinogenicity, of high molecular weight PAHs add to the potential toxicity of soils from petroleum landfarms.

The success of landfarming is dependent on the ability of microbial agents to metabolize contaminants, which is highly influenced by environmental factors. The effectiveness of microbial breakdown is affected by soil pH (Yeh and Novak 1994, Ijah and Antai 1995), sludge application rates (Li et al. 1995), oxygen diffusion in the soil (Huesemann and Truex 1996), fertilizer use, and soil temperature (Kelley 1983). Without the maintenance of proper soil conditions, biodegradation of petroleum waste may decrease from 4,644 mg/kg soil to < 100 mg/kg soil within one year (Line et al. 1996).

Frequent small applications of waste sludge produce higher degradation rates than single large applications (Dibble and Bartha 1979). A rate of 1,200 barrels/acre/year is given as the maximum amount to be treated in landfarming with individual applications of sludge not to exceed 150 barrels/acre (Kelley 1983). Microorganisms that perform the degradation process require certain environmental conditions to efficiently degrade petroleum waste. A critical condition is the soil pH. Ijah and Antai (1995) state that

highly acidic soils will decrease the degradation ability of microorganisms. Similarly, Yeh and Novak (1994) found petroleum degradation to occur only in soils with pH above 5.5.

A major inhibitory effect on oxygen diffusion is the moisture content of the soil (Huesemann and Truex 1996). Soils saturated with water severely reduce the oxygen diffusion coefficient and slow the process of biodegradation (Huesemann and Truex 1996). The addition of fertilizer can increase degradation rates by 2-3 times over the life of the treatment (Davis et al. 1998). Oil decomposition rates have been reported as averaging 14 barrels/ha/month without fertilization and 28 barrels/ha/month with nitrogen and phosphorus addition (Kelley 1983). Microbial breakdown also decreases at lower temperatures with minimal degradation occurring at temperatures below 21°C.

The size and conformation of the contaminants can also affect the rate of microbial degradation. A large percentage of petrochemical waste consists of hydrocarbon chains that are used as a substrate for microbial degradation. Hydrocarbon chains of 10-28 carbons in length provide an appropriate substrate for microbial degradation and are more readily consumed than longer chain and polyaromatic hydrocarbons (Line et al. 1996).

Landfarming has been used to treat petrochemical waste in a number of different sites throughout the world. For example, landfarming of petroleum waste from a refinery in Ghent showed hydrocarbon degradation of 15 g/kg/yr during the first 12 months (Genouw et al. 1994). However, the rate of degradation decreased to 4 g/kg/yr over the following 40 months. At these rates, PAH contamination would persist on that site for a minimum of 15 yrs. Duncan et al. (1999) described the landfarming of petrochemicals in

Oklahoma. Four years after the initial contamination, an average of 6,800 mg/kg of hydrocarbon remained in the soil.

Bioremediation techniques such as landfarming have been hailed as environmentally friendly, cost-effective, and efficient methods of reducing hazardous waste (Green and Moore 1985). With a properly designed bioremediation project, most petroleum waste organics can be degraded; however, the persistence of inorganic contaminants and carcinogenic PAHs make petrochemical waste a long-term problem for wildlife inhabiting abandoned landfarms (Milne et al. 1998).

It is reported that the market for waste treatment through bioremediation techniques, such as landfarming, will grow from $228 million in 1995 to $475 million in 2000 (Anonymous 1996). Future research in bioremediation may develop new techniques that will lower the cost and increase the efficiency of bioremediation. Uncertainty about the effects of contamination present in the environment during and after landfarming has led to the development of alternative methods such as controlled composting (Milne et al. 1998). Research has focused on the effects of environmental conditions on the rate of biodegradation (Dibble and Bartha 1979), but few studies have been conducted to determine the lingering effects of non-biodegradable products remaining after bioremediation.

EFFECTS OF CONTAMINATION

Contaminants are known to spread through trophic levels by ingestion and concentration in tissues (Batty et al. 1990). These processes lead to accumulation in the tissues of animals higher on the food web, and may alter animal physiology. Changes in physiology not only change an individual's ability to survive and reproduce, but may

affect the dynamics of the entire population living in the contaminated area (Lochmiller and Dabbert 1993).

Lochmiller (1996) proposed that contaminants may affect population dynamics through alterations in the immune function of individuals. The immune selection hypothesis proposed by Lochmiller (1996) suggests that immunosuppression from contaminants will make individuals more susceptible to disease, increasing mortality and affecting population structure. Congden et al. (2001) suggested that indirect effects from contamination might include increased energy expenditure for immune response and detoxification. The added energy expenditure would reduce the amount of energy available for reproduction. These changes in turn, affect community composition by creating a "bottom-up" chain of disturbance (Lampert and Sommer 1997).

Laboratory experiments on the effects of soil extracts from the Love Canal, NY dumpsite indicated that low birth weight, decreased maternal weight gain, and increased fetal resorption occurred in rats exposed to contaminated soil (Silkworth et al. 1986). Batty et al. (1990) found a similar effect on a wild population of white-footed mice (*Peromyscus leucopus*) living on polychlorinated-biphenyl contaminated sites. Additionally, densities of microtine rodents were found to be lowest immediately adjacent to a copper-nickel smelter in Russia and increased along a pollution gradient generated from aerial emissions of heavy metals and sulfur dioxide (Kataev et al. 1994). Not only have population changes been observed, but community differences have been noted as well (McMurry 1993). Decreased dominance by cotton rats and an absence of several uncommon rodent species typified contaminated sites in the tallgrass prairie

ecosystem of Oklahoma (McMurry 1993). It is clear that contaminants affect the health and status of individuals, populations, and communities living on contaminated sites.

Although studies have been done to understand the mechanisms that catabolize petroleum waste, few studies have examined the long-term effects of residual contamination on the plants and animals that inhabit abandoned landfarms.
The effect of chronic exposure to residual contaminants on mammalian immune systems is of importance, not only to wildlife, but also to humans living in areas surrounding these sites. If residual contaminants affect the immune systems of mammals living around landfarms, a reevaluation of landfarming as a safe method of waste disposal is warranted.

STUDY SITES

This study was funded by the United States Environmental Protection Agency (EPA) to evaluate the effects of chronic exposure to residual petrochemical waste. This study was conducted on 5 pairs of contaminated and reference sites, referred to as Units 1-5, throughout Oklahoma (Figure 1). Reference sites were located as close to petrochemical sites as possible to reduce climatological and vegetational variation. Each contaminated and reference pair was designated as a study unit. Three sites (Units 1-3) were abandoned landfarms with no further cleanup and two sites (Units 4 and 5) were abandoned landfarms that had undergone cleanup by the Environmental Protection Agency, including the removal and additional landfarming of contaminated soil. Each treatment site was accompanied by a matched reference site that had no history of contamination. All units therefore consisted of a petroleum landfarm and a matched reference site. All sites were located in the tallgrass prairie ecosystem and consisted of

disturbed grassland habitat. Sites were located in southwestern (Units 1 and 2), north-central (Unit 3), and eastern (Units 4 and 5) Oklahoma.

Units 1 and 2 consisted of 162 ha on an abandoned refinery that originally produced aviation fuel during the 1920's (Coleman 1997). Over the next 60 years, the refinery expanded and changed owners until 1980, when the refinery began producing automotive gasoline, diesel fuel, fuel oil, liquid propane gas, petrochemical feedstock, and petroleum coke with a capacity of 55,000 bbls of oil per day (Coleman 1997). Operation of the refinery ceased in July 1983, and treatment and storage of the refineries contents were handled under the Resource Conservation and Recovery Act (Coleman 1997). Unit 3 was located on recently abandoned landfarm plots inside an active refinery. Landfarming is still utilized within the refinery as a method of petroleum waste disposal, and little information is known as to the history of the site.

The landfarms at Units 4 and 5 were located on approximately 52 ha and operated as a petrochemical waste dump location for local oil companies from 1976 to 1980 (Kelley 1985). During that period, over 300,000 bbls of petroleum-based sludge were transported to the site from several sources. Site cleanup began in 1981 with landfarming used to treat contaminated soils (Kelley 1985). Sites were plowed and strip planted with 3-m wide grass strips interspersed with 8.5-m wide landfarm strips. Upon completion, the site was seeded with Bermuda grass (*Cynodon dactylon*), Chinese lespedeza (*Sericea lespedeza*), and legumes, such as sweetclover (*Melilotus* spp.; Kelley 1983). These species were chosen for their effectiveness in erosion control and as potential livestock forage.

Reference sites were determined by a number of factors that provided the closest match to each landfarm site by vegetation. However, due to the limited amount of public land available in Oklahoma, reference sites were located 1 to 30 km from their paired landfarm site. In addition to providing a location with similar vegetation characteristics, owners of the reference sites insured that grazing, burning, and mowing would not take place on or around the reference site for the duration of the study.

Oklahoma lies in an ecological transition zone and can have large climatological variability extending across the state. Temperature and rainfall during this study for the three study sites differed, with the southwest experiencing higher temperatures and less rainfall, north-central having colder temperatures, and eastern Oklahoma experiencing higher precipitation (Figures 2 and 3).

Soil metal and polyaromatic hydrocarbon (PAH) concentrations were measured by the Department of Plant and Soil Sciences as part of this study and the results are presented in Schroder et al. (2002), and a summary of these findings are presented below. Soils from all sites had detectable levels of all analyzed metals, with landfarms having increased concentrations of As (2 to 4-fold), Cr (3 to 85-fold), Cu (2 to 140-fold), Pb (2 to 150-fold), and Sr (1.5 to 14-fold, Table 1). Total F was also elevated on all landfarms reaching levels 6 – 34 times that of reference sites. The landfarms at Units 1 and 3 had the highest soil metal concentrations for Cr, Cu, Pb, and F.

Schroder et al. (2002) reported that PAHs and total petroleum hydrocarbons (TPHs) were commonly below detection levels (BDL = 0.004 mg/kg for PAHs and 0.10 mg/kg for TPHs) on reference sites, and were only slightly elevated on landfarms (Table 2). Individual PAHs elevated on landfarms above reference site concentrations were

naphthalene, acenapthene, phenanthrene, and benzo(g,h,i)perylene and reached levels more than 68 times that of background levels. The landfarms at Units 4 and 5 had PAH levels that were typically an order of magnitude lower than the other landfarms. The landfarms at Units 1 and 3 had the highest concentrations of PAHs. TPH levels were elevated on landfarms (1025.58 ± 212.32 mg/kg) compared to reference (31.72 ± 8.23 mg/kg) sites.

Although sites from this study were only exposed to petrochemical waste, differences in the refining process and petroleum products created great variation in the composition of waste applied to each site. As a result, individual sites differed in the composition and concentration of contaminants, with the landfarms at Units 1 and 3 showing the highest concentrations for most contaminants. Differences in contaminant profiles will undoubtedly affect the ability to detect toxic responses in animals and plants living on the landfarms and make comparisons between sites difficult at best. The low levels of TPH and PAHs found across all sites support landfarming as a potential bioremediation technique for organic contaminants, however, it is not known if the presence of potentially immunosuppressive PAHs and heavy metals at the concentrations observed will cause effects in wildlife living on abandoned landfarms.

This study will investigate these effects at the cellular, organ, population, and community level. In addition, an immunological assay will be adapted and evaluated for use as a new biomarker for immunotoxicity. I hypothesize that residual petrochemical contamination will affect individuals inhabiting abandoned landfarms. In particular, I hypothesize that contaminants will (1) alter immune function and hematology in cotton rats, (2) cotton rat populations inhabiting landfarms will have lower densities, survival,

and fecundity, and (3) the structure of rodent assemblages inhabiting landfarms will differ from rodent assemblages found on reference sites. The results of this study will be made available in a final report to the EPA and will be published in a series of peer-reviewed articles.

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Table 1. Concentration of metals in soils from landfarm and reference sites in Oklahoma. Data are presented as mean (mg/kg) and standard error (SE) for each study site (Unit) and are summarized from Schroder et al. (2002).

	Unit 1				Unit 2				Unit 3			
	Reference		Landfarm		Reference		Landfarm		Reference		Landfarm	
Metal	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
As	3.47	0.42	12.01	0.52	2.18	0.09	3.57	0.62	4.71	0.16	15.15	1.31
Ba	237.71	57.36	288.98	8.63	147.5	6.11	101.39	6.92	197.84	9.02	309.78	14.96
Cd	0.04	0.02	0.36	0.3	0.07	0.01	0.09	0.02	0.37	0.05	1.1	0.06
Cr	18.81	2.82	300.2	40.72	12.85	1.34	31.04	3.95	24.9	0.89	1652	218.01
Cu	6.14	1.13	94.54	8.22	7.1	0.96	11.42	1.49	10.02	0.81	954.85	95.89
F[1]	141.29	16.90	5545.5	686.04	126.15	10.27	2727.6	845.45	262.88	17.67	3026.1	761.45
F[2]	5.42	0.82	651.69	25.37	2.53	0.21	946.41	98.08	5.51	0.5	291.76	29.62
Ni	12.49	2.37	25.06	0.75	7.57	0.78	7.63	0.47	14.64	0.74	30.15	3
Pb	11.26	2.02	1491.9	150.04	6.41	0.39	54.59	9.36	11.43	1.03	1184.6	263.62
Sr	18.43	5.45	77.69	3.34	16.78	1.46	17.96	1.88	35.44	2.21	309.26	38.98
V	27.22	3.12	50.07	0.96	19.24	1.46	17.38	1.05	48.04	2.26	32.66	1.2
Zn	42.05	4.83	253.42	28.73	40.31	3.17	52.34	6.2	83.45	5.72	877.8	71.13

[1]Total F

[2]Bioavailable F

Table 1. Continued

	Unit 4				Unit 5			
	Reference		Landfarm		Reference		Landfarm	
Metal	Mean	SE	Mean	SE	Mean	SE	Mean	SE
As	3.72	0.07	7.55	0.68	3.10	0.28	9.53	1.96
Ba	142.90	4.03	210.76	15.25	145.18	9.25	250.08	22.61
Cd	0.13	0.01	0.19	0.03	0.04	0.02	0.02	0.02
Cr	18.69	0.38	110.64	18.83	21.49	1.58	53.06	7.63
Cu	5.26	0.22	21.32	3.06	5.65	0.38	12.57	1.11
F[1]	119.23	5.45	5169.28	1207.21	154.06	11.48	980.69	342.46
F[2]	5.53	0.44	843.47	90.65	3.50	0.26	222.62	94.86
Ni	9.00	0.12	15.76	2.22	11.07	0.99	18.36	1.93
Pb	13.99	3.20	29.78	4.33	7.97	0.24	18.74	1.45
Sr	19.35	0.75	38.44	2.55	21.42	1.74	31.90	2.48
V	30.57	0.53	39.03	3.31	30.29	2.29	51.24	3.22
Zn	48.48	0.98	308.39	47.34	49.79	3.52	154.34	25.5

[1]Total F

[2]Bioavailable F

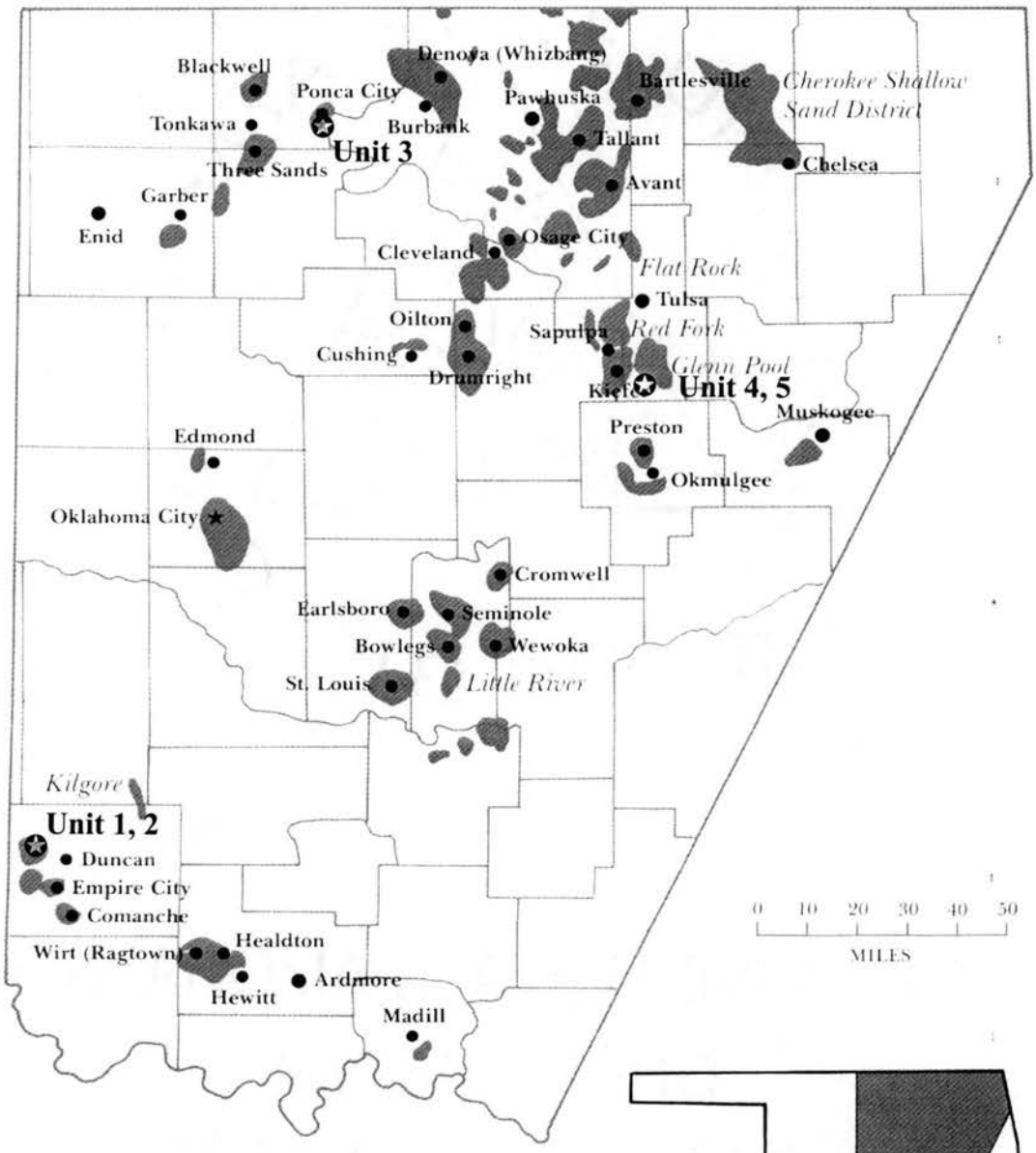
Table 2. Concentration of Polyaromatic hydrocarbons (PAHs) in soils from landfarm and reference sites in Oklahoma. Data are presented as mean (mg/kg) and standard error (SE) for each study site (Unit) and are summarized from Schroder et al. (2002). Contaminants with concentrations below detection level (0.004 mg/kg) are indicated by BDL.

	Unit 1				Unit 2				Unit 3			
	Reference		Landfarm		Reference		Landfarm		Reference		Landfarm	
Polyaromatic Hydrocarbon (PAH)	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Acenapthene	BDL	BDL	BDL	BDL	BDL	BDL	0.0047	0.0007	BDL	BDL	0.0050	0.0008
Acenapthylene	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	0.0067	0.0009
Anthracene	BDL	BDL	0.2777	0.0603	BDL	BDL	0.0262	0.0029	BDL	BDL	1.3713	0.3908
Benzo(a)anthracene	BDL	BDL	0.2713	0.0430	BDL	BDL	0.0502	0.0077	0.0093	0.0018	1.1863	0.1981
Benzo(a)Pyrene	0.0073	0.0002	0.5473	0.0848	BDL	BDL	0.0458	0.0093	0.0120	0.0018	1.0030	0.2719
Benzo(b)fluoranthene	0.0072	0.0002	0.2387	0.0445	BDL	BDL	0.0252	0.0025	0.0137	0.0012	1.2283	0.2670
Benzo(g,h,i)perylene	0.0043	0.0002	3.8087	0.6931	BDL	BDL	0.1303	0.0317	0.0092	0.0025	10.2403	2.0408
Benzo(k)fluoranthene	BDL	BDL	BDL	BDL	BDL	BDL	0.0130	0.0010	BDL	BDL	0.0903	0.0442
Chrysene	BDL	BDL	0.4677	0.0802	BDL	BDL	0.0743	0.0093	0.0137	0.0043	2.7043	0.6974
Dibenz(a,h)anthracene	0.0042	0.0002	0.4147	0.0693	BDL	BDL	0.3233	0.0038	0.0075	0.0006	0.9070	0.2295
Fluoranthrene	BDL	BDL	0.0287	0.0157	0.0043	0.0003	0.0098	0.0013	0.0047	0.0005	0.3007	0.0404
Fluorene	BDL	BDL	BDL	BDL	BDL	BDL	0.0043	0.0002	BDL	BDL	0.0358	0.0187
Indeno(1,2,3-CD)pyrene	0.0047	0.0004	0.4143	0.0637	BDL	BDL	0.0275	0.0037	0.0088	0.0004	1.4010	0.4040
Napthalene	0.0045	0.0003	0.6073	0.1114	0.0042	0.0002	0.0327	0.0040	0.0053	0.0004	0.5055	0.0780
Phenanthrene	0.0477	0.0225	0.5957	0.1275	BDL	BDL	0.1678	0.0807	0.0687	0.0166	1.6300	0.1787
Pyrene	BDL	BDL	0.4340	0.0629	BDL	BDL	0.0577	0.0072	0.0085	0.0021	1.3877	0.2329
Total Petroleum Hydrocarbons	23.5	13.5	418.67	147.71	24.6	8.6	760.33	328.19	77.0	1.0	2027.5	788.64

Table 2. Continued

	Unit 4				Unit 5			
	Reference		Landfarm		Reference		Landfarm	
Polyaromatic Hydrocarbon (PAH)	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Acenapthene	BDL	BDL	0.0060	0.0011	BDL	BDL	0.0042	0.0001
Acenapthylene	BDL	BDL	0.0777	0.0608	BDL	BDL	0.0047	0.0005
Anthracene	BDL	BDL	0.0792	0.0381	BDL	BDL	0.0145	0.0034
Benzo(a)anthracene	BDL	BDL	0.0238	0.0052	0.0047	0.0002	0.0127	0.0015
Benzo(a)Pyrene	0.0072	0.0002	0.0175	0.0066	0.0070	0.0010	0.0132	0.0018
Benzo(b)fluoranthene	0.0083	0.0002	0.0323	0.007	0.0075	0.0011	0.0170	0.0018
Benzo(g,h,i)perylene	BDL	BDL	0.8595	0.2498	BDL	BDL	0.1073	0.0223
Benzo(k)fluoranthene	BDL	BDL	0.0108	0.0022	BDL	BDL	0.0042	0.0002
Chrysene	BDL	BDL	0.0447	0.0097	BDL	BDL	0.0250	0.0049
Dibenz(a,h)anthracene	BDL	BDL	0.1063	0.0499	0.0052	0.0004	0.0305	0.0056
Fluoranthrene	BDL	BDL	0.0090	0.0010	BDL	BDL	0.0050	0.0004
Fluorene	BDL	BDL	0.0083	0.0019	BDL	BDL	0.0047	0.0005
Indeno(1,2,3-CD)pyrene	0.0058	0.0004	0.0607	0.0183	0.0063	0.0008	0.0258	0.0043
Napthalene	0.0067	0.0008	0.2545	0.0853	0.0048	0.0006	0.0528	0.0175
Phenanthrene	BDL	BDL	0.1478	0.0639	BDL	BDL	0.0303	0.0060
Pyrene	BDL	BDL	0.0578	0.0276	BDL	BDL	0.0140	0.0026
Total Petroleum Hydrocarbons	23.5	9.5	1,683.3	136.1	BDL	BDL	369.8	85.5

Figure 1. Location of study sites in relation to major oil deposits in Oklahoma. Study sites consisted of a matched pair of sites (Units 1 - 5): an abandoned landfarm and an uncontaminated reference site, they are represented by a circle-star symbol. Map adapted from Franks et al. (1981).



Early Oklahoma Oil Centers

Figure 2. Mean monthly precipitation recorded from the nearest mesonet weather station to Duncan, Mounds, and Ponca City, Oklahoma. Daily precipitation recordings are averaged to provide mean monthly precipitation values.

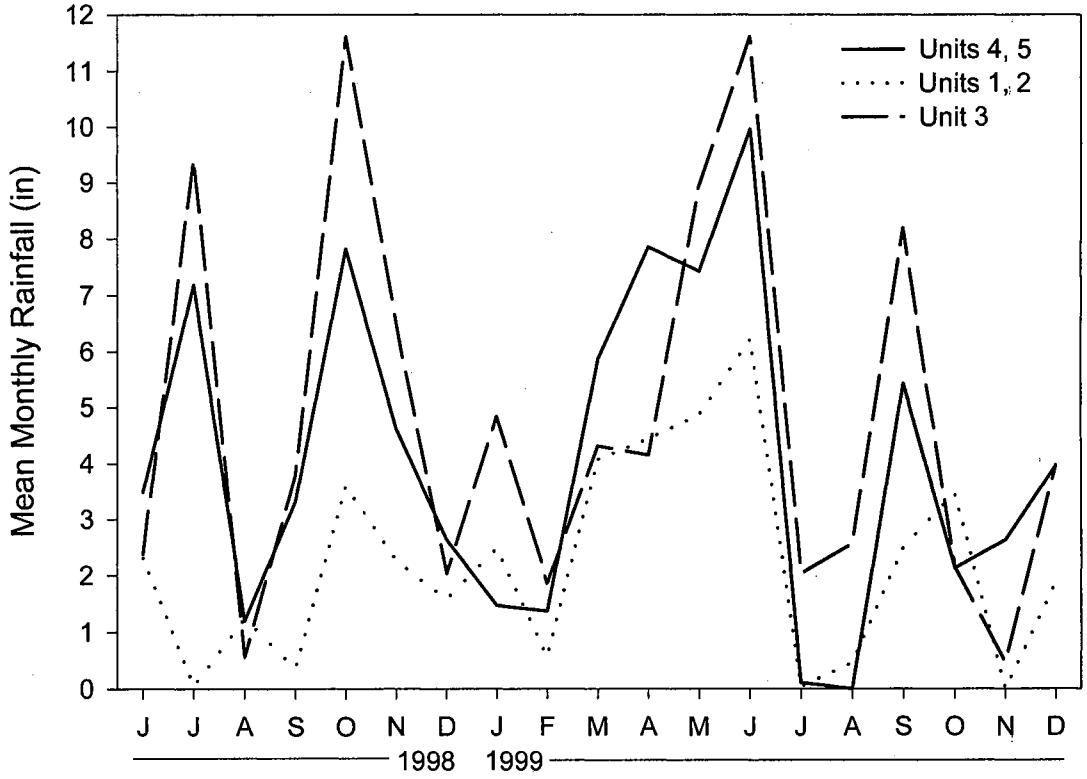
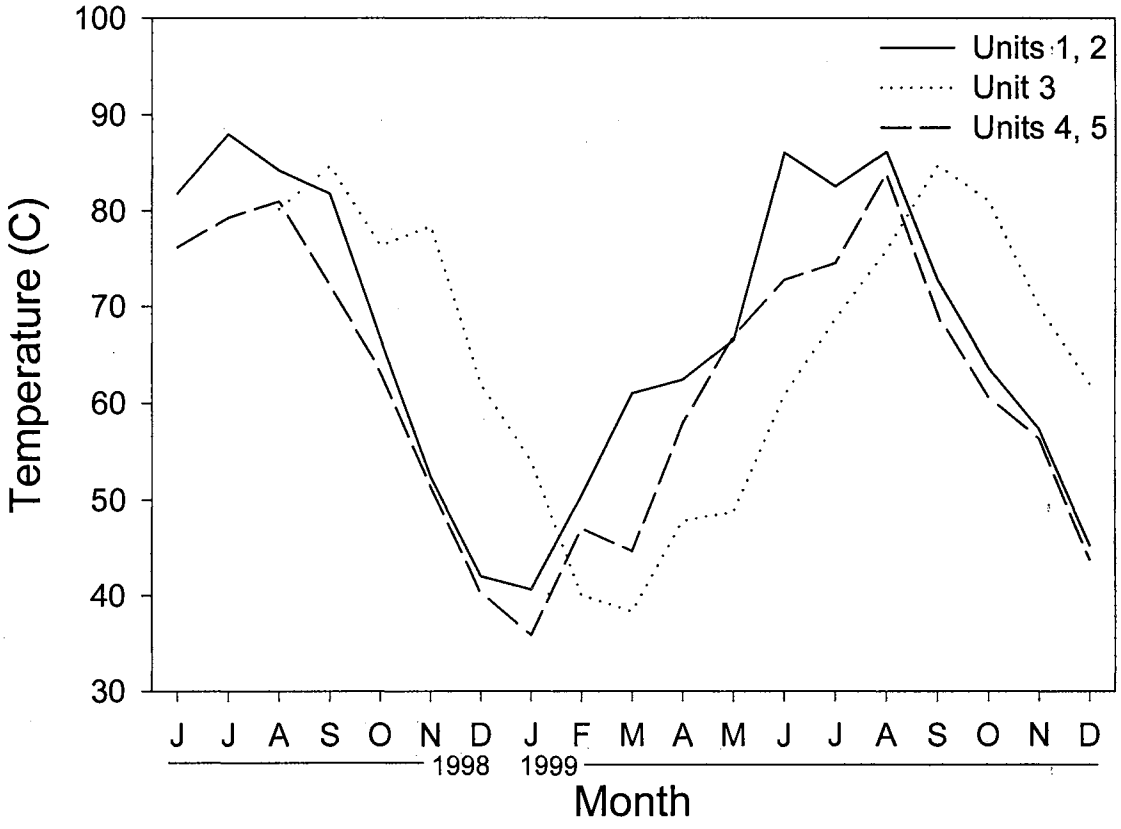

Mean Monthly Rainfall (in)
0
1
2
3
4
5
6
7
8
9
10
11
12
J J A S O N D J F M A M J J A S O N D
1998 1999
Units 4, 5
Units 1, 2
Unit 3

Figure 3. Mean monthly temperature recorded from the nearest mesonet weather station to Duncan, Mounds, and Ponca City, Oklahoma. Daily temperature recordings are averaged to provide mean monthly temperature values.


100
90
80
70
60
50
40
30
Temperature (C)
Units 1, 2
Unit 3
Units 4, 5
J J A S O N D J F M A M J J A S O N D
1998
1999
Month

CHAPTER II

ECOTOXICOLOGICAL RISKS ASSOCIATED WITH LAND TREATMENT OF PETROCHEMICAL WASTES: III. IMMUNE FUNCTION AND HEMATOLOGY OF COTTON RATS.

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RH: Immune function of cotton rats on landfarms

Abstract

Landfarming is a widely used method of treating petrochemical waste through microbial biodegradation. The effects of residual petrochemical contamination on wildlife, especially terrestrial mammals, are poorly understood. We used cotton rats (*Sigmodon hispidus*) as *in situ* models for the effects of petrochemical contamination on the immune system and hematology of mammals. Cotton rats were sampled seasonally (summer and winter) from 5 abandoned petrochemical landfarms (Units 1-5) in Oklahoma and from 5 ecologically matched reference sites for 2 years (1998-2000), and

returned to the laboratory for immunological and hematological assays. Rats inhabiting landfarms exhibited decreased relative spleen size compared to rats collected from reference sites, with the landfarm at Unit 1 showing the greatest reduction. Cotton rats collected from landfarms also had increased hemoglobin, hematocrit and platelet levels, and decreased blood lymphocytes during summer. During winter, an increase in the number of popliteal node white blood cells was observed from rats collected on landfarms. No difference was detected for lymphocyte proliferation in response to concanavalin A, pokeweed, or interleukin-2. Lymphokine-activated killer cell lytic ability showed a seasonal pattern, but no treatment differences. No differences between landfarm and reference sites were detected in the hypersensitivity reaction of rats given an intradermal injection of phytohemagluttinin (PHA-P). Comparisons within individual sites indicated that two sites (Units 1 and 3) had the greatest effects on immune function and hematology of cotton rats. Results of this study suggested that residual petrochemical waste affects the immune system and hematology of cotton rats living on abandoned landfarms during summer, and these effects are complicated by variation in the composition of contaminants found on individual petroleum sites.

INTRODUCTION

Petroleum refining generates a complex, concentrated waste sludge that varies in composition depending on the source. Petrochemical waste is produced from tank bottoms, separation units, equipment cleaning, and occasional spills, and is listed as a "hazardous material." Its disposal is under increasingly stringent regulations (Bryant and Moores 1991). Bioremediation techniques such as landfarming have been hailed as

environmentally friendly, cost-effective, and efficient methods of reducing hazardous waste (Green and Moore 1985). As a result, over half of the waste produced by the petroleum industry is disposed of in landfarms (American Petroleum Institute 1984). During landfarming, petrochemical waste is applied on or into soil where microbes metabolize the organic portion of the waste, decontaminating the site in the process.

Most petroleum waste organics can be degraded with a properly designed bioremediation project. However, bioremediation is of limited value to inorganic components of petroleum waste (Milne et al. 1998; Schroder et al. 1999). Asphaltines, resins, polyaromatic hydrocarbons (PAHs), and heavy metals are not readily biodegradable and may remain in the environment for prolonged periods (Milne et al. 1998). Even within the biodegradable fraction of petroleum waste, degradation is often slow and may take $\geq$ 25 yrs for a site to reach suitable decontamination levels (Genouw et al. 1994). Landfarms are often abandoned after waste has been applied, and become colonized by vegetation, making them suitable habitat for wildlife. The effects of residual contamination on wildlife inhabiting revegetated landfarms are poorly understood.

Contaminants may affect individuals directly by interfering with physiological processes. The immune system is particularly susceptible to direct effects from contaminants, and initiation of an immune response is energetically costly (Lochmiller and Deerenberg 2000). Lochmiller (1996) proposed that immunocompetence might be a key factor regulating wild herbivore populations. Individuals living in the wild typically experience numerous factors affecting their immune system including poor nutrition (Lochmiller et al. 1993b; Vestey et al. 1993; Davis et al. 1995), seasonal suppression

(Davis and Lochmiller 1994; Lochmiller et al. 1994; Lochmiller and Ditchkoff 1998), and age (Lochmiller et al. 1993a). Further challenges resulting from toxicant exposure may impair immune systems below the level needed to maintain individuals in good health.

The cotton rat (*Sigmodon hispidus*) is a good model for studying the effects of contamination on mammalian immune systems because of their abundance throughout the southern United States, relative ease in handling, transport, and housing, and their dominant place in the rodent community. Previous laboratory studies using cotton rats indicated a link between individual components of petrochemical waste and immunotoxicity. Because of their ability to bioaccumulate, organic contaminants are of particular interest. Adult cotton rats exposed to various doses of benzene and cyclophosphamide exhibited a wide range of effects from decreased thymus weight, low spleen weight, low hematocrit, and reduced lymphocyte numbers (McMurry et al. 1991). In addition to organics, exposure to metals can elicit immunotoxic effects. Reduced spleen mass was observed in cotton rats fed a diet containing Pb (McMurry et al. 1995), and exposure to low levels of As decreased hypersensitivity reactions by 30% (Savabieasfahani et al. 1998).

Studies of wild cotton rats inhabiting petroleum waste sites have documented effects at the genetic, cellular, and population levels. Increased incidences of chromosomal aberrations were detected in cotton rats from petrochemical-contaminated sites (McBee et al. 1987). Tooth lesions resulting from ameloblast necrosis has been associated with increased fluoride and has been identified in rats from contaminated sites (Paranpje et al. 1994; Schroder et al. 1999; Rafferty et al. 2000). Heavy metal exposure

has been linked to decreased splenocyte proliferation, leukocyte yield, and hepatic mass (McMurry et al. 1995). McMurry et al. (1999) also found decreased cellular counts for white blood cells, lymphocytes, and neutrophils in cotton rats from petrochemical contaminated sites. Rafferty et al. (2001) showed contaminant-related effects on serum protein levels, organ mass, and organ cellularity. Additionally, an increase in ovarian cell apoptosis was observed from rats inhabiting petrochemical-contaminated sites (Savabieasfahani et al. 1999). Lochmiller et al. (1999) also found increased activity in cytochrome P-450, an enzyme involved in detoxification, among cotton rats living on petrochemical contaminated sites.

We hypothesized that cotton rats exposed to residual contaminants found on abandoned landfarms would exhibit alterations in immune function and hematology. Specifically, we quantified functional aspects of the innate, humoral, and cell-mediated immune system. Standard hematological parameters were measured to provide an indication of the overall condition of the animal. Finally, changes at the organ level were measured through differences in the relative mass of secondary immune organs. The effect that residual contaminants have on mammalian immune systems is of importance, not only to wildlife, but also to humans living in areas surrounding these sites. If residual contaminants affect the immune system of mammals living on landfarms, a reevaluation of landfarming as a method of waste disposal may be warranted.

MATERIALS AND METHODS

Study Sites

This study was conducted on 10 sites throughout Oklahoma from the southwest to north-central. Five sites had some history of petrochemical contamination, and 5 were uncontaminated reference sites. Reference sites were located as close to petrochemical sites as possible to reduce variation from climate and vegetation. Contaminated sites for Units 1, 2, and 3 were abandoned landfarms with no further cleanup. Contaminated sites at Units 4 and 5 were abandoned landfarms that had undergone additional cleanup (removal of contaminated soil and reapplication of soils into a new landfarm) by the U. S. Environmental Protection Agency. All sites were located in the tallgrass prairie ecosystem and consisted of disturbed grassland habitat. Sites were located in southwestern (Units 1 and 2), north-central (Unit 3), and eastern (Units 4 and 5) Oklahoma. For detailed descriptions of the sites and their contaminant histories, see Schroder et al. (2002) in this issue.

Collection of Animals

Sixty-four Sherman live traps (Sherman Traps Inc., Tallahassee, FL) spaced at 10-m intervals were arranged in a square (8x8) or rectangular (4x16) grid as dictated by the shape of the area to be surveyed. Traps were baited with whole oats, set in the late afternoon, and checked the following morning. Cotton bedding was provided during winter months for added insulation. Twelve rats were collected from each site during summer (August) and winter (January – February) for 2 years. Unit 2 was only sampled during the first summer (1998) because no rats were found on the site during sampling after that period. No individuals < 80 g were used in this study. Rats were transported to Oklahoma State University for immunological trials and housed in standard plastic cages

(47 x 27 x 20 cm) with cob shavings for bedding. Water and rat chow (Purina, St. Louis, MO) were provided *ad libitum*.

Hematology

Blood was obtained from the retro-orbital sinus plexus of anesthetized rats with heparinized capillary tubes. A 40-µl sample of blood was placed into 10 ml of Serano diluent, mixed, and counted on an automated cell counter standardized for cotton rat blood. White blood cell count (WBC), red blood cell count (RBC), hemoglobin concentration (HGB), % hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and platelet count (PLT) were determined by the cell counter.

Whole blood smears were prepared by placing one drop of blood at the tip of a microscope slide and smearing the blood sample across the slide using a second slide. Blood smears were allowed to dry for 24 hrs, fixed with methanol, and stained using a combination of eosin and thiazine stain. The proportion of lymphocytes and neutrophils in 100 observed cells was counted using oil immersion (1,000x) on a light microscope.

Blood was drained from the retro-orbital plexus into a heparinized serum separation tube and centrifuged at 15°C, 1200 x g for 10 min. Serum was placed into an Eppendorf microcentrifuge tube and stored at -70°C until used for serum protein and immunoglobulin analysis. Total serum protein concentrations were determined using a standard Biuret method and compared against a human serum standard (Kingsley 1942). Total immunoglobulin concentrations were measured using an ammonium sulfate, sodium chloride precipitation assay (Bradford 1976). Absorbance of both total protein and total immunoglobulin solutions were read on a spectrophotometer at 550 nm (total

protein) and 555 nm (total immunoglobulins) following standardization with human serum protein.

Cotton rats were killed by cervical dislocation following exposure to methoxyfluorane (Mallinckrodt Veterinary Inc., Mundelein, IL) anesthesia. Spleens were removed aseptically, weighed, and placed in sterile petri dishes containing RPMI 1640 culture medium supplemented with 10% horse serum (RPMI-HS, pH 7.2), 1.025% L-glutamate (200mM), and 1.0% penicillin-streptomycin (10,000 U/ml-10 mg/ml). Spleen to somatic index (SSI), an index of relative spleen size, was calculated as the mass of the spleen (mg) divided by body mass (g) without the spleen.

Spleens were dissociated into individual cells by homogenization using a sterile glass tissue grinder with 5 ml of RPMI-HS medium. Cell solutions were allowed to settle for 7 min and the supernatant was decanted into sterile 16- by 125-mm screw-cap culture tubes. Cells were washed by centrifugation (8 min, 10°C, 220 x g) and resuspended in 5 ml of fresh RPMI-HS medium. Erythrocytes were lysed by washing cells in 5 ml of Tris-buffered ammonium chloride (0.83%, pH 7.2), followed by a wash with 5 ml RPMI-HS. Cells were counted using an automated cell counter, adjusted to a final concentration of 500,000-cells/90 µl, and maintained in RPMI-HS.

Paired popliteal nodes were removed from individuals, weighed, and placed into 3 ml of RPMI-HS. Popliteal nodes were homogenized by hand using a glass tissue grinder. The concentration of white blood cells and platelets found in popliteal nodes was measured by placing a 20-µl sample of the homogenate into 10 ml of Serano diluent for use in an automated cell counter (Serano System 9000, Allentown, PA).

Complement Activity

Complement activity, as determined through the classical pathway, was measured using protocols modified from Vestey and Lochmiller (1994). Serial 2-fold dilutions of cotton rat serum in vernal buffered saline (VBS) solution were placed in 96-well round-bottomed microtiter plates, making a 40 µl/well final volume. To each sample well, 25 µl each of 0.6% sheep red blood cells in VBS and heat-inactivated rabbit anti-sheep-red-blood-cell immunoglobulins was added. Microtiter plates were mixed, covered, and placed in a water-jacketed incubator (37°C) for 1.5 hrs. After incubation, plates were centrifuged at 220 x g for 5 min. Sixty µl of supernatant were transferred to new microtiter plates, and the sample absorbance determined at 414 nm. Two positive controls were used and consisted of 20% and 80% lysed sheep red blood cell solutions. In addition, a negative control consisting of 90 µl of VBS was used.

Lymphoproliferation

The use of concanavalin A and pokeweed mitogens was recommended for use on cotton rats by Lochmiller et al. (1993a) because of their consistent mitogenicity. Similarly, interleukin-2 has been used in lymphoproliferative assays for the cotton rat (McMurry et al. 1999). The use of concanavalin A, pokeweed, and interleukin-2 provides a measure of the complete lymphocyte response by preferentially stimulating T, B, and T and B lymphocytes respectively (Greaves and Janossy 1972, Hellstrom et al 1976).

The lymphoproliferative ability of cultured splenocytes was assessed *in vitro* by stimulation with 1 of 3 mitogens, following techniques modified from Lochmiller et al. (1993a). Lymphocytes were stimulated in triplicate with concanavalin A (5 µg/ml),

pokeweed (1.25 μg/ml), and recombinant human interleukin-2 (40 U/ml). Lymphocyte proliferation was assessed by incorporation of [^{3}H]-thymidine into the DNA of lymphocytes undergoing mitosis. Radioactivity was determined by counting the number of disintegrations/min (dpm) from incorporated [^{3}H]-thymidine. Cells were harvested onto nitrocellulose strips, allowed to dry overnight, placed into scintillation vials with 2 ml of scintillate, and read on a liquid scintillation counter (Packard 1600 TR, Merriden, CT). The lymphoproliferation index was calculated as the mean mitogenic dpm / mean control dpm.

Natural Killer Cell Activity

Tumorcidal activity of lymphokine-activated natural killer cells was assessed by measuring the ratio of target cell death in control and treatment samples as described by Lochmiller et al. (1998). Yac-1 murine tumor cells incorporated with [^{3}H]-thymidine (10 μCi/ml) were used as target cells for natural killer lytic activity. Target cells were harvested onto nitrocellulose strips and the radioactivity was measured with a liquid scintillation counter. The percent lysis of Yac-1 cells was measured as the mean DPM from natural killer cells divided by the mean DPM from control wells multiplied by 100.

Hypersensitivity

In vivo cell-mediated immune response was measured by hypersensitivity reaction to injections of phosphate buffer solution (PBS) and phytohemaglutinin (PHA-P) as described by Lochmiller et al. (1993b). Rats were injected with 1-cc of PBS (control) and PHA-P (stimulant). The difference between the initial and post-injection skinfold thickness was calculated for PBS and PHA-P, and the ratio of PHA-P to PBS reactivity was used as an index of immune response.

Statistical Analyses

Analysis of immune effects was performed using a 2 x 2 randomized block design. PROC MIXED was used to calculate F-test values with Satterthwaith's approximation used to calculate the degrees of freedom for the error term (SAS Institute 1994). Site*Treatment*Season was used for the error term, with site as a blocking variable, petrochemical treatment (reference and landfarm) as the main effect, and season (summer and winter) as the secondary effect. If there were no significant interactions, differences in the main effects were compared using the PDIFF option in the LSMEANS statement. Differences in terms with significant interactions were compared using the SLICE option in the LSMEANS. Site analyses also were performed to address variability of contaminants and immune responses found among the individual sites. PROC TTEST was used to determine differences between each petrochemical-contaminated and reference-site pair (SAS Institute 1996). All data are presented as means ± standard error, and all differences were considered significant at the $\alpha = 0.05$ level.

RESULTS

Relative spleen size was reduced in rats collected from landfarm sites (0.22 ± 0.008 mg/g) compared to reference sites (0.19 ± 0.006 mg/g, $F_{1,10} = 7.99$, $P = 0.02$, Figure 1). Within-site analyses of the spleen:somatic index showed the landfarm from Unit 1 (0.15 ± 0.008 mg/g) had smaller spleens than its reference site (0.21 ± 0.01 mg/g, $T_{91} = 3.09$, $P = 0.003$). Although spleen sizes were reduced in petroleum-treated sites, the number of splenocytes per mg of spleen did not differ ($F_{1,7.4} = 1.13$, $P = 0.32$, Table

1). However, during winter on Unit 4, rats from the petrochemical site showed an increase in splenocytes ($3.86 \pm 0.45 \times 10^3$ cells/mg) over the reference site ($2.85 \pm 0.26 \times 10^3$ cells/mg) that approached significance ($T_{35.9} = -1.94$, $P = 0.06$, Table 1).

A treatment*season effect was observed in white blood cells ($F_{1,6.82} = 9.02$, $P = 0.003$), with an increase in cells found in the popliteal node of rats collected from landfarms during winter (Table 1). Increases in popliteal node white blood cells were observed during winter from rats collected on petrochemical sites for Unit 1 ($3.35 \pm 0.49 \times 10^6$ cells/ml, $T_{36.3} = -2.19$, $P = 0.03$) and Unit 3 ($4.24 \pm 0.91 \times 10^6$ cells/ml, $T_{25} = -2.43$, $P = 0.02$) over their respective reference sites (2.13 ± 0.26 and $1.93 \pm 0.28 \times 10^6$ cells/ml). No treatment difference in popliteal node weight was detected, and no differences in the number of popliteal node white blood cells and platelets were detected for summer.

All differences in hematological parameters showed a treatment*season effect. Peripheral white blood cells were lower from rats collected on landfarms during summer months ($F_{1,10} = 3.97$, $P = 0.05$). However, red blood cells were elevated from rats collected on landfarms during summer ($F_{1,6.97} = 6.11$, $P = 0.04$). Hemoglobin ($F_{1,6.63} = 7.67$, $P = 0.006$, Table 2), platelet levels ($F_{1,9.2} = 14.04$, $P = 0.0002$), and hematocrit ($F_{1,10.3} = 3.95$, $P = 0.07$, Table 2) were elevated in cotton rats from landfarm sites for summer, with hematocrit levels approaching significance ($F_{1,10.3} = 3.95$, $P = 0.07$, Table 2). Hemoglobin, hematocrit, and platelet levels were 4.1, 4.3, and 18.3% higher for cotton rats from landfarm sites when compared to reference levels.

Within-site analyses for summer showed cotton rats from Unit 1 to have the greatest number of differences in hematological parameters. The number of red blood

cells ($6.36 \pm 0.12 \times 10^6$ cells μl^{-1}, $T_{46} = -2.82$, $P = 0.007$), hemoglobin concentration (13.62 ± 0.26 g dl^{-1}, $T_{46} = -2.23$, $P = 0.03$), and hematocrit (41.5 ± 0.8 %, $T_{46} = -2.15$, $P = 0.03$) were all greater on the petrochemical landfarm compared to the respective reference site values ($5.84 \pm 0.14 \times 10^6$ cells μl^{-1}, 12.82 ± 0.26 g dl^{-1}, and 39.01 ± 0.83 %), whereas white blood cell levels were decreased on the petrochemical site ($14.56 \pm 1.09 \times 10^3$ cells μl^{-1}) compared to reference values ($21.08 \pm 1.98 \times 10^3$ cells μl^{-1}, $T_{35.6} = 2.89$, $P = 0.007$, Table 2). Unit 1 was the only site with a decrease in white blood cell in rats collected from the landfarm. Unit 2 also had an increase in hemoglobin (13.41 ± 0.32 g dl^{-1}, $T_{22} = -2.39$, $P = 0.02$) and hematocrit (41.32 ± 0.92 %, $T_{22} = -2.84$, $P = 0.009$) on its petrochemical site compared to the respective reference values (12.51 ± 0.21 g dl^{-1} and 38.09 ± 0.66 %). The number of platelets on Unit 5 ($668 \pm 37 \times 10^3$ cells μl^{-1}) also increased compared to its reference site ($495 \pm 39 \times 10^3$ cells μl^{-1}, $T_{45} = 3.14$, $P = 0.003$).

No overall differences were detected during the winter for any of the hematological parameters (Table 2). Within sites, platelet levels were reduced in cotton rats on the petrochemical site at Unit 1 ($359 \pm 17 \times 10^3$ cells μl^{-1}) compared to rats from reference sites ($414 \pm 22 \times 10^3$ cells μl^{-1}, $T_{44} = 1.97$, $P = 0.05$). Unit 3 showed the most differentiation with reduced red blood cells ($5.54 \pm 0.12 \times 10^3$ cells μl^{-1}, $T_{45} = 3.34$, $P = 0.002$) and hematocrit (36.57 ± 0.61 %, $T_{45} = 2.57$, $P = 0.01$), and increased mean corpuscular volume (66.22 ± 0.69 *fl*, $T_{45} = -2.78$, $P = 0.008$) and mean corpuscular hemoglobin (22.09 ± 0.32 pg, $T_{40} = -2.66$, $P = 0.01$) compared to the respective reference values ($6.08 \pm 0.12 \times 10^6$ cells μl^{-1}, 38.74 ± 0.58 %, 63.82 ± 0.53 *fl*, and 20.97 ± 0.27 pg).

Mitogenic stimulation produced a 2-20-fold increase in the proliferation of splenocytes. Analysis of combined sites showed no difference in cellular proliferation

between cotton rats from landfarm and reference sites for any of the mitogens (Figure 2). However, because of the variability detected among the soil contaminants for each site (Schroder et al. 2002), site comparisons revealed that the proliferation index from concanavallin A in Unit 3 was reduced for rats from the landfarm site (7.67 ± 0.88) compared to the reference site (11.50 ± 1.72, $T_{48} = 2.08$, $P = 0.04$). Within-site analysis also showed an increase in the cellular proliferation index of rats from the landfarm at Unit 1 (4.17 ± 0.59) in response to interleukin-2 compared to rats from reference sites (2.74 ± 0.47, $T_{44} = -2.51$, $P = 0.02$).

No treatment effects were detected for natural killer lytic activity (Figure 3). Within-site comparisons revealed that Unit 3 (82.7 ± 1.2%, $T_{21} = -2.65$, $P = 0.02$) and Unit 4 (53.2 ± 3.8%, $T_{25} = 1.91$, $P = 0.06$) had increased lytic activity when compared to their respective reference sites (76.4 ± 2.0% and 43.5 ± 3.8%, Figure 3a). During winter, the trend was reversed with reference sites showing greater lytic activity (67.7 ± 4.6) than contaminated sites (49.8 ± 4.5%) at Unit 1 ($T_{20} = 2.69$, $P = 0.01$).

Cotton rats showed a 3-4 fold increase in the cell-mediated response to PHA-P stimulated side compared to the control injection (Figure 4). However, no differences were detected among petrochemical treatments or seasons ($F_{1,7.48} = 0.25$, $P = 0.63$, Figure 4). Unit 2 was the only site to show an increase in the cell-mediated hypersensitivity response on the contaminated site (4.2 ± 0.2) compared to its reference site (3.5 ± 0.2, $T_{22} = -2.79$, $P = 0.01$, Figure 4).

DISCUSSION

The results of our study show alterations in the immune function and hematology of cotton rats inhabiting some petrochemical landfarms. These effects showed a seasonal pattern, with most alterations occurring during summer. During summer, cotton rats living on landfarms had reduced spleen size, lower numbers of blood leukocytes, and increased numbers of red blood cells and platelets. In addition, hemoglobin concentration and hematocrit were elevated. The only parameter to show a change in winter was an increase in the leukocytes in popliteal node lymph nodes.

The lack of differences found during the winter may be due to seasonal differences in maintenance of the immune system and hematology. Decreases in immune function and hematology of cotton rats during winter has been observed for several parameters. Decreased splenocyte numbers, packed cell volume, peripheral leukocytes, lymphoproliferation, and spleen mass have been observed in winter (Davis and Lochmiller 1994; Lochmiller et al. 1994). Cotton rats from this study also showed a decrease in some parameters during winter, including size of lymph nodes and spleen, number of leukocytes and platelets in lymph nodes, and numbers of circulating platelets. In addition, natural killer cell activity was reduced during the winter. Reduction in the immune system during winter may reduce the ability to detect differences from contaminants because any further reduction in the immune function of an individual may result in infection and potentially increased mortality.

Petrochemical contamination affects individuals in a number of ways. However, variation in the composition and concentration of contaminants among sites makes it difficult to identify patterns of toxicity among populations of cotton rats. Sources of

variation include experimental and sampling error, interactions among contaminants, and habitat variation, especially with respect to cover and forage composition. Careful selection of reference sites that are similar in botanic composition and climate will reduce the variability between cotton rat populations and reduce sampling and experimental error. At the same time, choosing similar reference sites will reduce the variability due to differences in habitat quality and forage availability that would lead to confounding effects of nutrition.

The different mixtures of contaminants found among sites were likely the largest source of variability within our study. Each landfarm had a unique history of operation and was exposed to different contaminants (Schroder et al. 2002, this issue). As a result, exposure levels to cotton rats varied by site. Contaminant variability among sites may be enough to mask any patterns of toxicity that might exist. To identify this problem, we chose to include within-site analyses. It is important to note that individual site analyses using t-tests represents pseudoreplication and is not a proper use of this statistical technique. However, given the variability in the contaminants found on each site and their range of concentrations (Schroder et al. 2002), we thought it biologically important to identify patterns associated with immune effects and individual site contaminant profiles. Nevertheless, within-site analyses were effective in determining that Units 1 and 3 consistently showed the greatest effects on the immune and hematological system of cotton rats. Units 1 and 3 had higher levels of Pb, Cr, F, and Cd than other petrochemical sites, except for Unit 4 for F levels (Schroder et al. 2002). In addition, the landfarm at Unit 3 had the highest levels of polyaromatic hydrocarbons, followed by the landfarm at Unit 1 (Schroder et al. 2002).

The general lack of effects found in cotton rats collected from landfarms on Units 4 and 5 may be attributed to the way these landfarms were treated. Both landfarms at Units 4 and 5 had additional removal of contaminated soils from the site (Kelley 1983). Given this additional treatment, it is not surprising that the effect of contaminants would be reduced. The concentration of PAHs on the landfarms from Units 4 and 5, although still elevated over reference sites, were an order of magnitude lower than on the other landfarms, and Al, As, Ba, Cr, Cu, F, Pb, and Zn were the only elevated inorganics (Schroder et al. 2002).

Major organs involved in immunity or detoxification, such as the spleen and liver, are often used as biomarkers for toxicity. Because of the mixture of contaminants found on landfarms no individual contaminant can be singled out as a causative agent; however, controlled laboratory experiments have shown effects from individual components of petrochemical waste. In a laboratory study, spleen mass was reduced in male cotton rats exposed to lead levels of 100 ppm (McMurry et al. 1995). Rafferty et al. (2001) found that cotton rats inhabiting petrochemical sites did not show any effect on the size of spleens (Rafferty et al. 2001). However, Pb levels found on the sites studied by Rafferty et al. (2001) were less than 100 ppm. In contrast, mean Pb levels found on landfarms in this study averaged 556 ± 361 ppm (Schroder et al. 2002). Unit 1, the site with the highest Pb concentration, showed the greatest decrease in relative spleen mass. In addition to metals, organic contaminants have been shown to affect spleen size; however, exposure to organic contaminants in the laboratory resulted in an increase in the spleen mass of cotton rats (McMurry et al. 1991).

Although the size of the spleen was reduced in rats collected from petrochemical-contaminated sites, the number of splenocytes per unit of spleen did not differ. Thus, there was an overall reduction in the total number of splenocytes available to an individual for use in mounting an immune response. In addition, smaller spleen mass would reduce the amount of surface area available in the spleen to screen and remove old or damaged red blood cells and may be responsible for the increase in red blood cells found in rats from landfarms. An increase in apoptosis was observed in spleens collected from cotton rats inhabiting the landfarm at Unit 3 (unpublished data, David M. Janz, Oklahoma State University), and may contribute to the reduced spleen size observed in rats from landfarms.

Hematological values from this study were similar to studies of wild-caught cotton rats from Texas (Rattner et al. 1993) and Oklahoma (Robel et al. 1996; Rafferty et al. 2001). Differences in the hematology of cotton rats from landfarms in this study were found predominantly during the summer. McMurry et al. (1999) observed leukocyte counts that were 32 – 45% lower in rats from petrochemical-contaminated sites, similar to our results on Unit 1. McMurry et al. (1991) found alterations in several hematological parameters after cotton rats were exposed to organic contaminants in the laboratory; however, peripheral white blood cells increased with increasing exposure to benzene.

Effects of individual contaminants on the lymphoproliferation of cells are not well studied in wildlife. Lymphoproliferation varies by season (Davis and Lochmiller 1994) and with exposure to individual contaminants. No effect on proliferation was observed from cotton rats exposed to As under laboratory conditions (Savabieasfahani et al 1998);

however, exposure to Pb suppressed lymphoproliferation (McMurry et al. 1995). Additionally, laboratory exposure to low levels of benzo(a)pyrene elicited enhancement of lymphoproliferation while exposure to higher concentrations may suppress proliferation (Tomar et al. 1991). Although a reduction in proliferation has been observed in cotton rats exposed to petrochemical contamination (Propst et al. 1999), we observed no difference in the proliferation of lymphocytes between rats from landfarms and reference sites.

The innate branch of the immune system represents the first line of defense against infection. A major component of the innate immune system is the lytic ability of nonspecific lymphokine-activated killer cells in response to cells displaying non-self signals. Alteration of the innate immune system could affect individuals by making them more susceptible to infectious agents. Although McMurry et al. (1999) found no effect on the lytic ability of cotton rats inhabiting petrochemical-contaminated sites, killer cell activity from Unit 3 was increased in cotton rats from landfarms during summer. During winter, however, cotton rats from the landfarm at Unit 1 had lower lytic activity.

Cotton rats exposed to petrochemical waste have shown depressed cell-mediated hypersensitivity (McMurry 1993). Similarly, Propst et al. (1999) showed that rats exposed to petrochemical-contaminated soils had a 60% decrease in the hypersensitivity reaction. The results of the present study showed no differences in the responsiveness of cotton rats to intradermal injections of PHA. However, Unit 2 showed the opposite pattern, with a significant increase in the hypersensitivity response on the contaminated site during summer.

The results of this study show that cotton rats exposed to petrochemical contaminants exhibited some immunological and hematological changes. No definitive or patterned response to petrochemical contaminants was observed at the suborganismal level in this study. However, many contaminants can produce either immunoenhancement or suppression depending on their concentrations in the body. Contaminants also may exhibit interactions, producing synergistic or antagonistic effects on the immune system, leading to further complications in identifying patterns of immune response of wild mammals to contaminants.

Individuals may be affected indirectly through changes in their energy budget as a result of alterations in the immune system. The immune system comprises many levels of cells, tissues, and organs that interact with each other through chemical messengers. Individuals expend energy to maintain homeostasis in the immune system, and Congdon et al. (2001) suggests that individuals that are exposed to contaminants may have to expend additional energy to remove or isolate contaminants from the body. In addition, any damage from contaminant exposure such as cell loss must be replaced at an additional cost. These effects also may be enhanced by changes in the environment (i.e., reduced forage) caused by contaminants (Congdon et al. 2001). The additional energetic demands placed on the individual from contaminant exposure will reduce the energy available for growth and reproduction. This energetic trade-off may result in individuals that are affected by contaminants but do not show changes in the biomarkers that were measured. The costs of maintaining an effective immune system and mounting an appropriate immune response are described by Lochmiller and Deerenberg (2000). Continuous exposure to contaminants could strain individuals energetically and have

dramatic effects on population dynamics (Lochmiller 1996). Future studies into population and community effects should be conducted to identify the magnitude of indirect effects.

ACKNOWLEDGEMENTS

The research in this manuscript was funded by USEPA, Office of Research and Development, grant R826242-01-0. However, it has not been subjected to the Agency's required peer and policy review and therefore does not necessarily reflect the views of the Agency and no official endorsement should be inferred. Thanks to Dan Rafferty for help with collection of animals; Eric Webb, Terry Coffey, and Keri Snethan for help in the lab; Eric Hellgren for editorial work; and to Larry Claypool and Mark Payton for their help with statistical analyses.

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Table 1. Immunological parameters of cotton rats collected during the summer (n = 24) and winter (n = 24) from abandoned petrochemical landfarms (Landfarm) and sites with no history of petrochemical contamination (Reference) in Oklahoma. Means and standard errors (SE) are given for treatments with significant differences at the $P = 0.05$ level illustrated by different lettered superscripts. Within-site differences are indicated under the landfarm unit.

	Treatment Means								
	Reference		Landfarm		Within Site Effects[1]				
Season/Parameter	Mean	SE	Mean	SE	Unit 1	Unit 2	Unit 3	Unit 4	Unit 5
Summer									
Splenocytes ($x10^3$ / mg spleen)	2.76	0.14	3.16	0.44					
Popliteal Node (mg)	17.10	1.40	15.10	1.80					
Popliteal node white blood cells ($x10^6/mm^3$)	4.67	0.30	4.01	0.32	L < R				
Popliteal node platelets ($x10^3/\mu l$)	35.40	2.05	31.10	2.78	L < R				
Blood lymphocytes (%)	57.08	1.36	59.15	1.43					
Blood neutrophils (%)	41.35	1.34	39.82	1.44					
Serum total protein (g/dl)	5.59	0.06	5.76	0.06					
Serum total immunoglobulins (g/dl)	1.14	0.02	1.13	0.02					
Complement activity (CH_{50} U/ml)	68.63	3.75	89.89	12.49					
Winter									
Splenocytes ($x10^3$ / mg spleen)	3.67	0.40	3.36	0.16		NA			
Popliteal Node (mg)	6.00	1.00	8.00	0.50		NA			
Popliteal node white blood cells ($x10^6/mm^3$)	2.12	0.15^a	3.18	0.30^b	L > R	NA	L > R		
Popliteal node platelets ($x10^3/\mu l$)	13.44	1.10	21.54	2.32	L > R	NA	L > R		
Blood lymphocytes (%)	60.26	1.32	61.71	1.34		NA			
Blood neutrophils (%)	38.62	1.31	37.07	1.32		NA			
Serum total protein (g/dl)	5.18	0.17	5.41	0.17		NA	L > R		
Serum total immunoglobulins (g/dl)	1.10	0.02	1.10	0.02		NA			
Complement activity (CH_{50} U/ml)	85.35	7.41	76.056	6.80		NA			

[1]Sites with landfarm means greater than (L > R) or less than (L < R) reference sites are indicated; sites with no data are represented by NA.

Table 2. Hematological parameters of cotton rats collected during the summer (n = 24) and winter (n = 24) from abandoned petrochemical landfarms (Landfarm) and sites with no history of petrochemical contamination (Reference) in Oklahoma. Means and standard errors (SE) are given for treatments with significant differences at the $P = 0.05$ level illustrated by different lettered superscripts. Within-site differences are indicated under the landfarm unit.

	Treatment Means								
	Reference		Landfarm		Within Site Effects[1]				
Season/Parameter	Mean	SE	Mean	SE	Unit 1	Unit 2	Unit 3	Unit 4	Unit 5
Summer									
White blood cells ($x10^3$ μl^{-1})	20.22	1.08^a	16.18	0.62^b	L < R				
Red blood cells ($x10^6$ μl^{-1})	5.98	0.07^a	6.27	0.07^b	L > R				
Hemoglobin (g dl^{-1})	12.68	0.12^a	13.21	0.14^b	L > R	L > R			
Hematocrit (%)	38.55	0.39^a	40.21	0.43^b	L > R	L > R			
Mean corpuscular volume (*f*l)	64.68	0.34^a	64.20	0.33^a					
Mean corpuscular hemoglobin (pg)	21.29	0.13^a	21.11	0.12^a					
Mean corpuscular hemoglobin concentration (%)	32.91	0.07^a	32.88	0.07^a					
Platelets ($x10^3/\mu l$)	477.37	15.37^a	564.79	16.34^b					L > R
Winter									
White blood cells ($x10^3$ μl^{-1})	23.34	1.33^a	23.00	1.36^a		NA			
Red blood cells ($x10^6$ μl^{-1})	6.14	0.06^a	6.10	0.08^a		NA	L < R		
Hemoglobin (g dl^{-1})	12.94	0.12^a	12.83	0.12^a		NA			
Hematocrit (%)	38.83	0.30^a	38.71	0.38^a		NA	L < R		
Mean corpuscular volume (*f*l)	63.36	0.29^a	63.18	0.32^a		NA	L > R	L < R	
Mean corpuscular hemoglobin (pg)	21.10	0.13^a	21.00	0.13^a		NA	L > R	L < R	
Mean corpuscular hemoglobin concentration (%)	33.32	0.10^a	33.19	0.09^a		NA			
Platelets ($x10^3/\mu l$)	423.09	10.24^a	411.00	10.25^a	L < R	NA			

[1]Sites with landfarm means greater than (L > R) or less than (L < R) reference sites are indicated, sites with no data are represented by NA.

Figure 1. Relative spleen size of cotton rats in Oklahoma given as the spleen:somatic index (SSI). Individual means are presented for each petrochemical unit, and overall means for each petrochemical treatment type are shown as the total group. Petrochemical treatment types are landfarm and reference sites (no history of contamination). Means different at the $P = 0.05$ level are represented by different letters in the total group and by an asterisk within individual units.

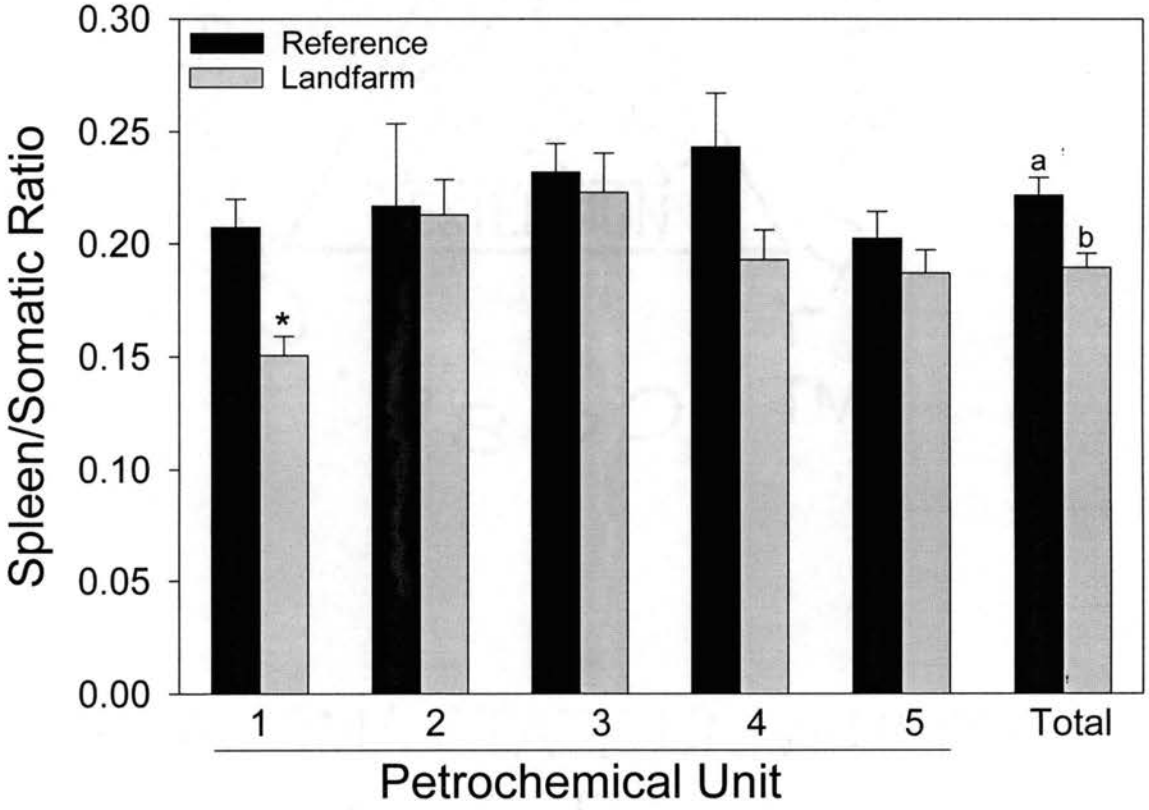

0.30
0.25
0.20
0.15
0.10
0.05
0.00
Spleen/Somatic Ratio
Reference
Landfarm
*
a
b
1
2
3
4
5
Total
Petrochemical Unit

Figure 2. Cotton rat lymphocyte proliferation index for three mitogens: (A) interleukin-2, (B) pokeweed, and (C) concanavalin A. Proliferation was measured by incorporation of [^{3}H]-thymidine and is given as the mean stimulated dpm / mean unstimulated dpm. Individual means are presented for each petrochemical unit, and overall means for each petrochemical treatment type are shown as the total group. Petrochemical treatment types are landfarm and reference sites (no history of contamination). Means significantly different at the $P = 0.05$ level are represented by an asterisk within individual units.

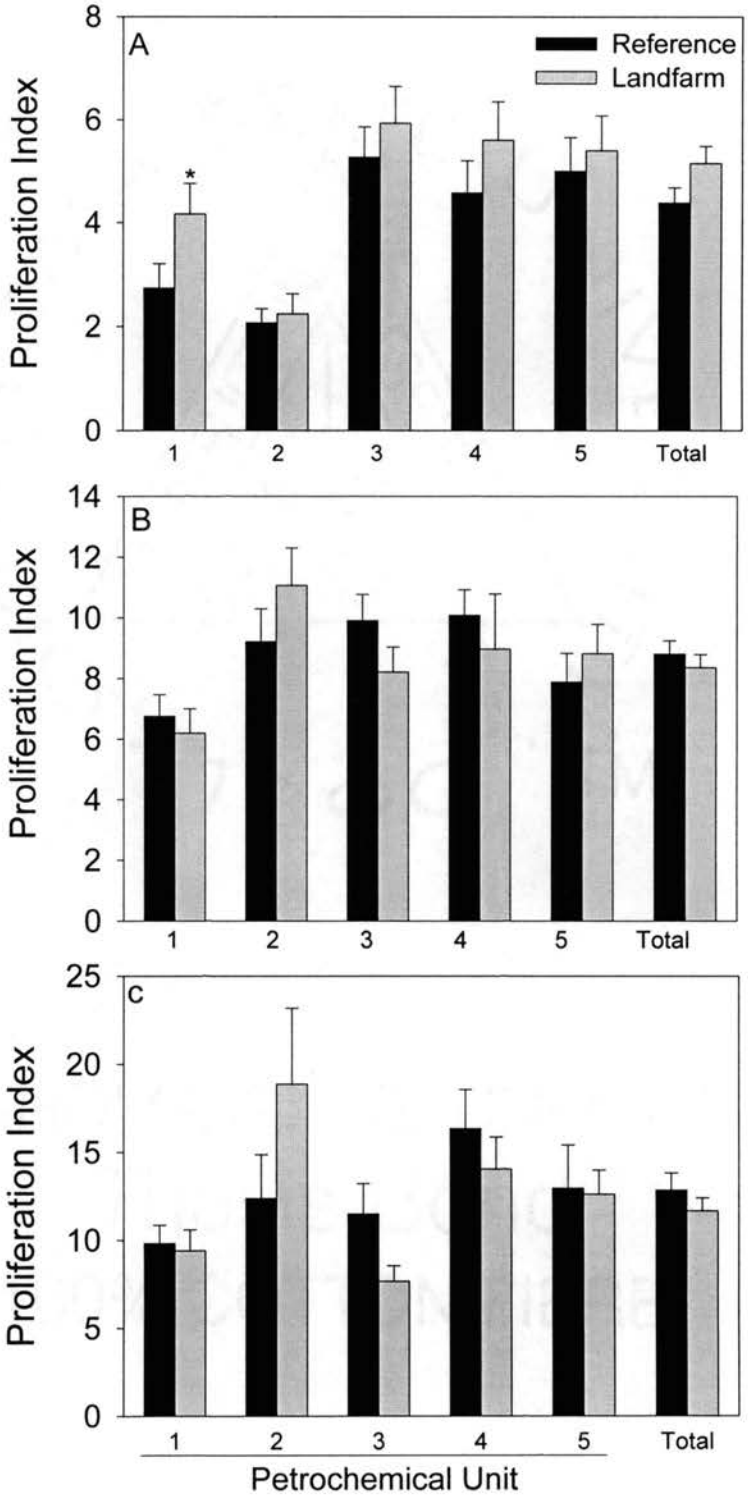

A
Reference
Landfarm
Proliferation Index
8
6
4
2
0
*
1
2
3
4
5
Total
B
Proliferation Index
14
12
10
8
6
4
2
0
1
2
3
4
5
Total
c
Proliferation Index
25
20
15
10
5
0
1
2
3
4
5
Total
Petrochemical Unit

Figure 3. Cotton rat lymphokine activated killer cell lytic ability (% lysis) against target Yac-1 tumor cells incorporated with [^{3}H]-thymidine. Lysis was measured as the mean dpm for wells with cotton rat killer cells added / mean dpm in control wells. Individual means are presented for each petrochemical unit, and overall means for each petrochemical treatment type are shown as the total group. Petrochemical treatment types are landfarm and reference sites (no history of contamination). Means significantly different at the $P = 0.05$ level are represented by different letters in the total group and by an asterisk within individual units.

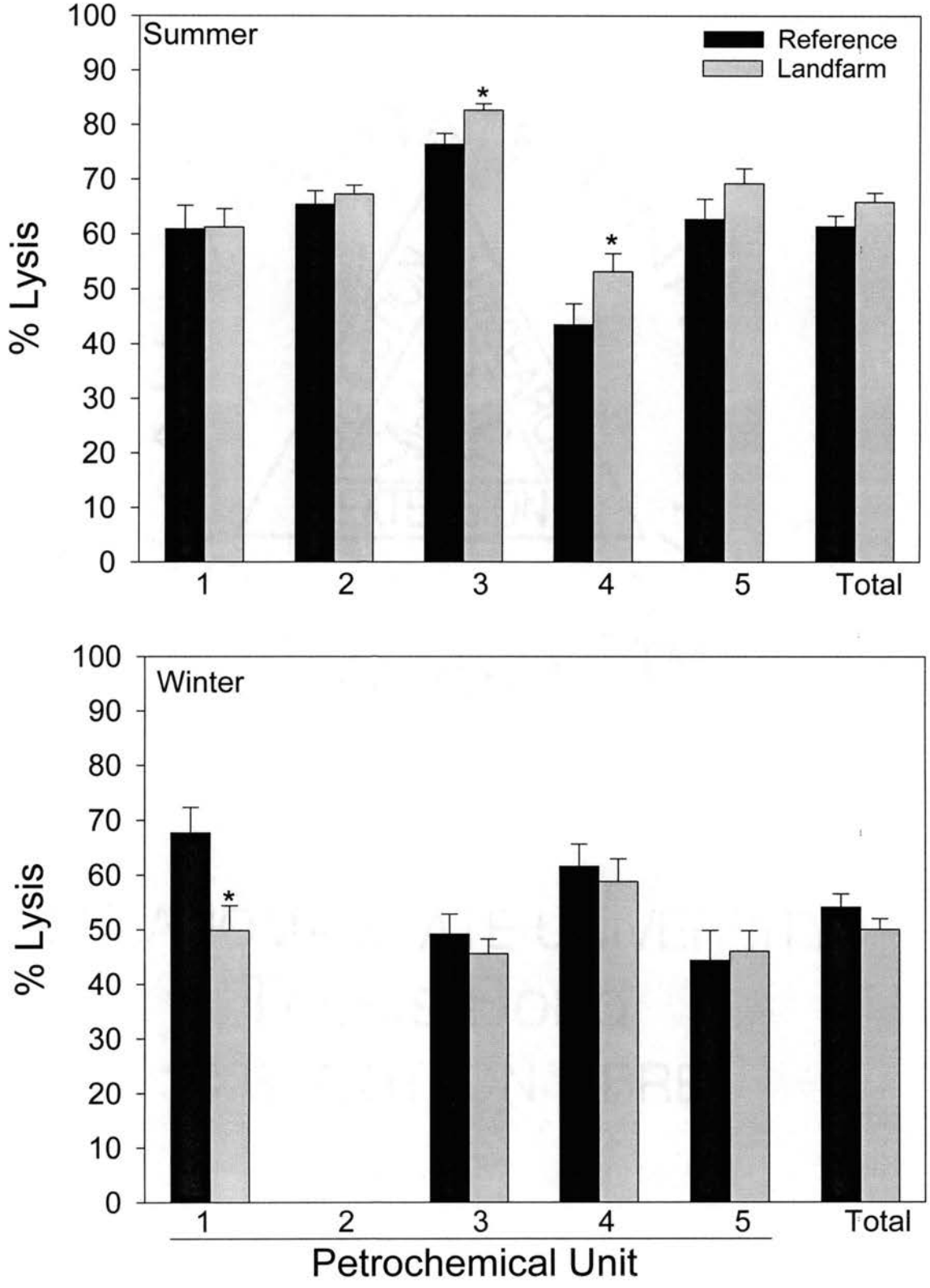
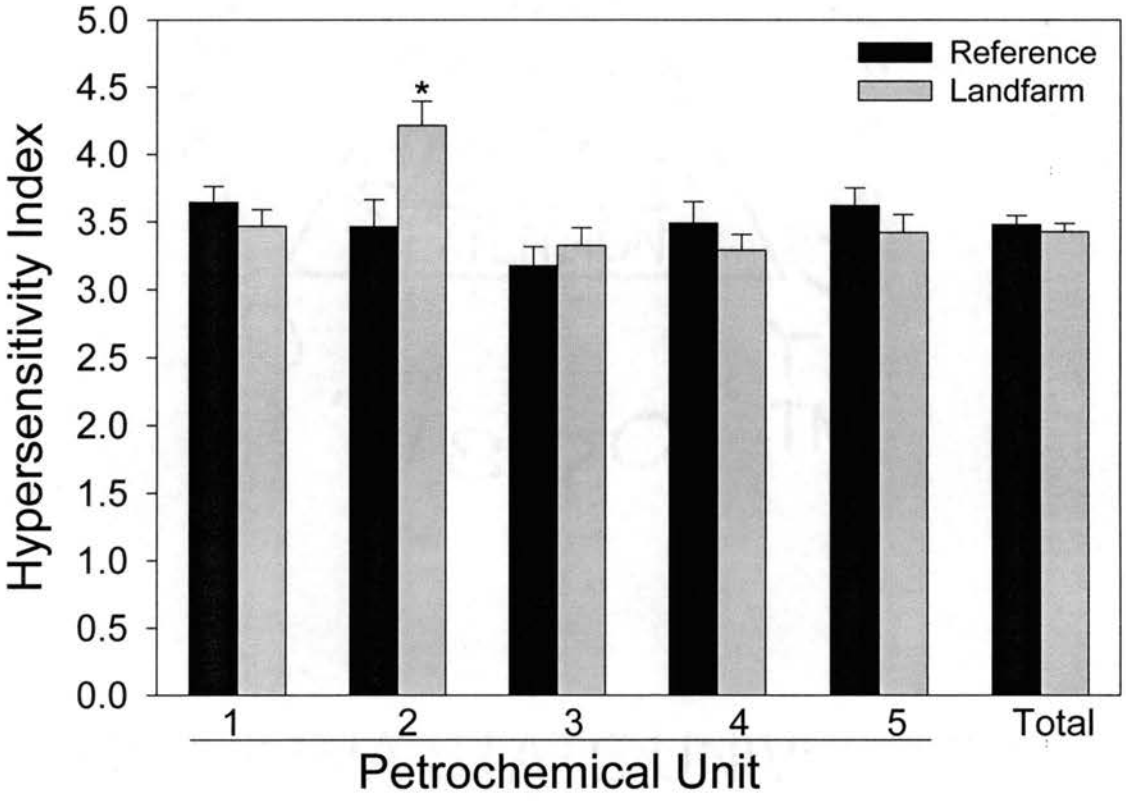

Summer
Reference
Landfarm
% Lysis
100
90
80
70
60
50
40
30
20
10
0
*
*
1
2
3
4
5
Total
Winter
% Lysis
*
1
2
3
4
5
Total
Petrochemical Unit

Figure 4. Cell-mediated hypersensitivity response of cotton rats to intradermal injections of phytohemagluttanin (PHA-P). Hypersensitivity index was calculated as the skinfold thickness of the PHA-P stimulated side / skinfold thickness of the unstimulated side. Individual means are presented for each petrochemical unit, and overall means for each petrochemical treatment type are shown as the total group. Petrochemical treatment types are landfarms and sites with no history of contamination (reference). Means significantly different at the $P = 0.05$ level are represented by different letters in the total group and by an asterisk within individual Units.


Hypersensitivity Index
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Reference
Landfarm
*
1
2
3
4
5
Total
Petrochemical Unit

CHAPTER III

POPULATION DYNAMICS AND REPRODUCTION OF COTTON RATS INHABITING ABANDONED PETROLEUM LANDFARMS IN OKLAHOMA

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Abstract. The immune selection hypothesis states that individuals exposed to immunosuppressive contaminants will be negatively affected with respect to survival and population density. Previous work on the sites in this study has shown bioaccumulation of contaminants and alterations in the immune system of cotton rats inhabiting landfarms. We compared population size, monthly survival, and reproduction of cotton rats (*Sigmodon hispidus*) inhabiting abandoned petrochemical landfarms (n = 5) and uncontaminated reference sites (n = 5) from these same sites. Populations were monitored during summer and winter for 2 yrs using mark-recapture techniques in association with program MARK. Toxicity indices were calculated for metal and polyaromatic hydrocarbon (PAH) soil contamination. After every season of trapping, a sample of cotton rats were returned to the laboratory to measure reproductive organ weight, placental scars/female, and number of embryos/female.

Cotton rat populations inhabiting abandoned landfarms experienced reduced population densities and lower monthly survival. Populations inhabiting landfarms reached maximum densities half that of reference populations. Survival was lowest in populations from landfarms during summer months. No relationship existed between

cotton rat density and survival and soil concentrations of Pb, Cr, F, and polyaromatic hydrocarbons. Cotton rat populations on landfarms had more variable sex ratios and lower proportion of juveniles than populations from reference sites. No differences were observed in the weight of epididymes, testes, uteri, or ovaries, and no difference in fecundity were detected. The lack of correlation between soil contaminants and population parameters suggests that alterations observed in populations from landfarms were not due to residual contamination. Habitat structure also differed between sites with landfarms composed of grassland interspersed with patches of bare ground. Differences in habitat structure provide a more plausible explanation for changes in cotton rat populations.

Key Words: *cotton rat*; *immune selection hypothesis*; *petroleum*; *population dynamics*; *reproduction*; Sigmodon hispidus; *survival.*

INTRODUCTION

Factors governing the regulation of animal populations have been the subject of much debate. It has been widely accepted that populations fluctuate due to the pressures exerted by extrinsic and intrinsic factors, and hypotheses developed to model population regulation incorporate one or more of these factors. Proposed extrinsic factors, which are external forces to the population that affect population growth, include rainfall (Shelford 1943), winter severity (Krebs and Myers 1974), interspecific competition (Pitelka 1973), forage nutritional quality (Lauckhart 1957) and quantity (Pitelka 1957), and vegetation cover (Goertz 1964). Intrinsic, or self-regulating, factors affect populations through a

bottom-up mode of regulation, and are based on changes at the individual level. Intrinsic regulatory mechanisms include behavior (Chitty 1967), physiological stress hypothesis (Christian 1950), dispersal (Lidicker 1962), aging (Boonstra 1994), intraspecific competition (Alder 1985), social fence hypothesis (Hestbeck 1982), ideal-free distribution (Fretwell and Lucas 1970), and recently, immune selection hypothesis (Lochmiller 1996). However, the realization that environments are highly variable and the similarity of many regulating factors have led to the development of multi-factorial models that incorporate both extrinsic and intrinsic factors (Hestbeck 1986).

Regardless of what factors are responsible, fluctuations in populations are ultimately dependent on changes in the probability of survival or reproduction of individuals. Because populations are collections of individuals, populations can only increase in size when the number of births exceeds deaths, and similarly populations decline when deaths exceed births. Although associations between populations and a number of regulatory factors have been observed, our understanding of specific mechanisms that affect survival and reproduction is poor. In particular, factors linking environmental stressors with physiological processes, and ultimately population regulation, have been difficult to measure. Difficulties associated with measuring physiological parameters in free-living populations, distinguishing between death and dispersal (Hilborn and Krebs 1976), and monitoring neonates and juveniles (Mihok et al. 1988, McShea and Madison 1987) has made it nearly impossible to identify physiological mechanisms regulating environmental-induced mortality.

The immune selection hypothesis (Lochmiller 1996) is an attempt to synthesize a mechanism for population regulation that incorporates factors at all levels. The focus of

this hypothesis is on immune function and the ability of individuals to maintain themselves in proper health. Lochmiller (1996) stated that environmental factors influence populations through their effect on host immunity. For example, extrinsic factors, such as poor food quality, negatively affect the immune system and will increase an individual's susceptibility to disease. Individuals unable to maintain their nutritional status may experience either decreased reproduction or increased mortality. Although the immune selection hypothesis synthesizes a number of regulatory factors and provides a possible mechanism that incorporates physiological parameters, measuring these factors in real populations is still difficult.

Lochmiller's (1996) immune selection hypothesis can be modified to include the effects of contamination on immune function (Figure 1). Contaminants may affect individuals either directly (e.g., through alterations in immune function), or indirectly by increasing the energetic costs of individuals, for example (Congden et al. 2001). Contaminant exposure increases maintenance costs by increasing energetic demands for metabolizing and excreting contaminants, countering immunosuppression, and increasing frequency of immune response resulting from pathogenic challenge (Lochmiller and Deerenberg 2000). According to the immune selection hypothesis, individuals exposed to contamination should experience alterations in immune function that may translate to increased mortality or decreased reproduction.

Many contaminants are known to affect the immune system directly through interactions with cellular receptors, such as the aryl hydrocarbon receptor (Whitlock 1993), or by replacing normal molecules and inhibiting proper physiologic function. Organic contaminants can be potent immunosuppressants (Johnson et al. 1982), reducing

antibody production (Dean et al. 1979) or selectively eliminating suppressor T cells (Shand 1979). In addition, exposure to metals can affect the immune system, and can produce contradictory results depending on the level of exposure. Exposure to low-level As (5 ppm) suppressed the immune system of cotton rats (Savabiesfahani et al. 1998); however, at higher concentrations, 1,000 ppm Pb has been shown to actually enhance immune function (McMurry et al. 1995). Although the end result of an impaired immune system is apparent, the effect of continual maintenance of a hyperactive immune system also would be detrimental to an individual.

Petroleum waste is a complex and highly concentrated mixture of hydrocarbons, metals, and caustics, and is generated in every process involved in petroleum refining (Bryant and Moores 1991). Bioremediation techniques such as landfarming have been hailed as environmentally friendly, cost-effective, and efficient methods of reducing hazardous waste (Green and Moore 1985). As a result, over half of the waste produced by the petroleum industry is disposed of in landfarms (American Petroleum Institute 1984). During landfarming, petrochemical waste is applied onto or into soil where microbes metabolize the organic portion of the waste, decontaminating the site in the process. However, bioremediation is of limited value to PAHs and heavy metals, which may remain in the environment for prolonged periods (Milne et al. 1998). Landfarms are often abandoned after waste has been applied and they become colonized by vegetation, making them suitable habitat for wildlife.

We monitored populations of cotton rat (*Sigmodon hispidus*) inhabiting abandoned petrochemical landfarms in Oklahoma and compared them to populations of cotton rats from uncontaminated reference sites. In particular, we used live-capture

methods to monitor population parameters (size, survival rates, male:female ratio, % juveniles, % females lactating or pregnant) and measure reproductive parameters (reproductive organ mass and fecundity) of rats collected from each site. We hypothesized that cotton rat populations inhabiting abandoned landfarms would show lower densities, increased mortality, and decreased reproduction compared to populations from reference sites.

METHODS

Study Sites

We conducted research on 5 paired sites (Units 1-5) throughout Oklahoma. Each pair of sites consisted of an abandoned petrochemical landfarm, and an uncontaminated reference site. Contaminated sites for Units 1, 2, and 3 were abandoned landfarms with no further clean up. Contaminated sites at Units 4 and 5 were abandoned landfarms that had undergone additional cleanup (removal of contaminated soil and reapplication of soils into a new landfarm) by the U. S. Environmental Protection Agency. Although reference and landfarm pairs were matched, differences in the structure of the plant community did exist. The landfarms at Units 1 and 2 were characterized by large patches of bare ground interspersed with plants characteristic of a disturbed site. Plants found on the landfarms were annual sunflower (*Aster annus*), western ragweed (*Ambrosia psilostachia*), cheatgrass (*Bromus tectorum*), Bermudagrass (*Cynodon dactylon*), and Japanese brome (*Bromus japonicus*). Both the landfarm and the reference site at Unit 3 were composed almost entirely of stands of johnsongrass (*Sorghum halapense*). Similar to Units 1 and 2, the landfarm at Unit 3 had patches of bare ground. However, the landfarms at Units 4 and 5 differed in the structure of the plant community compared

with the other landfarms. The landfarms at Units 4 and 5 had additional cleanup and the sites were seeded with non-native plants for use as potential cattle forage. As a result, the landfarms at Units 4 and 5 were composed primarily of Bermudagrass, cheatgrass, and fescue (*Festuca arundinacea*). The combination of Bermudagrass and fescue created a mat of grass approximately 10-cm thick covering the landfarm. Sites were located in southwestern (Units 1 and 2), north central (Unit 3), and eastern (Units 4 and 5) Oklahoma.

Reference sites were determined by a number of factors that provided the closest match to each landfarm site by vegetation. However, due to the limited amount of public land available in Oklahoma, reference sites were located 1 to 30 km from their paired landfarm site. In addition to providing a location with similar vegetation characteristics, owners of the reference sites insured that grazing, burning, and mowing would not take place on or around the reference site for the duration of the study. Reference sites for Units 1 and 2 were typical of old-field mixed-grass prairies and included ragweed, brome, Scribner's panicum (*Panicum oligosanthes*), and Chinese lespedeza (*Lespedeza cuneata*). Reference sites at Units 4 and 5 were abandoned fields that were composed of a mixture of prairie species including broom sedge (*Andropogon virginicus*), western ragweed, brome, broom weed (*Gutierrezia dracunculoides*), Scribner's panicum, silver nightshade (*Solanum elagantia*), and common yarrow (*Achillea millefolium*).

Although all landfarms were only used to treat petrochemical waste, the composition and concentration of waste applied at each site was different. Contamination remaining on landfarm sites consisted of two major classes: polyaromatic hydrocarbons (PAHs) and inorganics (heavy metals and fluoride). Schroder et al. (2002)

reports soil concentrations of contaminants found on each site in this study. Units 1 and 3 had higher levels of Pb, Cr, F, and Cd than other petrochemical sites, except that Unit 4 had higher F levels (Schroder et al 2002). In addition, the landfarm at Unit 3 had the highest levels of polyaromatic hydrocarbons, followed by the landfarm at Unit 1 (Schroder et al. 2002). The concentration of PAHs on the landfarms from Units 4 and 5, although still elevated over reference sites, were an order of magnitude lower than on the other landfarms, and Al, As, Ba, Cr, Cu, F, Pb, and Zn were the only elevated inorganics (Schroder et al. 2002). Soil concentrations from Schroder et al. (2002) were used to characterize individual sites based on their pattern of contamination.

An index of total soil PAH concentrations was calculated for each site. Many PAHs have been shown to act on organisms in a similar manner, and have been ranked according to their potency compared to benzo(a)opyrene (BaP; Nisbet and Lagoy 1992). These BaP rankings, termed toxic equivalents (T_{eq}), are used to describe the relative potencies of different PAHs (Compton and Sigal 1999). Soil PAH concentrations were measured on these sites by Schroder et al. (2002) and these were multiplied by T_{eq} and summed to obtain total BaP equivalents for each site. Mean Pb, Cr, and F soil concentrations reported by Schroder et al. (2002) also were used in analyses of soil contaminant levels. Seasonal relationships between cotton rat density and monthly survival and soil contaminants were tested using PROC REG in SAS (SAS 1994).

Collection of Animals

Sixty-four Sherman live traps (Sherman Traps Inc., Tallahassee, FL) spaced at 10-m intervals were arranged in a square (8x8) or rectangular (4x16) grid as dictated by the shape of the area to be surveyed. Traps were baited with whole oats, set in the late

afternoon, and checked the following morning. Cotton bedding was provided during winter months for added insulation. Populations of cotton rats were sampled monthly for four consecutive nights from each site during summer (June – August) and winter (December – February) for 2 years (1998 – 2000).

Captured animals were weighed (g), sexed, had their reproductive condition assessed, and capture location recorded. All animals were given a unique identification number by toe clipping and released. Reproductive condition was assessed through visual inspection or palpation as scrotal or non-scrotal for males and closed, open, pregnant, or lactating for females. Monthly male to female ratio was calculated for each site from trap data. In addition, monthly percent juveniles (< 60 g; Odum 1955) in the trappable population were calculated for each site. Sex ratios and the proportion of juveniles in the trappable population were used as descriptive data only.

Population Estimation

Estimates of population density were made using program MARK (White and Burnham 1999). Pollock's (1982) robust design data type under program MARK was used to analyze capture histories. Twelve primary sessions with 4 secondary occasions, for a total of 48 occasions, was used as the sampling structure. Primary sessions were separated by unequal time intervals, with consecutive summer and winter months separated by 1-month intervals. Seasons were separated by 4-month intervals (*e.g.*, Aug – Dec, Feb – Jun). Encounter histories were generated for each individual and grouped by site for analysis.

Estimated population parameters under the robust design were survival between primary sessions (S), probability of immigrating off study site (G''), probability of

remaining off the study site (G'), capture probability (p), recapture probability (c), and population size (N). To reduce the number of possible parameters, models were tested to obtain the simplest model for use in testing survival and population estimates. Both G" and G' were set to have no group or time effects (i.e., the dot model), and C was assumed to be equal to P. Factors used to build models of survival, density, and capture probability included group (g), time (t), treatment (trt), season (sea), and a difference in capture probability between the first night of trapping and subsequent nights of trapping (first). Individual landfarm and reference sites composed the group factor, and months were used as time categories. Models using a period indicate that no effects were specified for that parameter. The most parsimonious model obtained for use in further analyses was S(g*t)G"(.)G'(.)p(g*t)N(g*t), c=p. This model was used as the "global model" and was used for all further tests for survival and population estimates.

Reproduction

Twelve rats were collected from each site during summer (August) and winter (January – February) for 2 years to measure reproductive organs and parameters. Unit 2 was only sampled during the first summer (1998) because no rats were found on the site during sampling after that period. No individuals < 80 g were used in this study. Rats were transported to Oklahoma State University and housed in standard plastic cages (47 x 27 x 20 cm) with cob shavings for bedding. Water and rat chow (Purina, St. Louis, MO) were provided *ad libitum*.

Cotton rats were killed by cervical dislocation following exposure to methoxyfluorane (Mallinckrodt Veterinary Inc., Mundelein, IL) anesthesia. Epidymides, testes, uteri, and ovaries were removed, trimmed of fat, blotted dry, and weighed (g).

Any embryos found within females were removed, counted, blotted dry, and weighed. Number of placental scars along the uterus was counted for each female collected as an index of the number of embryos carried per female (Rolan and Gier 1967). Similarly, the number of corpora lutea in ovaries was counted as an index of ovulation rate (Zepp and Kirkpatrick 1976).

Plant Sampling

Plant cover was sampled on each site by placing 10 1-m^2 Daubenmire quadrats at randomly determined points on the site corresponding to intersections of an 8 x 8 grid (10-m spacing) during late spring 1999. The sampling grid was established for placement of live-traps for rodent sampling and covered the study area. Percent cover of each species and bare ground inside the quadrat was estimated. Quadrats were placed at randomly determined points on each study site. Mean percent cover and standard errors for each species were calculated for each treatment type (landfarm, reference).

Statistical Analyses

Comparisons involving organ size are confounded by problems associated with scaling. To account for scaling by body size, organ weights were analyzed using residual variation. A regression of body weight vs. organ weight was made using PROC REG and the residual variation values were exported to a new dataset. Residuals were then used for comparing treatment types using a 2 x 2 randomized block design. PROC MIXED was used to calculate F-test values with Satterthwaith's approximation used to calculate the degrees of freedom for the error term (SAS Institute 1994). Site*Treatment*Season was used for the error term, with site as a blocking variable, petrochemical treatment (reference and landfarm) as the main effect, and season (summer

and winter) as the secondary effect. If there were no significant interactions, differences in the main effects were compared using the PDIFF option in the LSMEANS statement. Differences in terms with significant interactions were compared using the SLICE option in the LSMEANS.

Site analyses also were performed to address variability of contaminants and immune responses found among the individual sites. PROC TTEST was used to determine differences between each petrochemical-contaminated and reference-site pair (SAS Institute 1994). Each landfarm had a unique history of operation and was exposed to different contaminants (Schroder et al. 2002). As a result, exposure levels to cotton rats varied by site. Contaminant variability among sites may be enough to mask any patterns of toxicity that might exist. To identify this problem, we chose to include within-site analyses. It is important to note that individual site analyses using t-tests represents pseudoreplication and is not a proper use of this statistical technique. However, given the variability in the contaminants found on each site and their range of concentrations (Schroder et al. 2002), we thought it biologically important to identify patterns associated with individual site contaminant profiles. All data are presented as means ± standard error, and all differences were considered significant at the $\alpha = 0.05$ level.

RESULTS

A total of 30,720 trapnights were evenly distributed across all sites during two years of trapping. A total of 3,562 individual cotton rats were captured 5,596 times during this study. The number of individual cotton rats trapped per site varied. Reference sites at Units 1 and 2 had higher numbers of individual cotton rats compared to

landfarms, with 592 and 431 individuals trapped on reference sites and 116 and 55 on the landfarms. On all other sites, the number of individuals trapped on landfarms was higher than reference sites. Individuals trapped on landfarms/reference sites were 420/350, 588/450, and 296/264 for Units 3, 4, and 5 respectively.

Goodness-of-fit tests were performed using RELEASE in program MARK. Because the robust design data type does not allow goodness-of-fit tests, encounter histories for all sites were combined into a single dataset with two groups (landfarm and reference) and 12 primary trap sessions, and goodness-of-fit was tested using the Cormak-Jolly-Seber data type. Test 2 was used to assess the assumption of capture homogeneity and indicated that cotton rats from landfarms met this assumption (χ^2 = 29.6, $P = 0.06$) but rats from reference sites did not ($\chi^2 = 37.4$, $P = 0.006$). Tests of homogeneity in survival indicated that neither rats from landfarms ($\chi^2 = 29.6$, $P = 0.06$) or from reference sites ($\chi^2 = 29.6$, $P = 0.06$) met the assumption. If individuals do not exhibit independence in trapability then estimated sampling variances will be underestimated, a situation called overdispersion. Within cotton rat structure, individuals that occupy a home range within the trap grid will be more likely to be trapped than individuals living outside the grid. This characteristic may violate the assumption of independence and will lead to overdispersion. Burnham et al. (1987) suggested that estimation of overdispersion (c-hat) is calculated from the summation of tests 2 and 3. If the assumption of independence is violated, then a c-hat value of > 1.0 will be observed. Burnham et al (1987) give a c-hat value of > 1.3 as evidence of significant overdispersion in the data. Calculated c-hat values for both sites at Units 1 and 3, landfarms at Units 2 and 5, and reference site at Unit 4 were 1.00. The reference site at Unit 2 and 5 and the

landfarm at Unit 4 showed a slight trend for overdispersion with c-hat values of 1.24, 1.03, and 1.33; however, data were not corrected for overdispersion and a c-hat value of 1.00 was used for the analysis.

Model selection led to the selection of two candidate models (Table 1). Both models had survival rates that varied by group (landfarm or reference site of each Unit) and month; however, the models differed in their criteria for estimating population size. The most parsimonious model selected used site and month to model population size (model 1, Table 1); whereas the second model varied population size by treatment (landfarm or reference) and month (model 2, Table 1). The selection of these two models indicated a difference in population size based on the application of petrochemical waste. In addition, cotton rat populations also varied because of characteristics unique to individual sites. Model selection did not support differences in survival based on the application of petrochemical waste as models with a treatment effect in survival.

A treatment by time (model 2, Table 1) effect was observed for cotton rat densities; with cotton rat density being lower on landfarms compared to reference sites (Figure 2). Time effects showed cotton rat populations increasing during summer months and decreasing during winter months, with differences in cotton rat density between landfarm and reference sites most apparent during summer. Cotton rat populations inhabiting landfarms reached maximal densities approximately half (56.1 ± 6.1 rats/ha) that of reference sites (86.2 ± 6.2 rats/ha) during summer. Maximal densities during winter did not differ between cotton rats inhabiting landfarms (36.9 ± 18.0 rats/ha) and reference sites (37.5 ± 8.8 rats/ha). Populations of cotton rats on all sites were reduced during winter for both years of sampling. Mean monthly density of cotton rats inhabiting

reference sites was calculated to be 52.3 ± 7.0 rats/ha during summer and 22.8 ± 5.8 rats/ha in winter, whereas cotton rat densities from landfarms were 30.1 ± 10.2 in summer and 20.0 ± 9.4 rats/ha in winter.

A group*time interaction was selected to describe monthly survival by both of the top models (Table 1). Mean monthly survival was lower in cotton rats inhabiting landfarms (Figure 3), although this effect varied by landfarm unit. Monthly survival was highly variable in cotton rats inhabiting the landfarms on Units 1 and 2 (Figure 3a, b). Rats from these landfarms experienced severe declines in monthly survival during August. Cotton rats inhabiting the landfarm at Unit 3 also had decreased survival across August and into December; however, monthly survival for the remainder of the study was similar to reference levels (Figure 3c). Survival of cotton rats inhabiting the landfarm at Unit 4 followed a similar pattern as rats from the reference site, except during summer 2000 (Jun – Aug) when survival was higher, and in winter (Dec – Jan) when survival was lower. Survival of cotton rats from both landfarm and reference sites fluctuated greatly. Reference sites from all units, except Unit 5, showed similar trends in monthly survival, remaining at or above 0.40. The reference site at Unit 5 experienced declines in monthly survival during the winter (Dec – Feb) of 1999.

Treatment differences in monthly survival was greatest during July and August 1998, with monthly survival for cotton rats inhabiting landfarms being 0.69 ± 0.10 and 0.41 ± 0.16, respectively, and survival from rats inhabiting reference sites being 0.97 ± 0.20 and 0.68 ± 0.03, respectively. Winter survival was similar between treatments for 1998-1999, but survival differed during January 2000, with rats from landfarms (0.64 ± 0.17) having lower monthly survival compared to rats from reference sites (0.94 ± 0.05).

Mean monthly survival of cotton rats inhabiting reference sites was 0.73 ± 0.05 during summer and 0.76 ± 0.10 in winter, and 0.59 ± 0.14 in summer and 0.60 ± 0.14 in winter for cotton rats from landfarms.

Density (Figure 4) and monthly survival (Figure 5) of cotton rats inhabiting landfarms was not correlated with soil concentrations of Pb, Cr, F, or BaP. Regression results indicate that soil contaminant concentrations are not related to cotton rat survival or density. Monthly survival showed no relationship during summer or winter with BaP ($F_{1,4} = 0.02$, $P = 0.88$ – Summer, $F_{1,4} = 0.01$, $P = 0.92$ – Winter) or Pb ($F_{1,4} = 0.11$, $P = 0.76$ – Summer, $F_{1,4} = 0.001$, $P = 0.95$ – Winter). Cotton rat density decreased with increasing soil concentrations of both BaP and Pb during winter, however, the results were not significant ($F_{1,4} = 0.98$, $P = 0.38$ – BaP, $F_{1,4} = 1.43$, $P = 0.30$ – Pb). Survival and density also were tested against Cr and F soil levels, but no relationship was observed.

Sex ratio of cotton rats for both sites followed a similar pattern until February 1999 (Figure 6a). At this point, cotton rat populations inhabiting landfarms skewed toward females whereas reference sites had a higher proportion of males. Reference sites maintained the male dominance in the population for the remainder of the study, fluctuating between 1.2 and 2.0 M:F. After February 1999, sex ratios of cotton rat populations inhabiting landfarms fluctuated to a greater extent than populations inhabiting reference sites.

The percent of juveniles in the trappable population differed between cotton rat populations inhabiting landfarms and reference sites (Figure 6b). Peaks in the proportion of juveniles in the population were observed in populations from reference sites during

late winter and mid-summer. However, in populations inhabiting landfarms, the proportion of juveniles decreased during these periods.

Reproductive organs from cotton rats showed a strong seasonal difference in mass. Both males and females had reduced organ weights during winter, compared to summer. Size of epididymes did not differ between cotton rats inhabiting landfarms and those from reference sites (Figure 7). Individual site analysis also did not show any significant differences. However, a seasonal difference was observed with cotton rats collected during winter (0.43 ± 0.03 g) having lower epidymides weights ($F_{1,11.5} = 19.11$, $P = 0.001$) than rats collected in summer (0.27 ± 0.02 g). Paired testes weight also did not show a difference between rats collected from landfarms and reference sites (Figure 8). No differences in paired testes weight were detected between rats from each Unit. Paired testes weight also was reduced during winter, 0.85 ± 0.04 g compared to 1.77 ± 0.13 g for summer; however, the difference was not significant.

Females showed the same pattern in reproductive organ weight as males. Uterus weight was not different between rats collected from landfarms and those from reference sites (Figure 9). Uterus weight was reduced in winter (0.07 ± 0.02 g) compared to summer (6.39 ± 1.53 g, $F_{1,10} = 76.9$, $P < 0.0001$). Paired ovary weight did not show an overall treatment effect or within site effects. However, ovaries also were reduced in size during winter (14.6 ± 1.8 g) compared to summer (53.0 ± 2.8 g).

Reproduction in cotton rats also showed seasonality as no pregnant or lactating females were captured during winter. During summer, there were no differences in the proportion of pregnant or lactating females inhabiting landfarms or reference sites (Figure 10). However, individual site analyses showed that rats from the landfarm at

Unit 2 had a higher proportion of pregnant females (83.3%, $\chi^2 = 5.82$, $P = 0.01$) than those from reference sites (16.7%). Mean number of embryos/female also did not differ between rats from landfarms and reference sites; however, the landfarm at Unit 2 showed an increase in the number of embryos/female ($T_{10} = -2.26$, $P = 0.05$, Figure 11a). The landfarm at Unit 3 also showed an increase in the number of embryos/female; however, the difference only approached significance ($T_{22} = -1.75$, $P = 0.09$). No embryos were found in females collected during winter. No overall difference was detected in mean number of placental scars/female between rats collected from landfarm and reference sites; however, on Unit 2, reference rats had more scars/female (15.2 ± 3.3 scars) than those from landfarms (0.7 ± 0.7 scars, $T_{5.4} = 4.28$, $P = 0.006$, Figure 11b).

The composition of plant species varied between landfarms and reference sites (Table 2). Species richness did not differ between landfarm (16 species) and reference sites (15 species). Species present only on reference sites were broomsedge bluestem (*Andropogon virginicus*), Scribner's panicum (*Panicum oligosanthes*), old-field threeawn (*Aristida oligantha*), and plantain (*Plantago* spp.). Monocots composed the majority of plants found on landfarms with non-native grasses, especially bermudagrass (*Cynodon dactylon*) and fescue (*Festuca arundinacea*), being the most abundant. Japanese brome (*Bromus japonicus*) was a common grass growing as an even mat in the understory of all sites. Bare ground was more common on landfarms (18.8 ± 4.9%) compared to reference (8.0 ± 2.6%) sites.

DISCUSSION

Differences existed between populations of cotton rats inhabiting sites contaminated with petrochemical waste and those from uncontaminated reference sites,

as predicted by the immune selection hypothesis. Populations of cotton rats inhabiting abandoned landfarms reached lower densities and experienced lower monthly survival rates than their counterparts from reference sites. Although survival was lower throughout the year, differences in survival and population density were most notable during summer months, with winter showing little differentiation in population structure. Along with a reduction in survival, we observed a decrease in the proportion of juveniles from cotton rats inhabiting landfarms, suggesting a reduction in reproduction. However, mass of reproductive organs, % pregnant, or % lactating did not show any differences between cotton rats from either treatment.

The cotton rat was developed as a biomonitor for the effects of contamination by Elangbam et al. (1989) because of their phylogenetic similarity to humans. Cotton rats are widely distributed across much of the southern United States, inhabiting grass-dominated habitats (Cameron and Spencer 1981), and are easily accessible species for study. In addition, they are easy to capture, have short generation times, occur in relatively large numbers, and live in close proximity to humans (Elangbam et al. 1989). Since the development of cotton rats as biomonitors, they have been used to investigate the effects of contaminants on P-450 enzyme induction (Elangbam et al. 1989, Lochmiller et al. 1999), dental fluorosis (Paranjpe et al. 1994), immune function (McMurry et al. 1999), host resistance to pathogens (Jones et al. 2000), chromosome structure (McBee et al. 1987), histopathology (Rattner et al. 1993), and hematopoesis (Kim et al. 2001a,b).

The foundation of the immune selection hypothesis is that contaminants must accumulate in individuals and exhibit some form of toxicity, directly or indirectly, on the

individual. Although no data are presented in the present article on bioaccumulation or immunotoxicity, Schroder et al. (2002) and Carlson et al. (2002) presented data collected from rats inhabiting the same sites as this study that showed accumulation of heavy metals and fluoride in tissues and exposure to polyaromatic hydrocarbons. In addition, Wilson et al. (Chapter II) showed that cotton rats collected from certain landfarms in this study exhibited immunotoxicity and alterations in hematological parameters. As a result, we conclude that rats inhabiting petrochemical landfarms in this study accumulate contaminants and exhibit immunotoxicity.

We observed a marked difference in the seasonal response of populations inhabiting reference sites and landfarms. Populations inhabiting petrochemical landfarms had densities that were lower than reference sites, and this pattern was magnified during summer. Populations on both sites reached higher densities during the first summer of trapping (June – August 1997), and dropped off during the following winter. During 1997, Oklahoma experienced a severe drought followed by a colder-than-average winter. Cotton rat populations are known to be sensitive to the previous year's rainfall, with population densities being positively correlated with rainfall (Windberg 1998). In addition, the colder conditions during the following winter may have exacerbated these poor conditions reducing populations even more. Goertz (1964) found that populations of cotton rats experienced severe declines during winters with long periods of below-freezing temperature, frozen ground, and snow cover.

Maximum densities and treatment effects reported here are similar to densities reported by Elangbam et al. (1989) for cotton rats from contaminated (21 rats/ha) and uncontaminated sites (68 rats/ha) in Oklahoma. Across their range, maximum densities

for cotton rat populations are highly variable (Cameron and Spencer 1981). Although populations of cotton rats inhabiting landfarms were reduced in this study, Flickinger and Nichols (1990) found that cotton rats inhabiting waste-contaminated sites in southern Texas did not have lower population sizes. They reported the largest concentrations of cotton rats on waste sites, and suggested that cotton rats may be a poor choice for an indicator species (Flickinger and Nichols 1990). However, Elangbam et al. (1989) and McMurry et al. (1999) found a decrease in cotton rat densities on contaminated sites, and Dmowski et al. (1998) found that small mammal populations were lower on a thallium-contaminated site compared to an uncontaminated site. The relationship of site contamination to population density was shown by Rowley et al. (1983), who monitored vole (*Microtus* spp.) populations at increasing distances from the hazardous waste site at Love Canal, New York. They found that as the distance from the contaminated site increased, population size also increased. The consensus from this work is that contamination negatively affects populations of small mammals inhabiting contaminated sites. In populations with limited dispersal, reductions in population size can occur through a combination of two mechanisms: increased mortality and decreased reproduction.

We found that cotton rat populations inhabiting landfarms showed decreased survival. The trend was similar to population size with the greatest difference in survival during summer. Swiergosz et al. (1998) found that voles chronically exposed to cadmium had mortality levels that were more than double that of uncontaminated voles. Rowley et al. (1983) found that voles inhabiting a contaminated site had increased mortality rates. Because of the difficulty in monitoring individual causes of mortality

(Rose and Dueser 1980), we cannot identify the source of mortality in either set of populations. However, the observed decrease in survival may be due to a number of reasons. Contaminants may be directly related to increased mortality by disruption of normal physiological mechanisms preventing individuals from functioning. Cotton rats inhabiting contaminated sites have the added cost of detoxification and must increase their foraging time to accommodate their added energetic demands (Congden et al. 2001). Individuals unable to consume enough forage to meet these extra costs would be subject to mortality. In addition, the extra time needed to forage exposes them to additional predation pressures. Finally, immunosuppressive effects of contaminants may increase individual's susceptibility to pathogens and may result in direct mortality from disease (Lochmiller and Deerenberg 2001).

The second factor potentially reducing population density is reproduction. Elangbam et al. (1989) found that mean testes weight did not differ between cotton rats collected from reference and contaminated sites. However, reductions in testis weight have been noted in rodents from sites contaminated with polychlorinated biphenyls (Batty et al. 1990). Swiergosz et al. (1998) found that voles exposed to cadmium did not show a difference in testes weight, but did affect tissue morphology and testes function. No differences in the mass of reproductive organs from males or females were detected in this study between cotton rats from landfarms and those from reference sites. In addition, no differences were observed in the number of embryos or the proportion of pregnant or lactating females observed on the sites. These results suggest that the reproductive ability of individuals inhabiting landfarms is not being affected.

Although we did not find a difference in reproductive structures, there was a reduction in the proportion of juveniles in populations inhabiting landfarms. Elangbam et al. (1989) and McMurry et al. (1999) found that cotton rat populations inhabiting contaminated sites had a lower proportion of juveniles than populations from reference sites. Because fecundity did not differ between treatment sites, a reduction in the proportion of juveniles most likely represents increased juvenile mortality. However, because juveniles are typically excluded from the trappable population (Mihock et al. 1988), survival rates calculated in this study represent adult survival. As a result, juvenile survival may be lower than that of adults but was not estimable.

The reduction in population densities and survival observed in cotton rats inhabiting petrochemical landfarms supported the predictions made by the immune selection hypothesis; however, we established no clear link of the effects of contaminants on immune function observed in previous studies to alterations in the population dynamics observed in this study. In fact, we found no relationship between Pb, Cr, F, and Bap soil concentrations and cotton rat survival and density. As a result, it is possible that cotton rat populations inhabiting petrochemical landfarms are not affected by residual contamination and that indirect effects may be responsible for the declines in density and survival. It is likely that cotton rat populations are responding to differences in habitat structure, most notably plant composition.

Plant species composition and the amount of bare ground differed between landfarms and reference sites. Cameron (1977) found that cotton rat populations are positively correlated with the abundance of grasses, and reproductive activity is reduced in habitats dominated by dicots. Lidicker et al. (1992) suggested that cover is an

important habitat characteristic for cotton rats, and implicates predation risk as the reason that cotton rats prefer areas with overhead cover. Wiegert (1972) found that predation was a contributing factor to fall-winter population declines in cotton rats. The process of landfarming is inherently disruptive to habitats. Development of a landfarm typically begins with clearing land of all vegetation. After waste has been applied, the site will be left for succession, or seeded to prevent erosion. Habitat on Units 1, 2, and 3 resulted from succession after landfarming and were a patchwork of bare ground interspersed with prairie species. However, landfarms at Units 4 and 5 were seeded and had dense mats of fescue and Bermudagrass. These differences in habitat structure offer a valid alternative to the immune selection hypothesis (Congdon et al. 2001). In fact, cotton rat populations on landfarms from Units 1, 2, and 3 were dramatically reduced, often reaching densities < 10 rats/ha, whereas those from landfarms on Units 4 and 5 had densities closer to reference levels.

Reductions in density and monthly survival of cotton rats inhabiting petrochemical landfarms suggest that contaminants affect population dynamics. In addition, previous studies have found that cotton rats from these landfarms had altered immune and hematological parameters. However, no connection between contaminant effects at the cellular and population level was observed. In fact, we found that soil contaminant levels were not related to population dynamics or fecundity. Instead, cotton rat populations appeared to be affected by changes in habitat composition from physical disturbance associated with the process of landfarming.

ACKNOWLEDGEMENTS

The research in this manuscript was funded by USEPA, Office of Research and Development, grant R826242-01-0. However, it has not been subjected to the Agency's required peer and policy review and therefore does not necessarily reflect the views of the Agency and no official endorsement should be inferred. We thank Gary White, Dave Anderson, and Ken Burnham for their help in developing models for use in program MARK, and Dan Rafferty, Eric Webb, Terry Coffey, and Charlotte Deerenberg for their help in trapping.

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Table 1. Model selection for survival and population density of cotton rats inhabiting petrochemical landfarms (n = 5) and reference sites (n = 5) in Olahoma. Recapture rates were assumed equal to initial capture rates. S = survival, G" = probability of emmigration, G' = probability of remaining outside study area, P = probability of first capture, and N = populations size.

Model [A]	AICc[B]	Delta AICc	AICc weight	Parameters	Deviance
1 S(g*t)G"(.)G'(.)P(t)N(g*t)	4098.07	0.00	0.65	262	2321.73
2 S(g*t)G"(.)G'(.)P(t)N(trt*t)	4099.30	1.23	0.35	178	2505.13
3 S(Trt*t)G"(.)G'(.)P(t)N(g*t)	4139.80	41.73	0.00	179	2543.50
4 S(trt*t)G"(.)G'(.)P(t)N(trt*t)	4149.37	51.30	0.00	96	2727.64
5 S(trt*t)G"(.)G'(.)P(t)N(trt*t)	4149.37	51.30	0.00	96	2727.64
6 S(t)G"(.)G'(.)P(t)N(t)	4196.03	97.97	0.00	73	2821.74
7 S(sea*Trt)G"(.)G'(.)P(t)N(g*t)	4249.47	151.40	0.00	161	2691.48
8 S(t)G"(.)G'(.)P(first)N(t)	4283.94	185.87	0.00	49	2958.73
9 S(g*t)G"(.)G'(.)P(sea,t)N(g*t)	4369.28	271.21	0.00	223	2678.23
10 S(g)G"(.)G'(.)P(g)C(g)N(g)	4551.47	453.40	0.00	40	3244.55
11 S(g)G"(.)G'(.)P(g)N(g)	4584.80	486.73	0.00	31	3296.12
12 S(.)G"(.)G'(.)P(.)C(.)N(.)	4914.94	816.87	0.00	6	3676.60

[A] Models are defined by differences in group (g), time (t), treatment (trt), null (.), season (sea), and night of first capture (first).

[B] Akaike's Information Coefficient

Table 2. Percent cover of plant species found on landfarm (n = 5) and reference (n = 5) sites in Oklahoma. Mean and standard error (SE) are presented. Species not present on sites are indicated with an *. Total percent cover may sum to > 100% due to vertical stratification of species. Each site was sampled with 10 randomly placed 1-m^2 quadrats per site.

		Landfarm		Reference	
Species	Common Name	Mean	SE	Mean	SE
Monocots					
Andropogon virginicus	Broomsedge Bluestem	*	*	12.1	3.9
Bromus japonicus	Japanese Brome	26.2	6.0	27.9	6.1
Bromus tectorum	Cheatgrass	5.4	2.5	*	*
Cynodon dactylon	Bermudagrass	53.2	6.8	1.7	1.2
Festuca arundinacea	Fescue	37.5	7.1	*	*
Panicum oligosanthes	Scribner's Panicum	*	*	2.3	1.9
Panicum virgatum	Switchgrass	0.1	0.1	3.0	3.0
Dicots					
Achillea millefolium	Common Yarrow	0.2	0.2	0.1	0.1
Ambrosia psilostachya	Western Ragweed	8.4	2.5	7.3	2.3
Antennaria neglecta	Field Pussy-toes	0.2	0.1	0.3	0.1
Aristida oligantha	Old-field Three Awn	*	*	29.2	7.2
Aster ericoides	White Aster	1.2	0.8	4.4	1.9
Aster annus	Annual Sunflower	0.2	0.1	*	*
Aster spp.	Late Summer Purple Aster	0.1	0.1	10.2	2.8
Gallium spp.	Bedstraw	0.5	0.3	*	*
Gutierrezia dracunculoides	Common Broomweed	0.2	0.2	0.2	0.1
Haplopappus ciliatus	Wax Goldenweed	0.6	0.6	*	*
Plantago spp.	Plantain	*	*	6.4	1.7
Solidago spp.	Goldenrod	2.6	1.2	1.0	0.5
Trifolium spp.	Clover	0.2	0.1	0.1	0.1
	Bare Ground	18.8	4.9	8.0	2.6
	Moss	0.1	0.1	0.2	0.1

Figure 1. Simplified representation of the immune selection hypothesis proposed by Lochmiller (1996). The immunotoxic effects of anthropogenic contamination have been added to the model. Arrows indicate direction of effect between parameters.

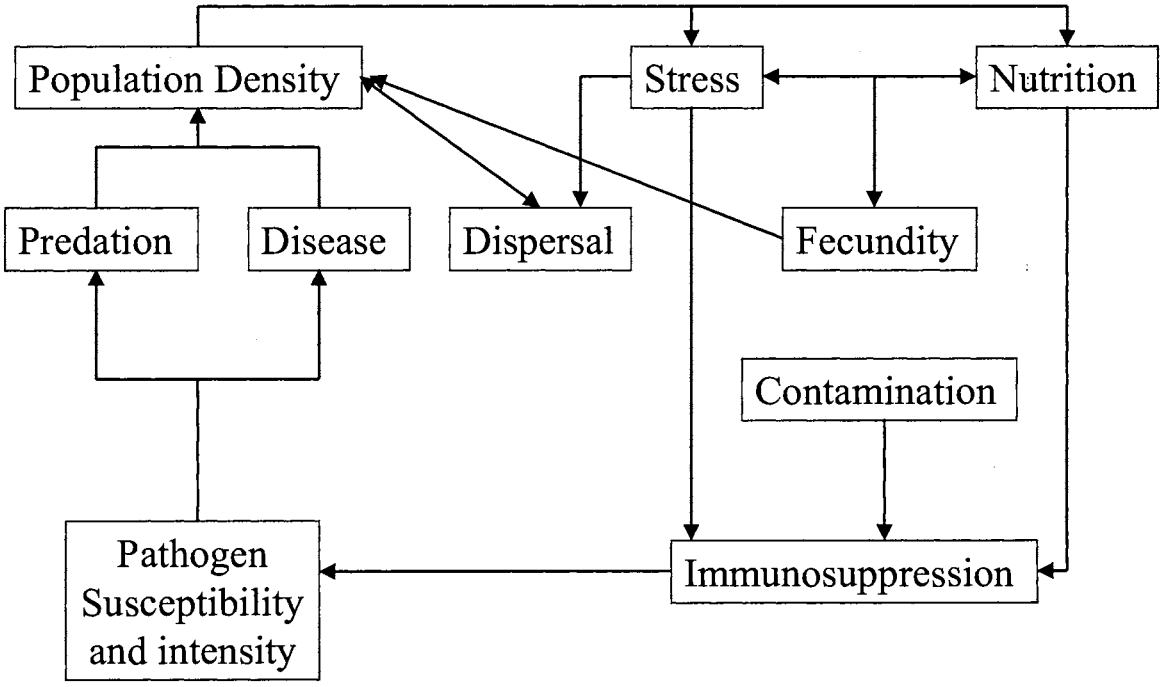

Population Density
Stress
Nutrition
Predation
Disease
Dispersal
Fecundity
Contamination
Pathogen Susceptibility and intensity
Immunosuppression

Figure 2. Mean monthly density of cotton rat populations inhabiting petrochemical-contaminated landfarms (n = 5) and uncontaminated reference sites (n = 5) in Oklahoma. Population estimates were obtained using live recapture data and program MARK. Populations were monitored from June 1998 to February 2000.

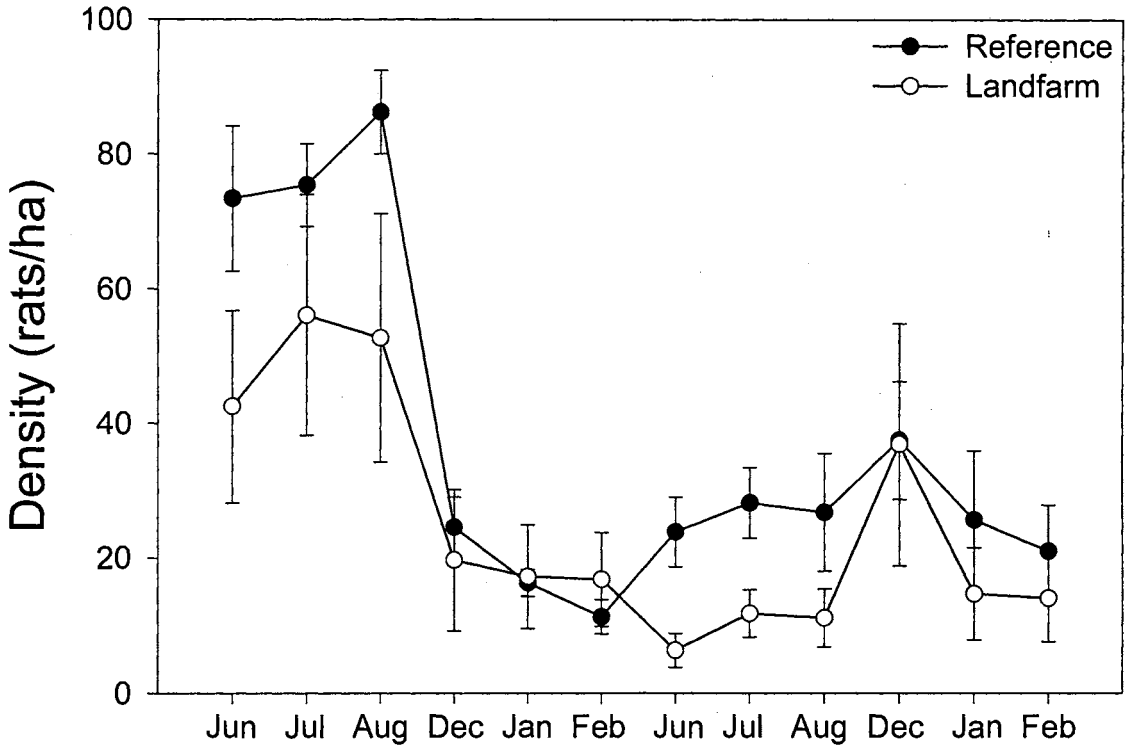
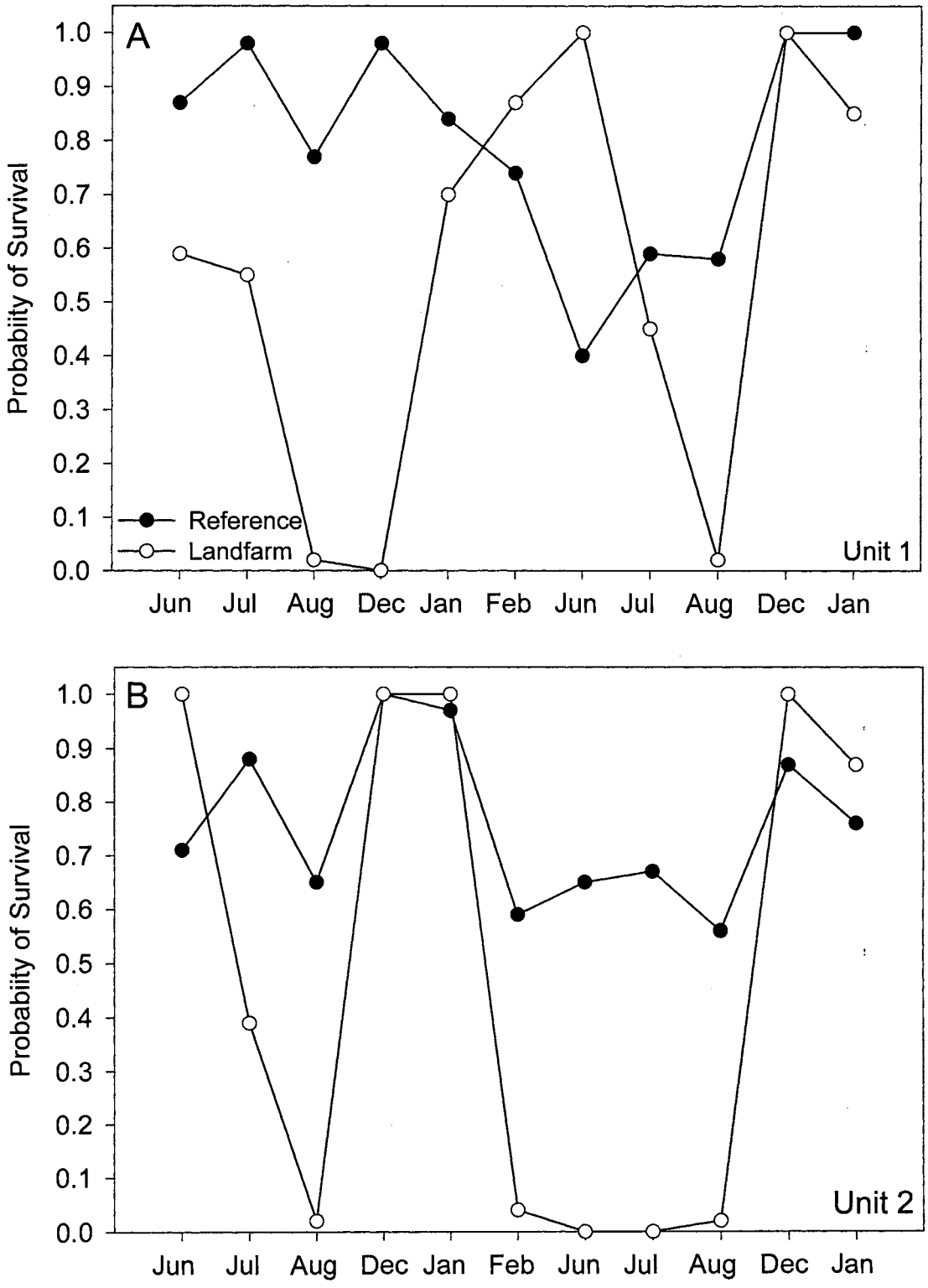
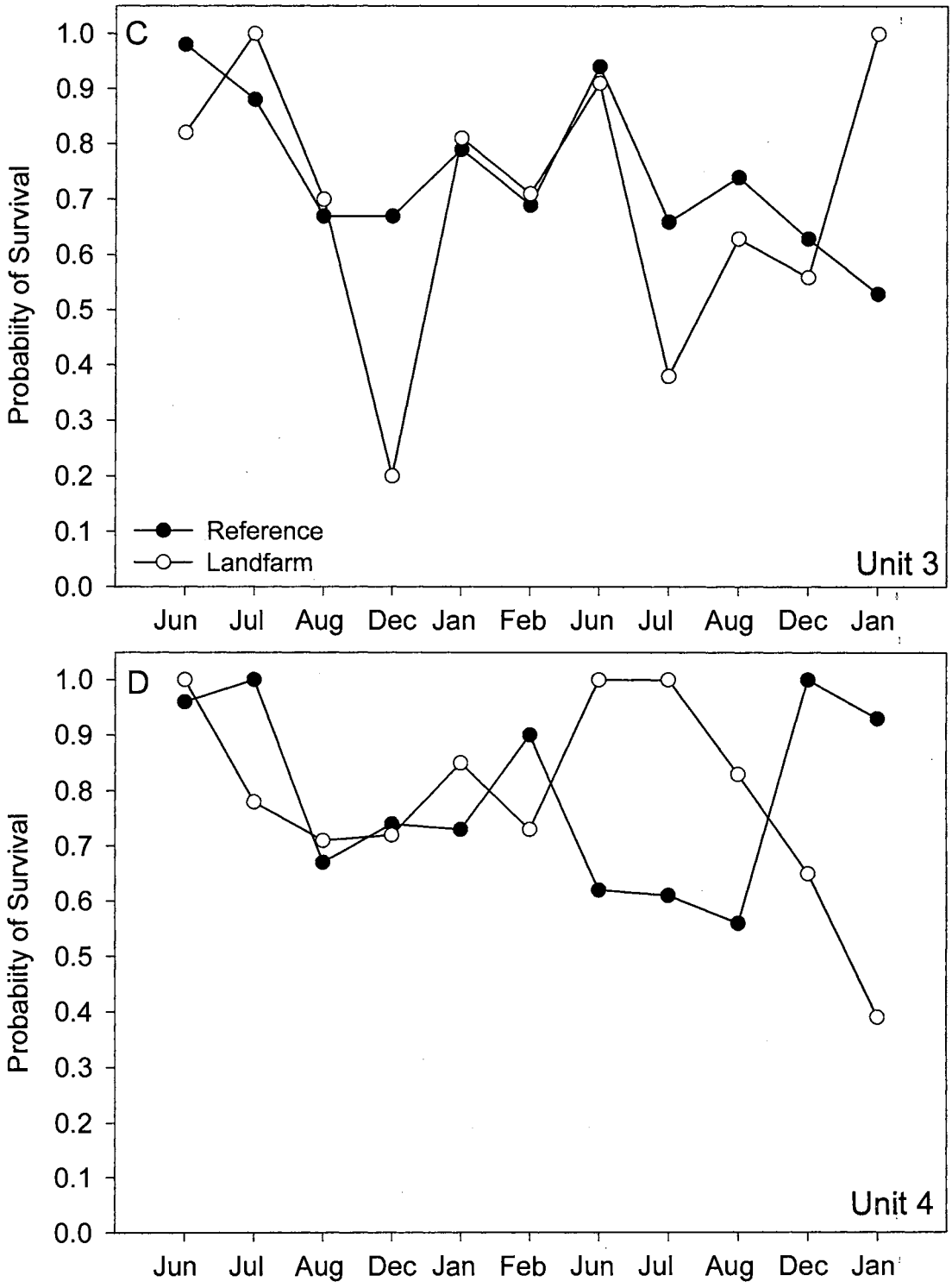

Density (rats/ha)
100
80
60
40
20
0
Reference
Landfarm
Jun
Jul
Aug
Dec
Jan
Feb
Jun
Jul
Aug
Dec
Jan
Feb

Figure 3. Mean monthly survival for cotton rats inhabiting petrochemical landfarms and reference sites from Units 1 (A), 2 (B), 3 (C), 4 (D), and 5 (E) in Oklahoma. Survival estimates were obtained using live recapture data and program MARK. Populations were monitored from June 1998 to February 2000.


A
Probability of Survival
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Reference
Landfarm
Unit 1
Jun
Jul
Aug
Dec
Jan
Feb
Jun
Jul
Aug
Dec
Jan
B
Probability of Survival
Unit 2


C
Probability of Survival
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Reference
Landfarm
Unit 3
Jun
Jul
Aug
Dec
Jan
Feb
Jun
Jul
Aug
Dec
Jan
D
Probability of Survival
Unit 4

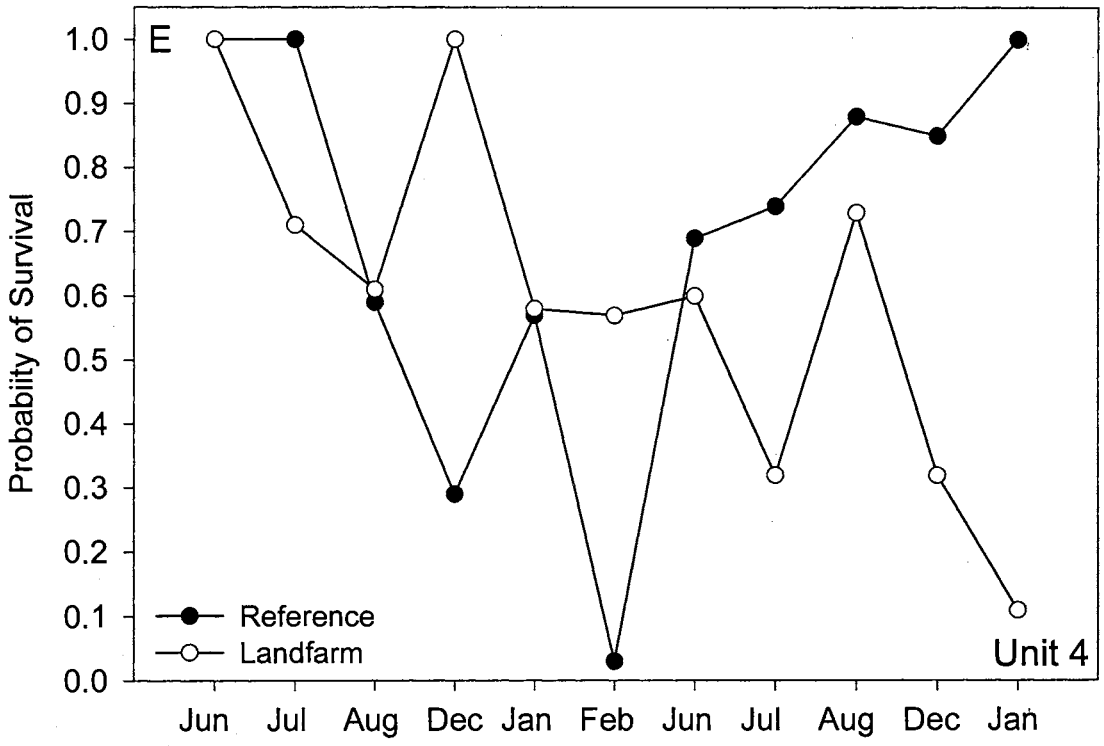
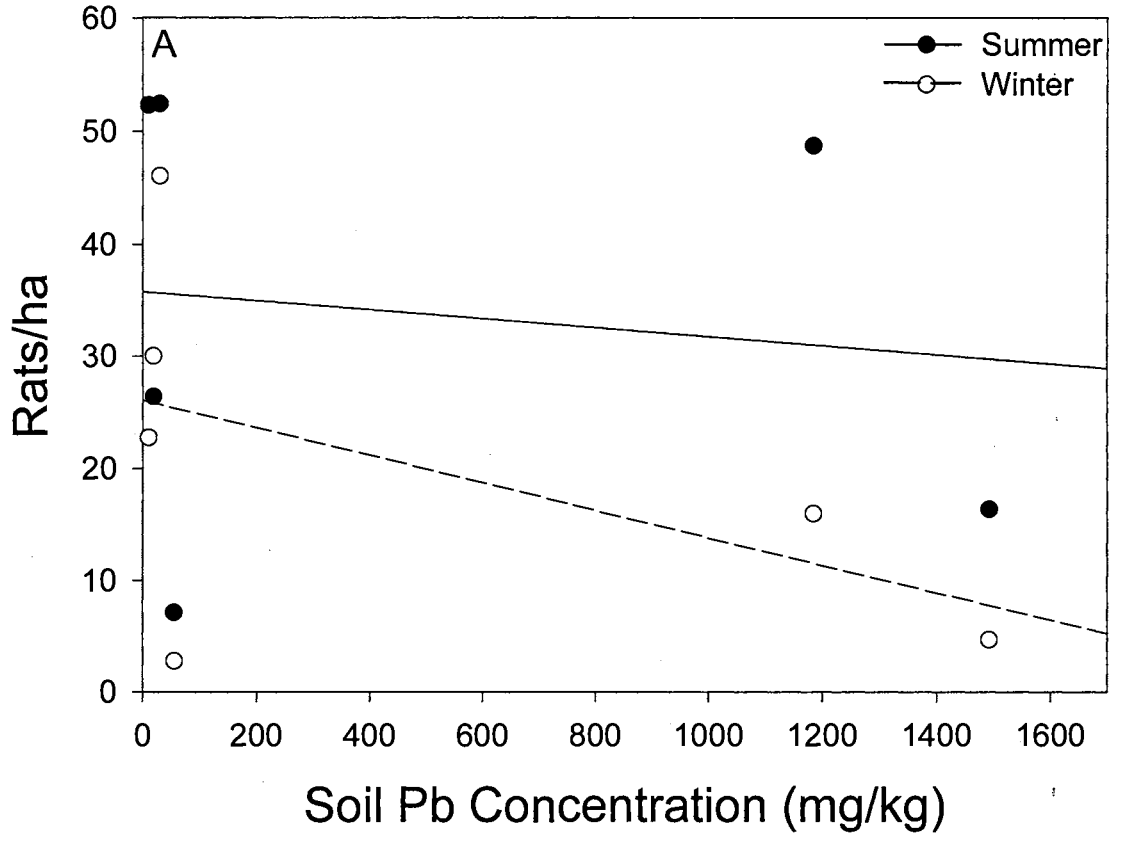

E
Probability of Survival
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Reference
Landfarm
Unit 4
Jun
Jul
Aug
Dec
Jan
Feb
Jun
Jul
Aug
Dec
Jan

Figure 4. Cotton rat density in relation to soil Pb (A) and benzo(a)pyrene (BaP) equivalent (B) concentrations from populations inhabiting petrochemical landfarms and reference sites in Oklahoma.


A
Summer
Winter
Rats/ha
60
50
40
30
20
10
0
0
200
400
600
800
1000
1200
1400
1600
Soil Pb Concentration (mg/kg)

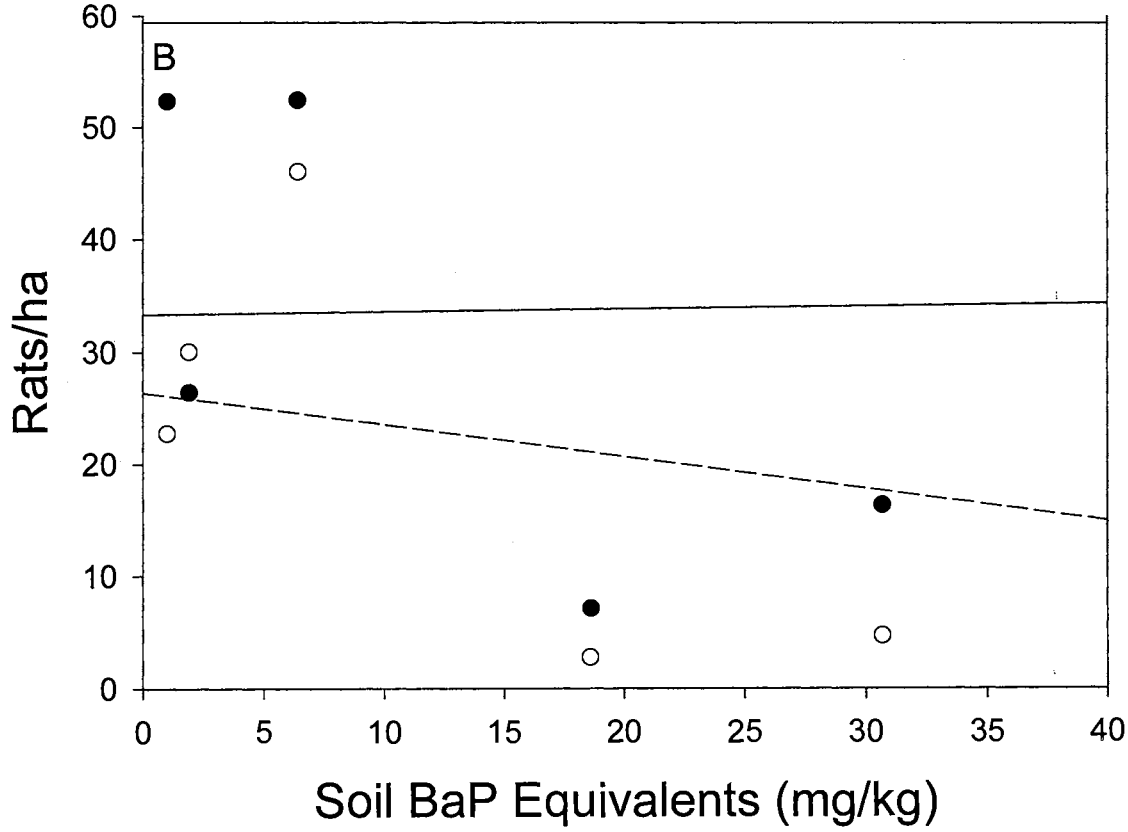
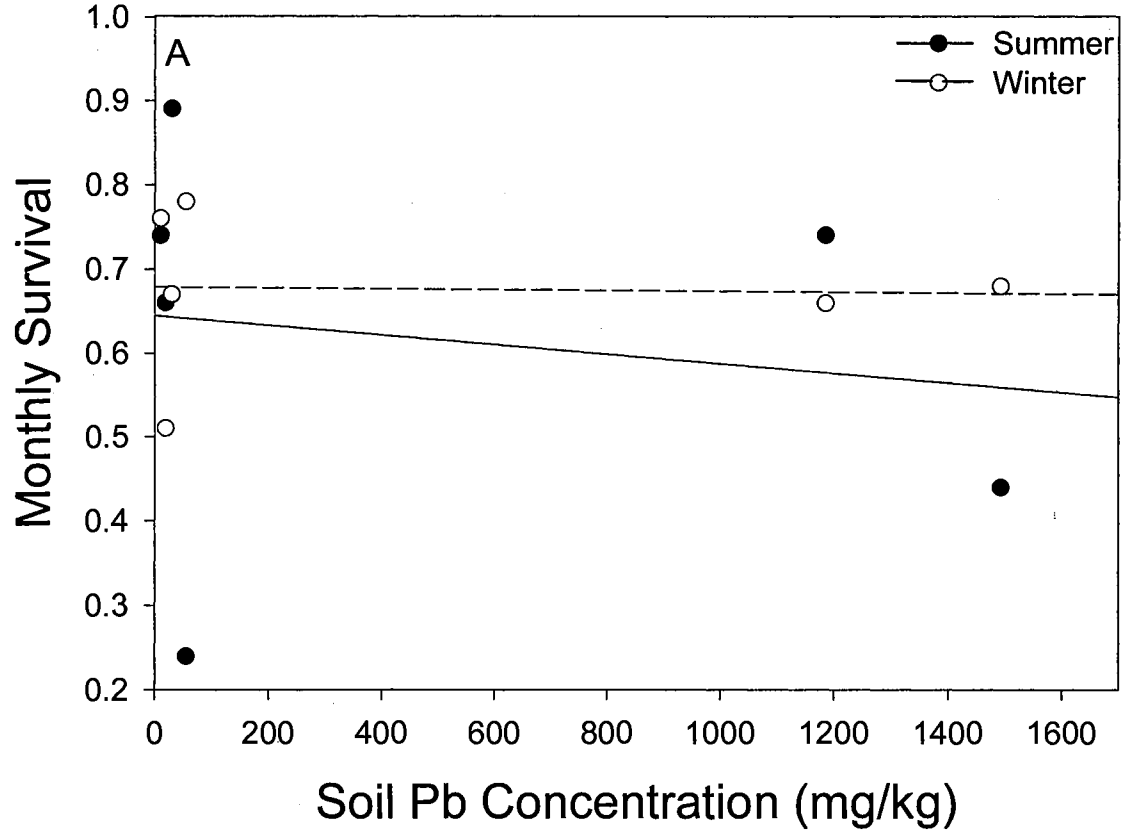

B
Rats/ha
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
Soil BaP Equivalents (mg/kg)

Figure 5. Cotton rat survival in relation to soil Pb (A) and benzo(a)pyrene (BaP) equivalent (B) concentrations from populations inhabiting petrochemical landfarms and reference sites in Oklahoma.


A
Summer
Winter
Monthly Survival
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0
200
400
600
800
1000
1200
1400
1600
Soil Pb Concentration (mg/kg)

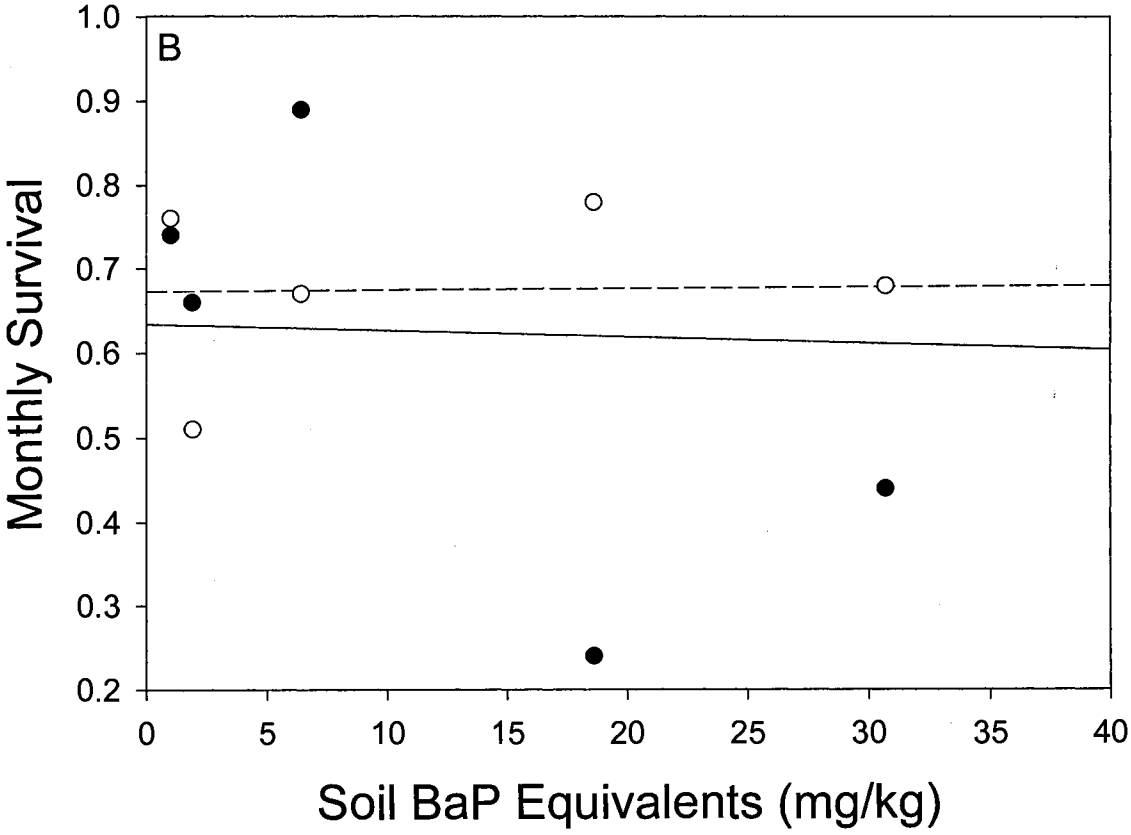

B
Monthly Survival
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0
5
10
15
20
25
30
35
40
Soil BaP Equivalents (mg/kg)

Figure 6. Sex ratio (A) and percent juveniles (B) of cotton rat populations inhabiting petrochemical landfarms (n = 5) and uncontaminated reference sites (n = 5) in Oklahoma. Cotton rats weighing < 60 g were considered juveniles.

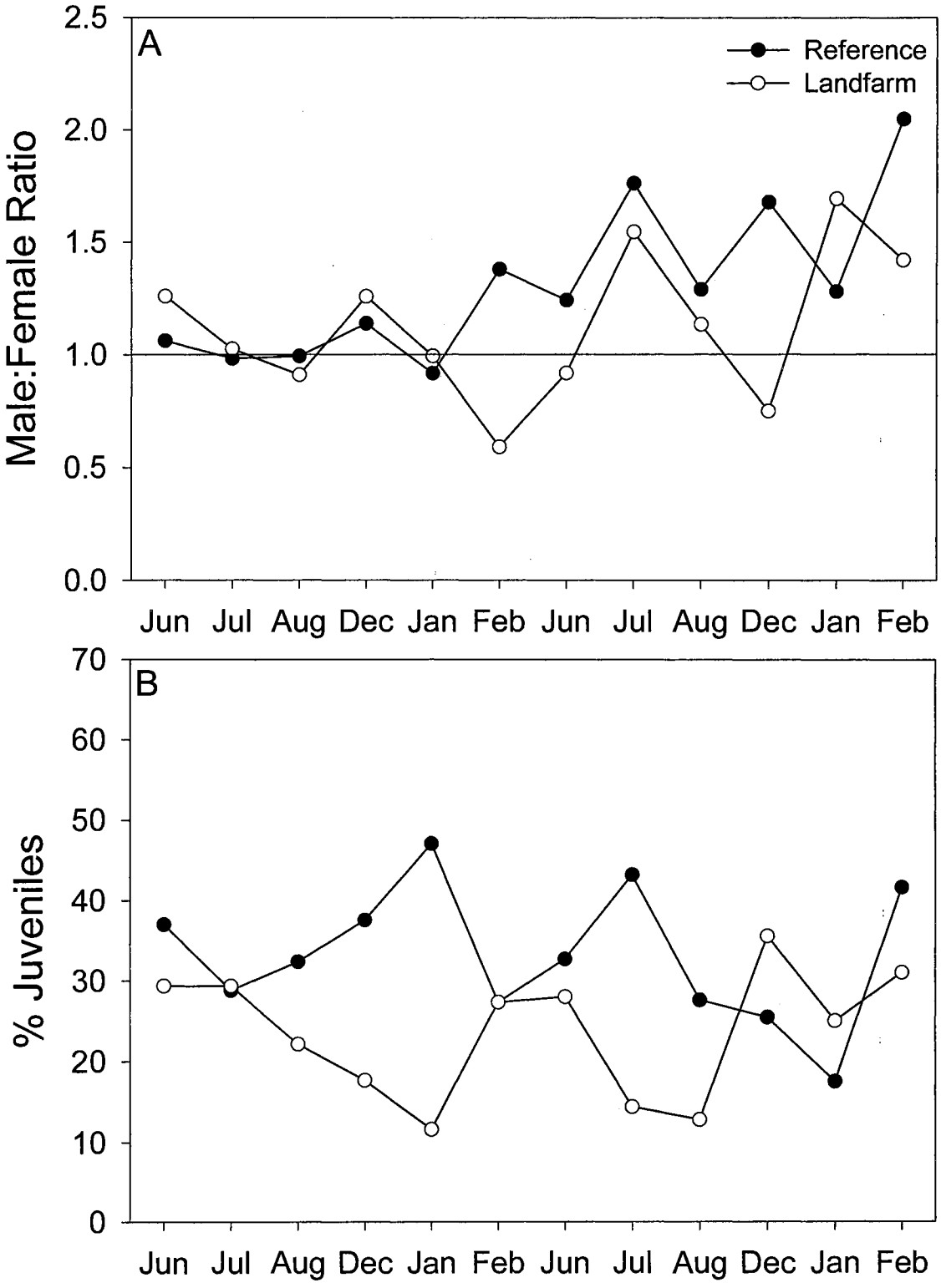

A
Reference
Landfarm
Male:Female Ratio
2.5
2.0
1.5
1.0
0.5
0.0
Jun Jul Aug Dec Jan Feb Jun Jul Aug Dec Jan Feb
B
% Juveniles
70
60
50
40
30
20
10
0
Jun Jul Aug Dec Jan Feb Jun Jul Aug Dec Jan Feb

Figure 7. Weight of cotton rat epididymes (g), during summer and winter, collected from 5 study sites (Petrochemical Units 1 – 5) in Oklahoma. Treatment differences are presented by the total group.

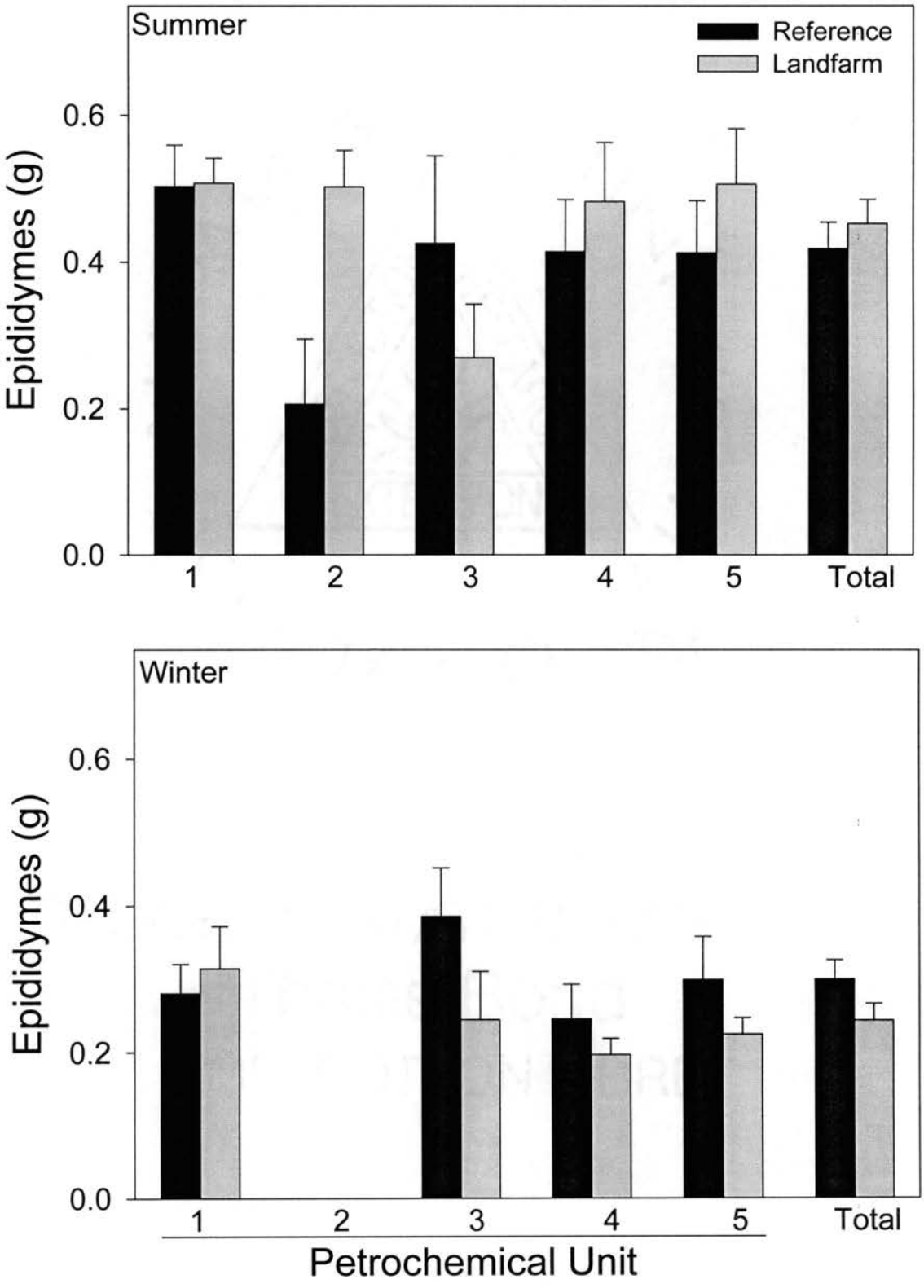

Summer
Reference
Landfarm
Epididymes (g)
0.0
0.2
0.4
0.6
1
2
3
4
5
Total
Winter
Epididymes (g)
0.0
0.2
0.4
0.6
1
2
3
4
5
Total
Petrochemical Unit

Figure 8. Weight of cotton rat testes (g), during summer and winter, collected from 5 study sites (Petrochemical Units 1 – 5) in Oklahoma. Treatment differences are presented by the total group.

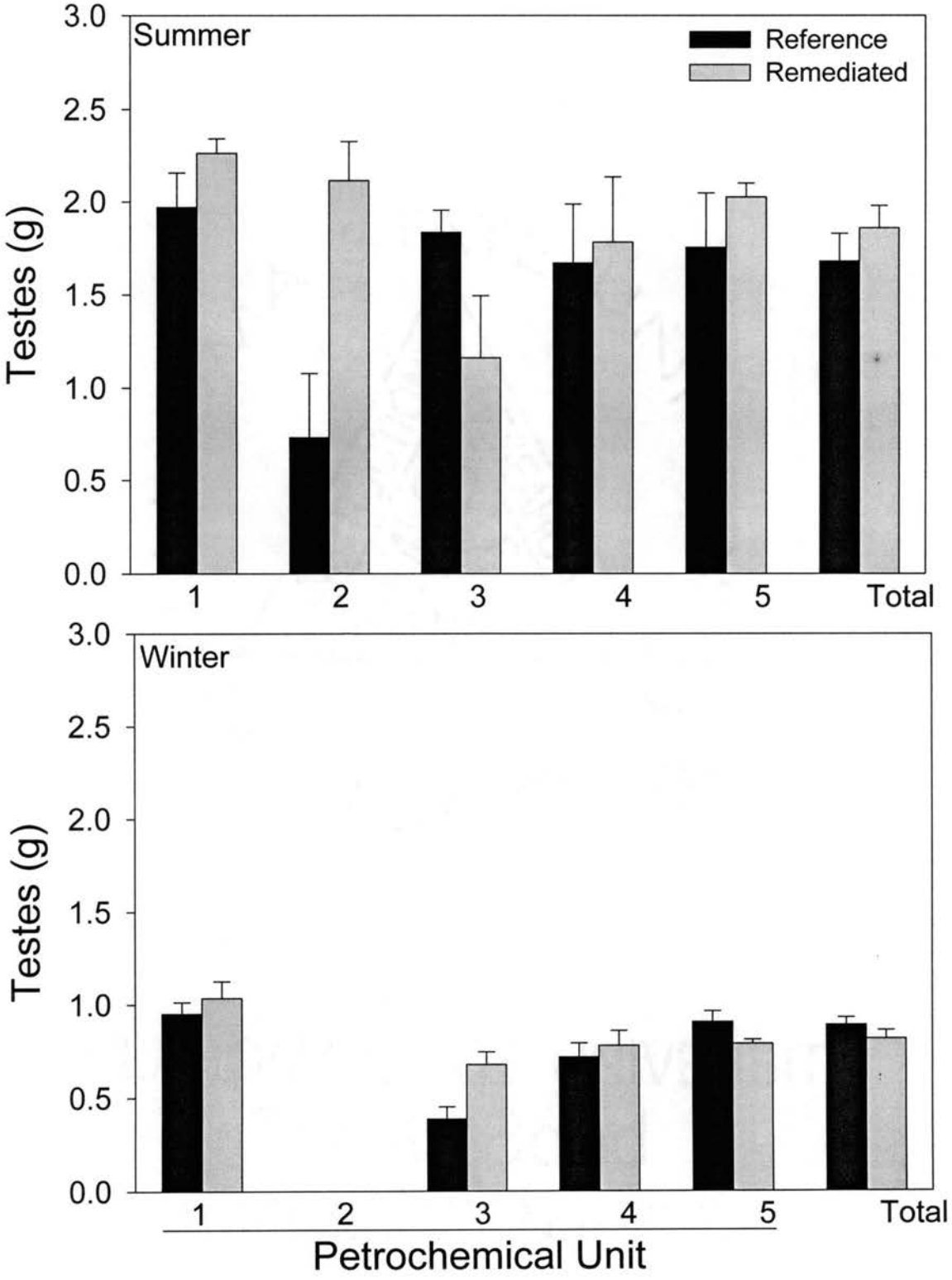

Summer
Reference
Remediated
Testes (g)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
Total
Winter
Testes (g)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
Total
Petrochemical Unit

Figure 9. Uterus weight (g) of cotton rats during summer and winter collected from 5 study sites (Petrochemical Units 1 – 5) in Oklahoma. Treatment differences are presented by the total group.

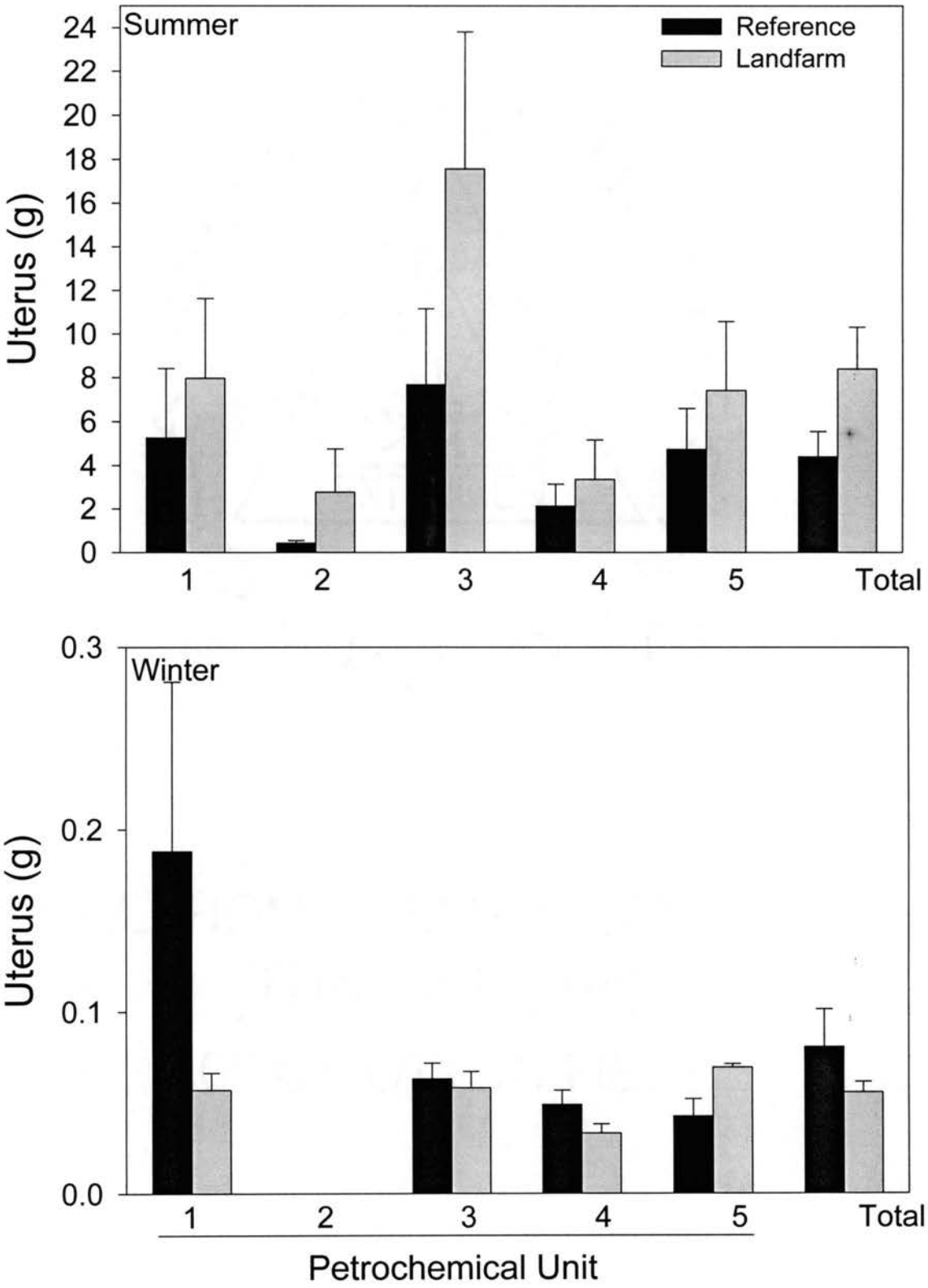

Summer
Reference
Landfarm
Uterus (g)
24
22
20
18
16
14
12
10
8
6
4
2
0
1
2
3
4
5
Total
Winter
Uterus (g)
0.3
0.2
0.1
0.0
1
2
3
4
5
Total
Petrochemical Unit

Figure 10. Percent of pregnant (A) and lactating (B) female cotton rats captured each month on reference and petrochemical-contaminated landfarms in Oklahoma. No pregnant or lactating females were observed during winter (December – February).

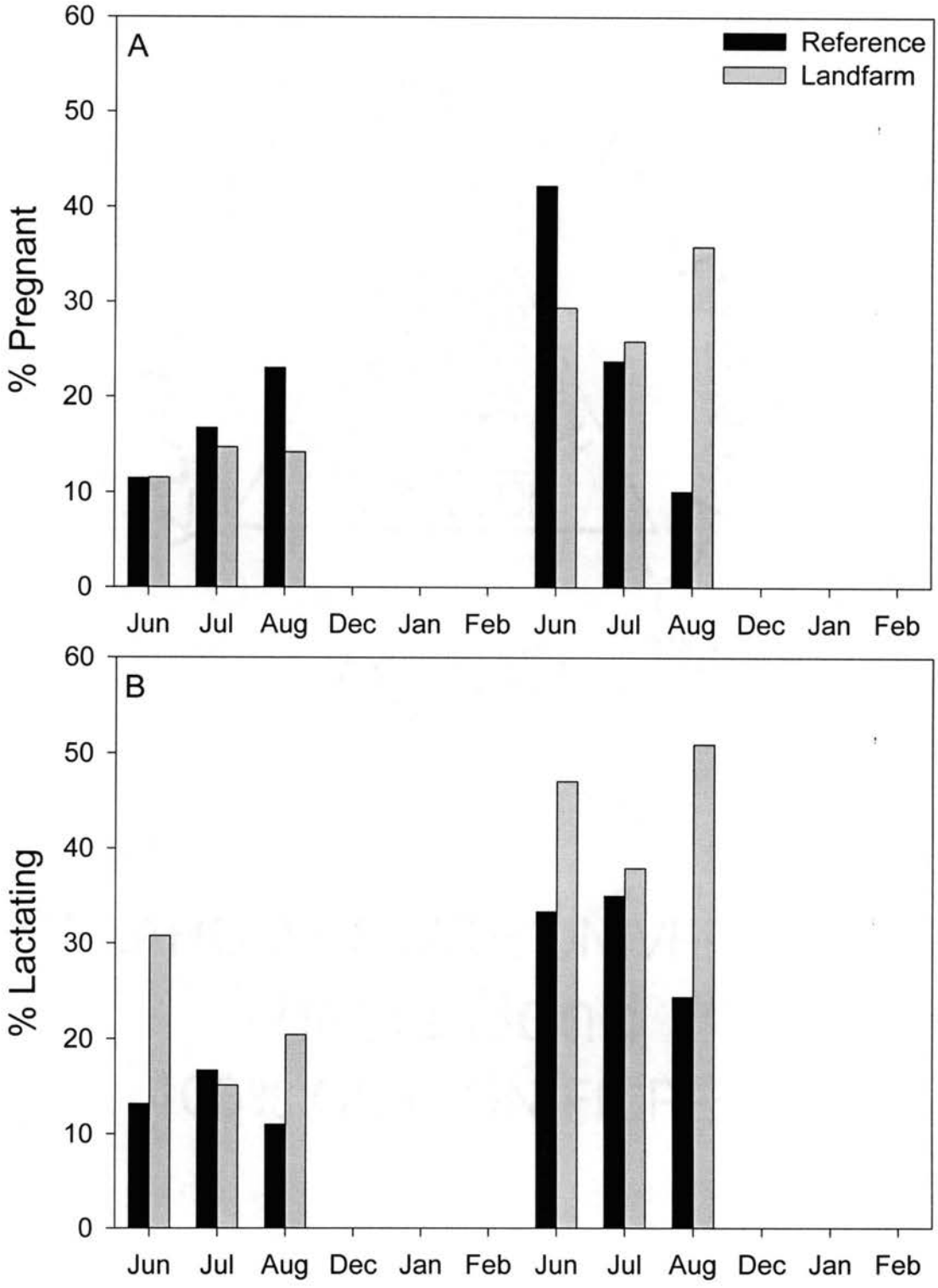
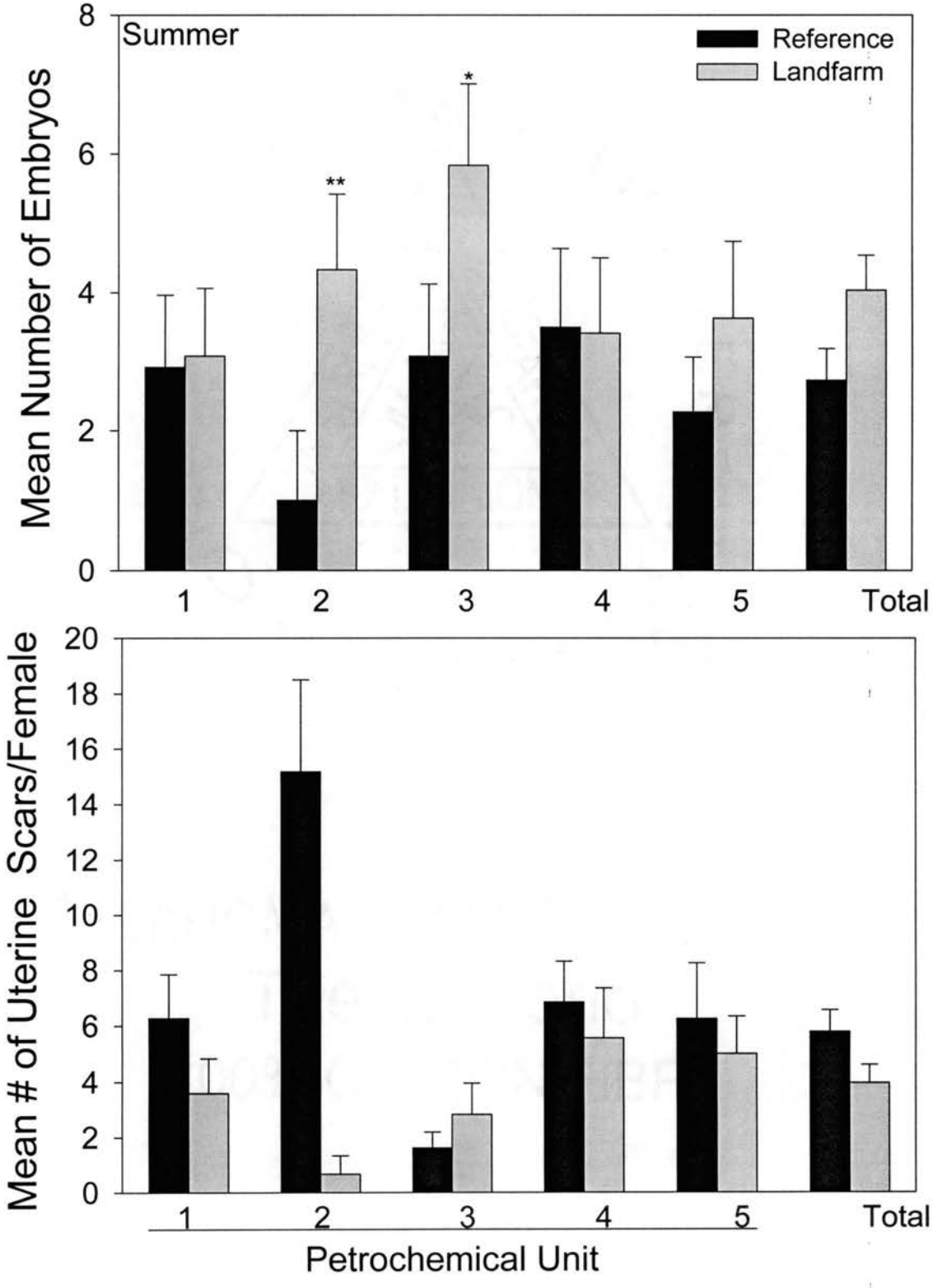

A
Reference
Landfarm
% Pregnant
60
50
40
30
20
10
0
Jun
Jul
Aug
Dec
Jan
Feb
Jun
Jul
Aug
Dec
Jan
Feb
B
% Lactating
60
50
40
30
20
10
0
Jun
Jul
Aug
Dec
Jan
Feb
Jun
Jul
Aug
Dec
Jan
Feb

Figure 11. Mean number of embyos (A) and placental scars (B) per female cotton rat collected from 5 study sites (Petrochemical Units 1 – 5) during summer in Oklahoma. Treatment differences are presented by the total group.


Summer
Reference
Landfarm
Mean Number of Embryos
0
2
4
6
8
**
*
1
2
3
4
5
Total
Mean # of Uterine Scars/Female
0
2
4
6
8
10
12
14
16
18
20
1
2
3
4
5
Total
Petrochemical Unit

CHAPTER IV

DYNAMICS OF RODENT ASSEMBLAGES INHABITING ABANDONED PETROLEUM LANDFARMS IN OKLAHOMA

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Studies on the effects of contamination on wildlife have been focused at the individual level. Biomarkers have been used to monitor changes in the health of individuals exposed to contaminants; however, little attention has been given to the effects of chronic exposure at the population or community level. I studied rodent assemblages from uncontaminated (reference) sites and abandoned petrochemical landfarms to identify alterations in community structure and composition. Reference sites showed typical rodent assemblage structure dominated by hispid cotton rats (*Sigmodon hispidus*) and fulvous harvest mice (*Reithrodontomys fulvescens*). Assemblages inhabiting landfarms also were dominated by cotton rats; however, harvest mice were replaced by deer mice (*Peromyscus maniculatus*). Contaminated sites also were characterized by an increase in house mice (*Mus musculus*) and an absence of voles (*Microtus* spp.). Cotton rat abundance had a negative impact on rodent diversity on both reference and landfarm sites, but was more intense on landfarm sites. Species diversity varied seasonally on both landfarms and reference sites, with peaks in diversity associated with periods of low cotton rat abundance. Community evenness was indirectly affected by the abundance of cotton rats. The results of this study suggest that

rodent assemblages were impacted by landfarms either directly through effects of contaminants on the health of individuals or indirectly by changes in the habitat.
Key words: cotton rats, contamination, diversity, evenness, *Reithrodontomys fulvescens*, *Sigmodon hispidus*.

Populations of rodent species fluctuate with variations in environmental conditions. Precipitation, which directly affects seed and forage production, has been correlated with rodent population densities (Shelford 1943, Krebs and Myers 1974, Windberg 1998). Species with different life histories will exhibit different patterns in population growth under the same environmental conditions (Windberg 1998), and have evolved strategies for coexistence with other species in a varying environment. As a result, fluctuations in the population structure of individual species will affect community structure as well. Contamination from anthropogenic sources contributes additional stressors that may alter community dynamics.

Petroleum refining generates a complex and highly concentrated waste product. The use of landfarms in the treatment and disposal of petrochemical waste has increased in recent years, and it is estimated that over half of all waste produced by refineries is treated in landfarms (American Petroleum Institute 1984). During landfarming, waste is spread onto a prepared bed of soil. Microbes in the soil metabolize portions of the organic component of waste, detoxifying the site in the process. However, microbes are not effective against certain polyaromatic hydrocarbons and inorganic compounds, leaving residual waste in the soil that may be available for bioaccumulation.

Contaminants associated with petrochemical refining have been reported to cause a number of adverse affects on individuals inhabiting abandoned landfarms. Effects from petrochemical waste include chromosomal aberrations (McBee et al. 1987), dental lesions (Paranjpe et al. 1994, Rafferty et al. 2000), alterations in immune function and hematology (Rafferty et al. 2001), and increased detoxification enzyme activity (Lochmiller et al. 1999). Effects of contaminants at the individual level can be translated into changes at the population and community level through direct (e.g., increased mortality) or indirect effects, such as increased energetic demands through costs associated with detoxification (Congden et al. 2001).

Changes in rodent assemblages have been noted on sites exposed to various types of contamination. McMurry (1993) found sites contaminated with heavy metals and hydrocarbons had lower species richness, but increased species diversity due primarily to increased house mouse (*Mus musculus*) populations. In addition, there was a decrease in the proportion of juveniles found on contaminated sites. Batty et al. (1990) also found a decrease in the number of juvenile and subadult white-footed mice (*Peromyscus leucopus*) on sites contaminated with polychlorinated biphenyls. Lower densities and species richness in rodent assemblages also were found in sites contaminated with thallium (Dmowski et al. 1998).

The generation and disposal of contamination will be a persistent and increasingly prominent problem to wildlife. Most studies have focused on finding biomarkers to quantify effects at the individual level. However, few studies have focused on the effects of chronic exposure at the population and community level. Responses at these levels represent the integration of effects at the individual level. My objectives were to identify

alterations in the rodent assemblage inhabiting abandoned petrochemical landfarms. Specifically, I measured differences in species diversity, community evenness, and species overlap between rodent assemblages living on landfarms and reference sites. In addition, I investigated the importance that cotton rats, the dominant rodent in the system, have in structuring the rodent assemblage on contaminated and uncontaminated sites. I hypothesized that rodent assemblages inhabiting petroleum-contaminated sites would shift in species composition toward more opportunistic species and will support lower population abundances compared to reference sites.

METHODS

This study was conducted on 10 paired sites throughout Oklahoma (Units 1-5). Five sites were abandoned landfarms that had some history of petrochemical contamination, and 5 were uncontaminated reference sites. Reference sites were located as close to landfarms as possible to reduce variation from climate and vegetation. Contaminated sites for Units 1, 2, and 3 were abandoned landfarms with no further cleanup. Contaminated sites at Units 4 and 5 were abandoned landfarms that had undergone additional cleanup (removal of contaminated soil and reapplication of soils into a new landfarm) by the U. S. Environmental Protection Agency. All sites were located in the tallgrass prairie ecosystem and consisted of disturbed grassland habitat. Sites were located in southwestern (Units 1 and 2), north central (Unit 3), and eastern (Units 4 and 5) Oklahoma.

Rodent assemblages were sampled on all sites using mark-recapture techniques. All rodent assemblages were surveyed monthly during winter (December – February) and

summer (June – August) for two years (June 1999 – February 2001) with each seasonal trap session consisting of 3, 4-day periods separated by 3-week intervals. Sixty-four Sherman live traps (Sherman Traps Inc., Tallahassee, FL) spaced at 10-m intervals were arranged in a square (8 x 8) or rectangular (4 x 16) grid as dictated by the shape of the area to be surveyed. Traps were baited with whole oats, set in the late afternoon, and checked the following morning. Cotton bedding was provided during winter months for added insulation.

Captured individuals for each species were sexed, weighed, given a unique identification number by toe clipping, and released. Because several species were represented by low incidences of capture, the minimum number known alive (MNKA) estimate was used for population abundance (Krebs 1966). Birth, death, and immigration were assumed to be zero for all sites during each 4-day period of trapping. Mean minimum longevity was calculated for each species by averaging the time, in months, between the first and last capture of each individual that was captured at least twice (Wilkins 1995). Mean minimum longevity values were calculated for each site. Monthly species richness was calculated for every landfarm and reference site as the number of species captured during the 4-day trapping session.

Rank abundance analysis (Begon et al. 1996) was used to determine differences in the structure of rodent assemblages from landfarms and reference sites. The log of the proportion of individuals was used as the measure of abundance and species were assigned a rank from 1 (most abundant) to 9 (least abundant). The relationship between species rank and abundance was tested between rodent assemblages inhabiting landfarm and reference sites using multiple linear regression with PROC REG (SAS 1994).

Horn's (1966) index of similarity (R_0) was used to measure the amount of overlap between rodent assemblages from landfarms and reference sites. This index generates a value ranging from 0 when sites do not share any species in common to 1 when sites are identical with respect to proportional species composition. Horn's index of similarity is based on the abundance of individuals found on each site and is relatively unaffected by low sample sizes, making it useful for MNKA data (Krebs 1999).

Individual landfarms were contaminated with a unique set of contaminants. To identify patterns in rodent assemblage composition between individual sites, a cluster analysis was performed using a similarity matrix generated for each combination of site pairs (Krebs 1999). The similarity matrix was constructed using similarity values obtained from the complement of the Canberra metric (Krebs 1999). The Canberra metric is not biased by the dominance of a single species; however, data points that are missing a species from both sites are inestimable and were therefore dropped (Krebs 1999). A phenogram of rodent assemblage similarity by site was constructed using single linkage clustering (Krebs 1999).

Differences in rodent assemblages in relation to cotton rat abundance were measured using procedures given by Brady and Slade (2001). Shannon's diversity index (H') was calculated from the proportional abundance of individuals of each species. The relationship between species diversity and cotton rat abundance was determined for landfarms and reference sites using linear regression. Treatment differences were compared using an F-test with dummy variables for treatment. To determine if fluctuations in cotton rat abundance affected species diversity, linear regression was performed on cotton rat abundance and Shannon's evenness (J') with and without

inclusion of cotton rats in the evenness calculations as described by Brady and Slade (2001).

RESULTS

Rodent assemblages, as described by rank-abundance analysis, differed between landfarms and reference sites (Fig. 1; $F_{2,15} = 3.62$, $P = 0.05$). Rank-abundance analysis, which incorporates species richness, diversity, and evenness, showed rodent assemblages inhabiting landfarms to have a more even distribution of species (slope = -0.11, $R^2 = 0.82$, $P < 0.001$) compared to those from reference sites (slope = -0.13, $R^2 = 0.90$, $P < 0.001$). Increased abundance of incidental species and a reduction in the number of cotton rats on landfarms were responsible for the change toward a more even assemblage.

Although cotton rats were the most abundant species on both landfarms and reference sites, their effect on species diversity and evenness was not equal. Higher abundances of cotton rats on reference sites may have artificially caused rodent assemblages on reference sites to appear to have lower diversity and evenness, thereby masking any real differences in the rank abundance analysis. Each component was tested separately to determine if any treatment effects were present for species richness, diversity, and evenness. Species richness showed a treatment effect ($F_{1,96} = 4.86$, $P = 0.03$), but no effects by month; with reference sites having slightly higher mean monthly species richness (3.77 ± 0.16 species/mo) than landfarms (3.28 ± 0.16 species/mo). Species diversity was negatively associated with cotton rat abundance on both reference (slope = -0.005, $R^2 = 0.63$, $P < 0.0001$) and landfarm (slope = -0.005, $R^2 = 0.29$, $P < 0.0001$) sites (Fig. 2). However, the negative effect of cotton rat abundance on rodent

diversity was more intense on landfarms compared to reference sites ($F_{2,116} = 5.04$, $P = 0.008$).

Cotton rat abundance initially appeared to affect total rodent evenness; however, the effect of cotton rat abundance on rodent evenness was an artifact of the comparatively large number of individuals (Fig. 3). Both landfarms (slope = -0.006, $R^2 = 0.40$, $P < 0.0001$) and reference (slope = -0.008, $R^2 = 0.77$, $P < 0.0001$) sites showed a negative relationship between community evenness and cotton rat abundance when cotton rats were included in the evenness calculations. However, when cotton rats were excluded from the evenness calculations, the effect of cotton rat abundance on evenness disappeared from both reference (slope = -0.0004, $R^2 = 0.002$, $P = 0.97$) and landfarms (slope = 0.00003, $R^2 < 0.001$, $P = 0.79$, Fig. 3b). Similarly, a treatment difference was apparent when cotton rats were included in the evenness calculations ($F_{2,108} = 3.05$, $P = 0.05$, Fig. 3a). The effect of cotton rat abundance on community evenness was less intense on rodent assemblages from landfarms as on reference sites (Fig. 3a). The treatment differences that were present when cotton rats were included in the evenness calculations were no longer significant when cotton rats were excluded from evenness calculations ($F_{2,90} = 0.18$, $P = 0.84$, Fig. 3).

Horn's index of overlap (R_0) indicated that sites were similar in proportional species abundance (Fig. 4). Overlap varied temporally, with rodent assemblages on landfarms and reference sites being most similar during the summer of 1999 and peaking in July ($R_0 = 0.88$). The following winter, the similarity of rodent assemblages reached its lowest point in January ($R_0 = 0.52$). Cluster analysis of individual site similarity produced three major clusters of sites (Fig. 5). Reference sites at Units 1 and 2 showed

the greatest similarity (0.82) with all reference sites, except that from Unit 4, clustering together with a similarity of 0.59. The reference site at Unit 4 was most similar to the landfarm at Unit 5 (0.74), and only shared a similarity index of 0.57 with the other reference sites. Landfarms at Units 3, 4, and 5 clustered together with the reference site at Unit 4 with a similarity index of 0.60. The final cluster was formed by the landfarms at Units 1 and 2 (similarity = 0.55) and had a similarity index of 0.54 when compared to all other sites.

Cotton rats were the most abundant species on both reference and landfarm sites (Table 1). Cotton rat abundance was greater on reference sites during the first summer, but then remained at a similar level as contaminated sites for the remainder of the study (Fig. 6). Cotton rat abundance peaked during the summer months on reference sites; however, the peak was more than half that of the first summer. Similarly, harvest mice were more abundant on reference sites (Table 1). Harvest mice had peaks in abundance during winter months with populations from reference sites reaching greater abundances than those from landfarms (Fig. 7a). However, plains harvest mice (*Reithrodontomys montanus*) were found as incidental species on both landfarms and reference sites (Table 1).

Two species of white-footed mice were found among the sites (Table 1), the deer mouse and the white-footed mouse (*Peromyscus leucopus*). Deer mice were found in low numbers on both sites, but tended to be more abundant on landfarms, increasing during the winter of 2000-2001 (Fig. 7c). White-footed mice also were found in higher numbers on reference sites and showed peaks in their abundance during summer (Fig. 7d). Voles were found only on reference sites from Units 1, 2, and 3 (Table 1; Fig. 7e). Captures of

voles consisted of two species, prairie vole (*Microtus ochrogaster*) and pine vole (*M. pinetorum*). Conversely, house mice, although trapped on both landfarms and reference sites, were found predominantly on landfarms and had abundances that peaked in winter (Fig. 7b). Pocket mice (*Chaetodipus hispidus*) also were found in higher abundances on landfarms (Table 1) and peaked in summer (Fig. 7f). Rice rats (*Oryzomys palustrus*) were not trapped on any site until December 2000, and may represent a temporary range expansion, as they have never been reported in Tulsa County, Oklahoma. Eastern woodrats (*Neotoma floridana*) were caught as incidentals on sites in reference and landfarm sites.

No differences in mean minimum longevity were observed for most rodent species inhabiting landfarms and reference sites. However, treatment effects for mean minimum longevity were observed in cotton rats and house mice (Table 2). Cotton rats inhabiting petrochemical landfarms (2.20 ± 0.09 mo) had a minimum longevity that was a month shorter than cotton rats inhabiting reference sites (3.91 ± 0.15 mo). House mice inhabiting landfarms (1.84 ± 0.19 mo) had longer longevity values than their counterparts from reference sites (1.00 ± 0.00 mo).

DISCUSSION

The structure of rodent assemblages differed between those inhabiting reference sites and landfarms. Rank abundance analyses present a combined measure of diversity, richness, and evenness (Begon et al. 1996). Rodent assemblages inhabiting landfarms showed a trend towards a more even distribution of rodents compared to those inhabiting reference sites. The differences in the structure of the assemblages between sites was

primarily due to a reduction in cotton rat abundance and increased abundances in incidental species found on landfarms, both contributing to increased evenness.

The dominance of cotton rats in prairies may influence the diversity and species composition of the rodent assemblage through direct or indirect interactions. Glass and Slade (1980a) found that as cotton rat densities increased, interspecific competition with voles increased. In addition, increases in aggression toward other species occurred during the reproductive period of cotton rat populations (Glass and Slade 1980b). Glass and Slade (1980b) also found that vole populations increased in size following local extinction of cotton rats, suggesting a form of ecological release.

The results of this study also suggest that cotton rat abundance may influence species diversity negatively. Diversity was negatively associated with cotton rat density on both reference and landfarm sites in this study. However at the same cotton rat abundance, contaminated sites had lower species diversity, indicating that further reductions in rodent diversity on landfarms may be due to direct or indirect affects of residual contamination. The additional stress of contaminants may impact individuals directly through decreased reproduction (Linzey 1987), increased energetic costs (Lochmiller and Deerenberg 2000), impaired immune function (Rafferty et al. 2001), or indirectly by reducing plant diversity (Xiong et al. 1997) and decreasing plant biomass (Stoughton and Marcus 2000). In addition, accumulation of contaminants varies across species (Dmowski et al. 1998) and may be responsible for differences in the response of species observed at the population and community level.

Diversity changed temporally on both landfarms and reference sites, and was in response to cotton rat abundance (Fig. 6). Comparing monthly cotton rat abundance to

monthly diversity showed that months with low diversity were associated with peaks in mean monthly abundance of cotton rats. This effect was also observed in rodent assemblages from Kansas with diversity negatively associated with cotton rat abundance during periods of high cotton rat density (Brady and Slade 2001). Cotton rat populations on landfarms did not reach abundance levels observed on reference sites and should not have reduced rodent diversity as much as on reference sites. However, rodent diversity was lower on landfarms at every level of cotton rat abundance. Because reference sites had larger fluctuations in cotton rat populations, there was more variability in rodent diversity on reference sites.

Diversity measures can be affected by changes in either species richness or evenness (Brady and Slade 2001). Although mean monthly richness was slightly higher on reference sites, the influence of cotton rat abundance on evenness was the most interesting. The lack of treatment effects when cotton rats were excluded from evenness calculations suggests that cotton rat populations inhabiting landfarms are affected to a greater extent than other rodent species, and that the observed treatment effect was the result of reduced cotton rat numbers and not a real change in community structure.

Reductions in cotton rat populations and the absence of voles on landfarms suggested that cotton rats and voles were most affected by contamination. McMurry (1993) also noted the absence of voles from contaminated sites. Dmowski et al. (1998) found that of the rodents inhabiting a thallium-contaminated site, voles (*Clethrionomys* sp.) had the highest level of contaminant uptake. Rowley et al. (1983) found that voles (*Microtus pennsylvanicus*) inhabiting the contaminated site at Love Canal, NY experienced increased mortality, and reduced liver and adrenal weights. Rowley et al.

(1983) found that voles inhabiting a contaminated site had a steeper survivorship curve and increased mortality rates. Decreased survival resulted in a 50% decrease in minimum life expectancy for voles from contaminated sites (Rowley et al. 1983). We also observed a 50% decrease in the minimum longevity of cotton rats from petrochemical landfarms. Cotton rats from landfarms only lived an average of 2.2 months compared to 3.9 months for rats from reference sites. However, the longevities of cotton rats from both reference sites and landfarms in this study meet or exceed longevities from other studies. Mean minimum longevity values have been reported to be 1.8 months for cotton rats from Texas (Wilkins 1995). Cameron (1977) lists 1.96 and 2.2 months as the expectation of future life for males and females respectively.

It has been shown that voles are not any more sensitive to acephate than other rodent species, and that the observed increase in susceptibility may be due to differences in their behavior or habitat preference compared to other rodents (Rattner and Hoffman 1984). As a result of their foraging behavior, they may have increased exposure, but do not exhibit increased toxicity at any given concentration of contaminants. Cotton rats and voles are grassland specialists that live in close association with soil, consuming a diet that is primarily composed of grasses (Cameron and Spencer 1982, Stalling 1990). About 2.8% of the dry matter collected from cotton rat stomachs and caeca is soil (Garten 1980). This association may increase their exposure to contaminants through ingestion of soils during feeding. Omnivorous rodents, such as white-footed mice, may not be exposed to contaminated soils as much as herbivores.

One possible explanation for the apparent effect of landfarms on cotton rats is an alteration in habitat structure. Landfarming represents a significant disturbance to the

habitat, both chemically and physically. In addition to the addition of contaminants to the habitat, most landfarms are cleared of all vegetation and experience complete mixing of the topsoil during tilling. After completion of landfarms, they are seeded with native or non-native plant species or are left for succession by species from surrounding habitats. This disturbance in the flora inhabiting landfarms may represent a significant factor in determining the structure of rodent assemblages on landfarms. In this study, cotton rats are the dominant member of the rodent assemblage, reducing diversity as cotton rat abundance increased. As a result, changes in cotton rat abundance would have the most impact on the structure of rodent assemblages. Cotton rat densities are positively associated with increasing plant cover (Cameron 1977), and may be due to increased risk of predation associated with open habitats (Lidicker et al. 1992). Landfarms in this study had increased proportion of bare ground compared to reference sites (Chapter III). Alterations in rodent assemblages on landfarms may reflect the effects of decreased plant cover, reducing cotton rat abundance, and allowing secondary species to increase in abundance.

Rodent assemblages from landfarms had lower cotton rat abundances with an increase in the abundance of secondary species. Diversity of rodent species decreased with cotton rat abundance on both sites; however, species diversity was affected to a greater extent on landfarms. Although species diversity was altered on petrochemical landfarms, community evenness did not show any differences between landfarms and reference sites. Diversity varied temporally on both landfarms and reference sites with peaks in diversity associated with periods of low cotton rat abundance. Reference sites showed a typical structure observed in studies from south Texas to Kansas and were

dominated by cotton rats and harvest mice. Rodent assemblages on contaminated sites also were dominated by cotton rats; however, harvest mice were of lesser importance. Contaminated sites also were characterized by an increase in house mice and an absence of voles. Structure of rodent assemblages was impacted by landfarms either directly through effects of contaminants on the health of individuals or indirectly by changes in the habitat. Further study needs to be done to investigate the effects of chronic exposure at the population and community level.

ACKNOWLEDGEMENTS

The research in this manuscript was funded by USEPA, Office of Research and Development, grant R826242-01-0. However, it has not been subjected to the Agency's required peer and policy review and therefore does not necessarily reflect the views of the Agency and no official endorsement should be inferred. Thanks to Dan Rafferty and Eric Webb for help with trapping animals; Terry Coffey for help in the field; Eric Hellgren and David Janz for editorial work; and to Larry Claypool and Mark Payton for their help with statistical analyses.

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Table 1. Total number of unique individuals by rodent species captured during June 1999 - Feb 2001 on reference and landfarm sites in Oklahoma. Total captures are listed by site for each petrochemical and reference pair (Units 1-5). Units 1 and 2 are located in southwest, Unit 3 in northcentral, and Units 4 and 5 in eastern Oklahoma. A total of 30,720 trapnights were evenly distributed among the sites.

	Unit 1		Unit 2		Unit 3		Unit 4		Unit 5	
Species	Reference	Landfarm	Reference	Landfarm	Reference	Landfarm	Reference	Landfarm	Reference	Landfarm
Sigmodon hispidus	592	116	431	55	350	420	450	588	264	296
Reithrodontomys fulvescens	56	29	29	21	58	35	97	20	123	42
Reithrodontomys montanus	0	0	0	7	0	0	2	0	0	2
Peromyscus maniculatus	7	189	6	86	62	1	7	4	18	5
Peromyscus leucopus	37	24	88	41	28	4	17	0	32	3
Chaetodipus hispidus	6	21	1	51	0	0	0	0	0	0
Neotoma floridana	0	1	0	0	2	0	0	0	1	0
Mus musculus	1	7	1	1	1	117	0	0	1	2
Microtus spp.	20	0	24	0	1	0	0	0	0	0
Oryzomys palustris	0	0	0	0	0	0	11	17	7	15

Table 2. Mean minimum longevity (mo) of species captured during the study (June 1999 - Feb 2001). Longevities were calculated as the time in months from first capture to time of last capture and are listed by site for each petrochemical and reference pair (Units 1-5). Significant differences at the P = 0.05 level are indicated with an asterisk.

	Reference		Landfarm	
Species	Mean	SE	Mean	SE
Sigmodon hispidus	3.91	0.15	2.20*	0.09
Reithrodontomys fulvescens	1.62	0.12	1.60	0.22
Reithrodontomys montanus	1.00	0.00	1.25	0.12
Peromyscus maniculatus	1.51	0.16	1.83	0.14
Peromyscus leucopus	1.90	0.19	1.65	0.31
Chaetodipus hispidus	2.00	1.00	1.59	0.16
Neotoma floridana	1.00	0.00	1.00	0.00
Mus musculus	1.00	0.00	1.84*	0.19
Microtus spp.	1.63	0.26		
Oryzomys palustris	1.20	0.14	1.10	0.06

Fig. 1. Rank abundance patterns of rodent communities inhabiting abandoned petrochemical landfarms (dashed) and uncontaminated reference (solid) sites in Oklahoma. The log of the proportion of individuals (P_i) of each species is ranked from most abundant (1) to least abundant (8).

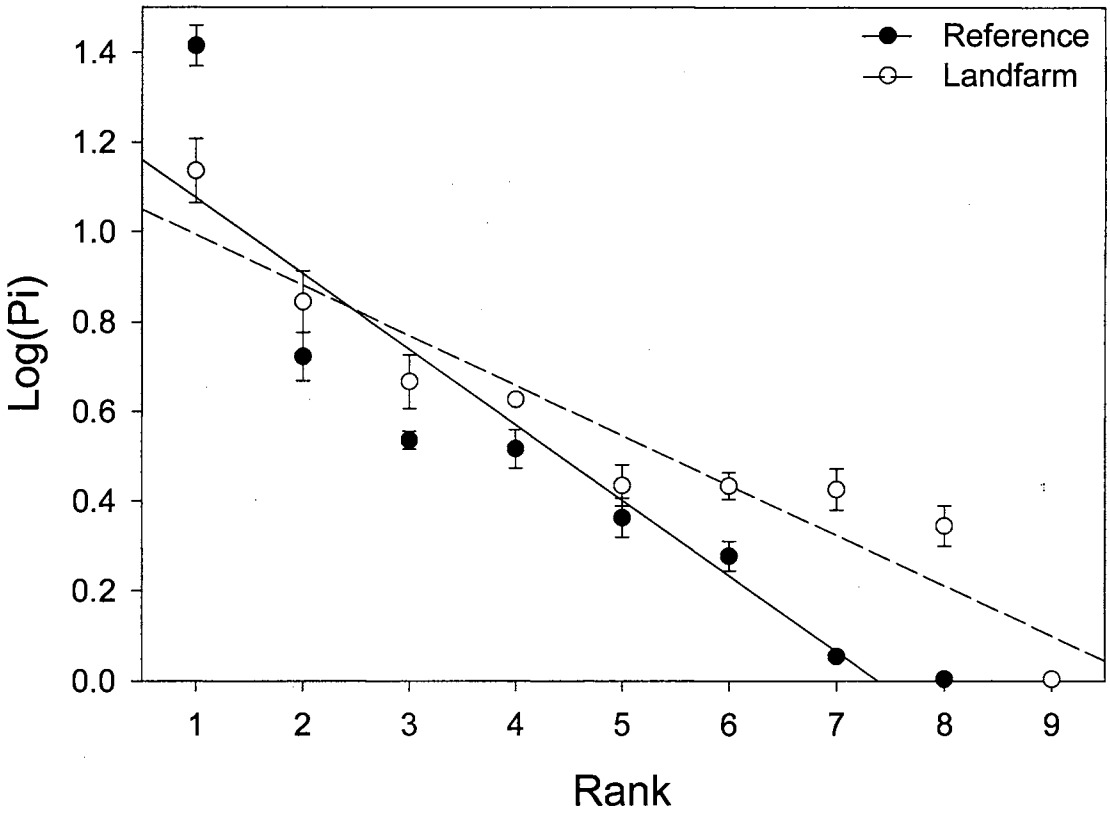

Reference
Landfarm
Log(Pi)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
4
5
6
7
8
9
Rank

Fig. 2. Shannon's diversity index in relation to cotton rat (*Sigmodon hispidus*) abundance for communities inhabiting abandoned petrochemical landfarms (n = 5) and uncontaminated reference sites (n = 5) in Oklahoma.

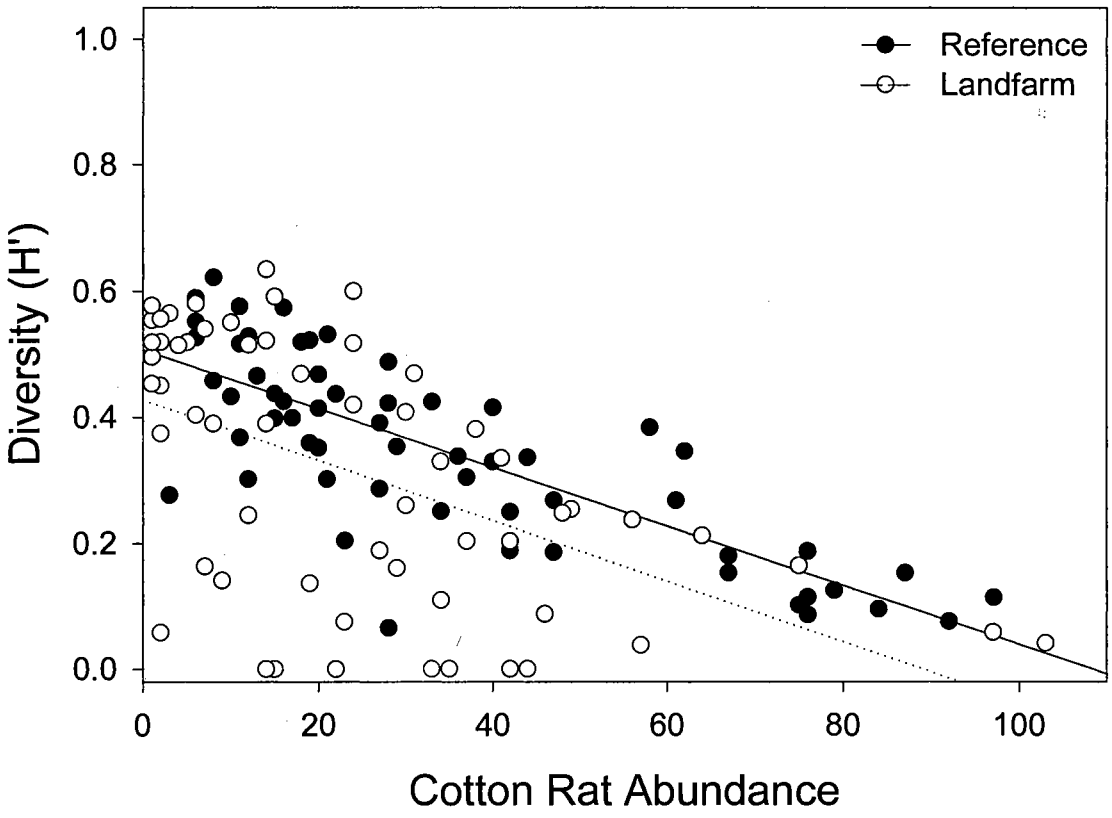

Reference
Landfarm
Diversity (H')
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Cotton Rat Abundance

Fig. 3. Shannon's community evenness in relation to cotton rat (*Sigmodon hispidus*) abundance for communities inhabiting abandoned petrochemical landfarms (n = 5) and uncontaminated reference sites (n = 5) in Oklahoma. Community evenness was measured with (A) and without (B) cotton rats included in evenness calculations.

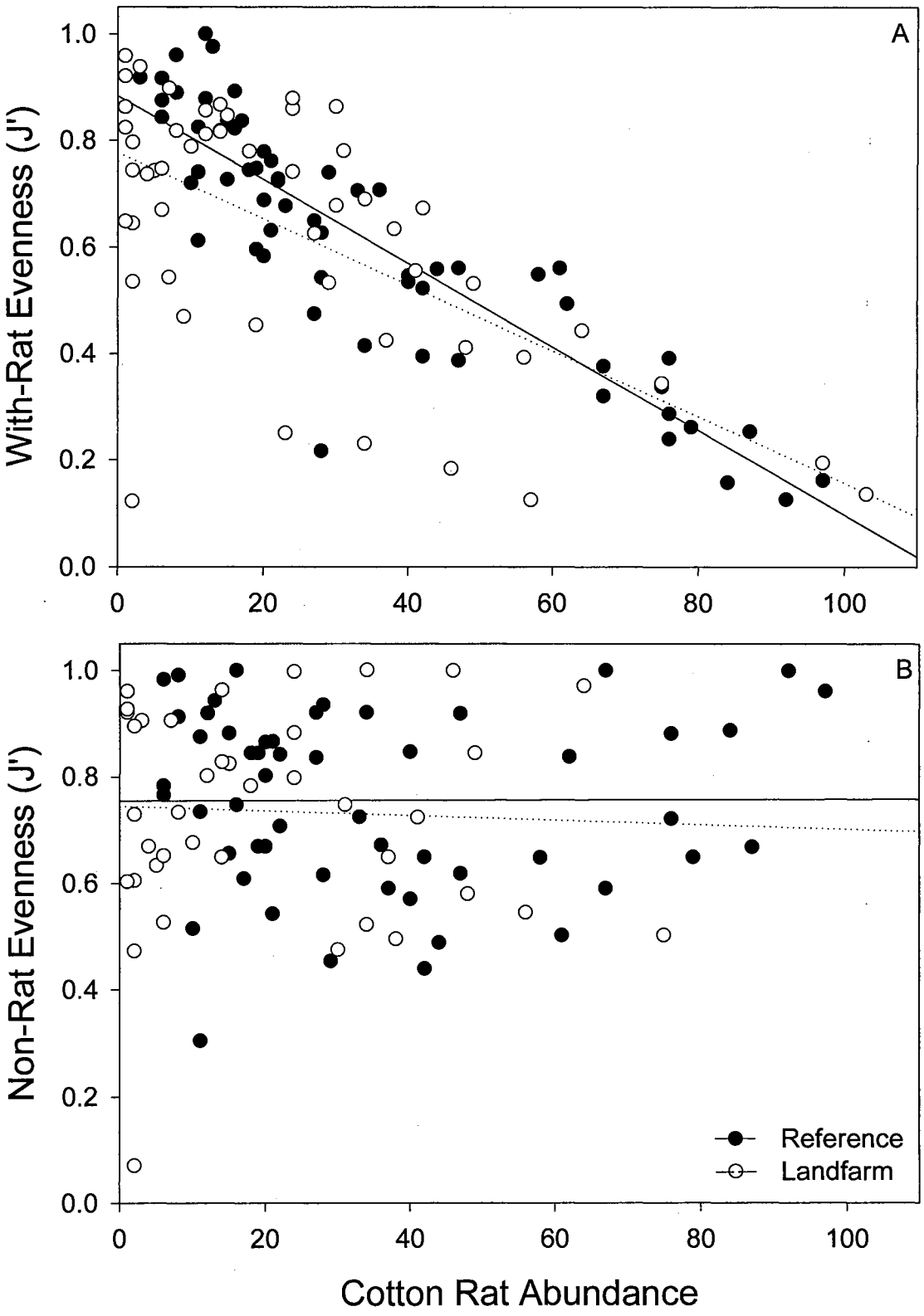

A
With-Rat Evenness (J')
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
B
Non-Rat Evenness (J')
Reference
Landfarm
Cotton Rat Abundance

Fig. 4. Horn's index of community overlap between rodent assemblages inhabiting abandoned petrochemical landfarms (n = 5) and uncontaminated reference sites (n = 5) in Oklahoma, June 1999-February 2001.

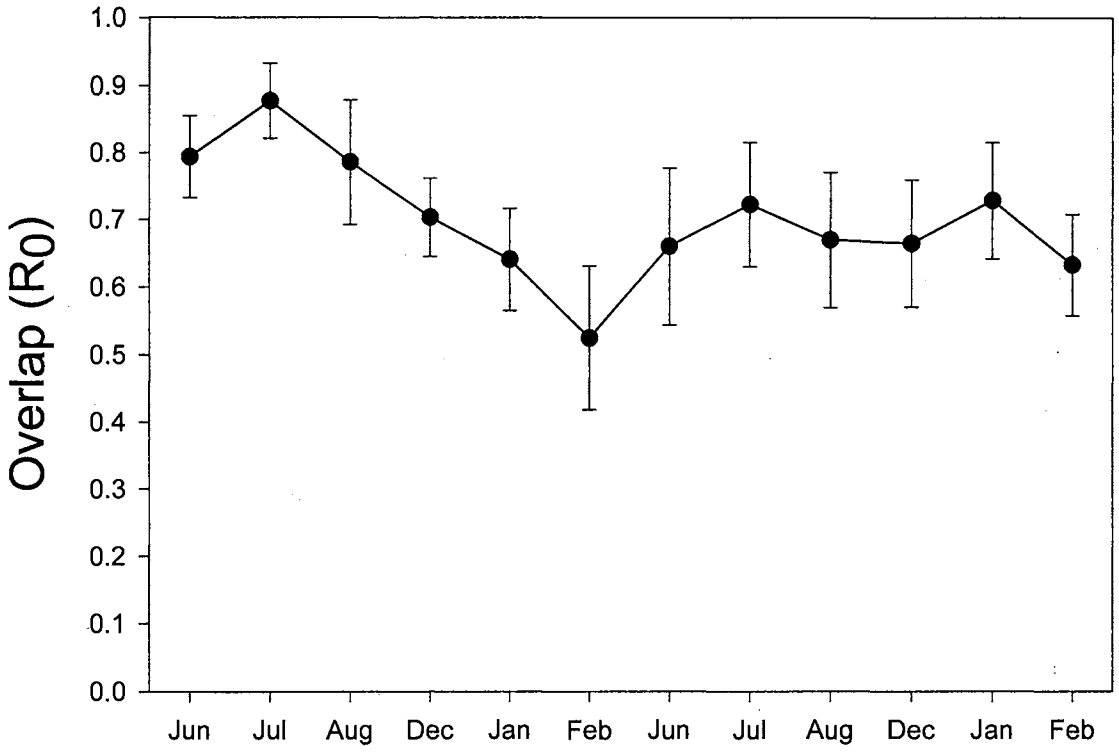

Overlap (R0)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Jun
Jul
Aug
Dec
Jan
Feb
Jun
Jul
Aug
Dec
Jan
Feb

Fig. 5. Similarity of rodent communities among individual study sites. Units consisted of paired sites: a landfarm (L) and a reference site (R). Individual units were located in southwesten (Units 1 and 2), northcentral (Unit 3), and eastern (Units 4 and 5) Oklahoma. The phenogram was constructed using single linkage clustering based on the complement of the Canberra metric (Krebs 1999).

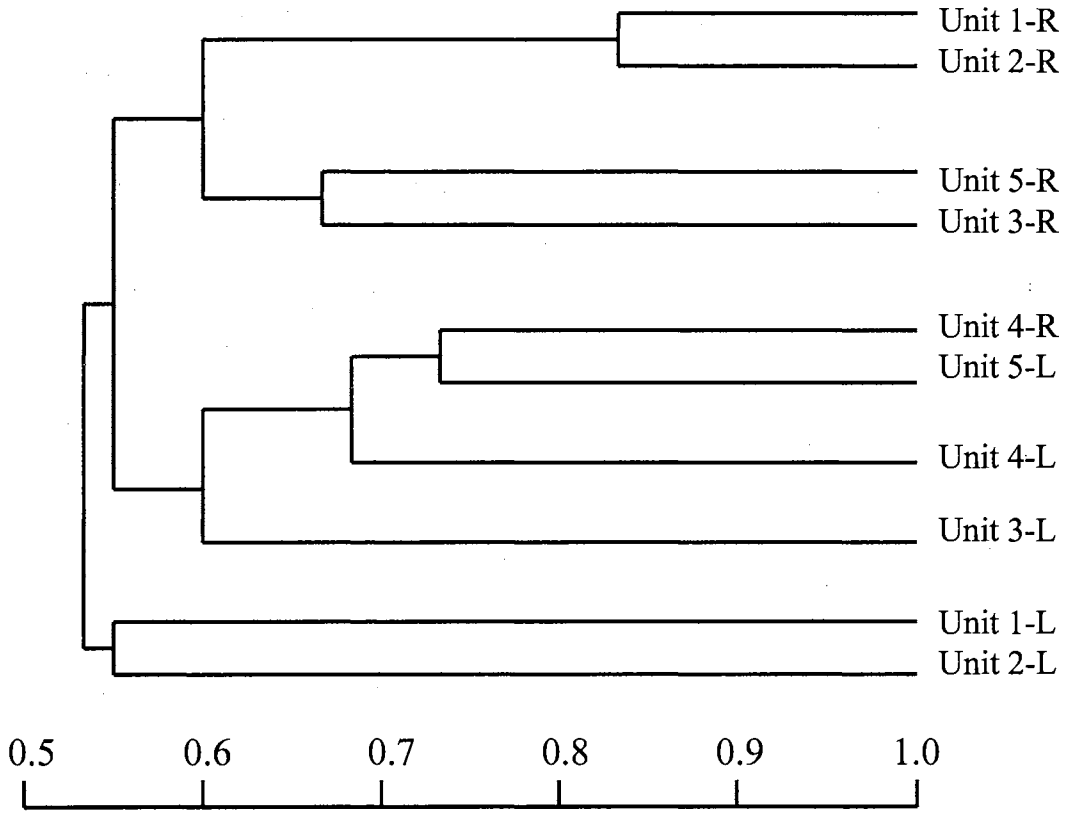

Unit 1-R
Unit 2-R
Unit 5-R
Unit 3-R
Unit 4-R
Unit 5-L
Unit 4-L
Unit 3-L
Unit 1-L
Unit 2-L
0.5
0.6
0.7
0.8
0.9
1.0

Fig. 6. Shannon's diversity index (dashed) in association with cotton rat (*Sigmodon hispidus*) abundance (solid) for communities inhabiting uncontaminated reference sites (A) and petrochemical landfarms (B) in Oklahoma, 1999-2001.

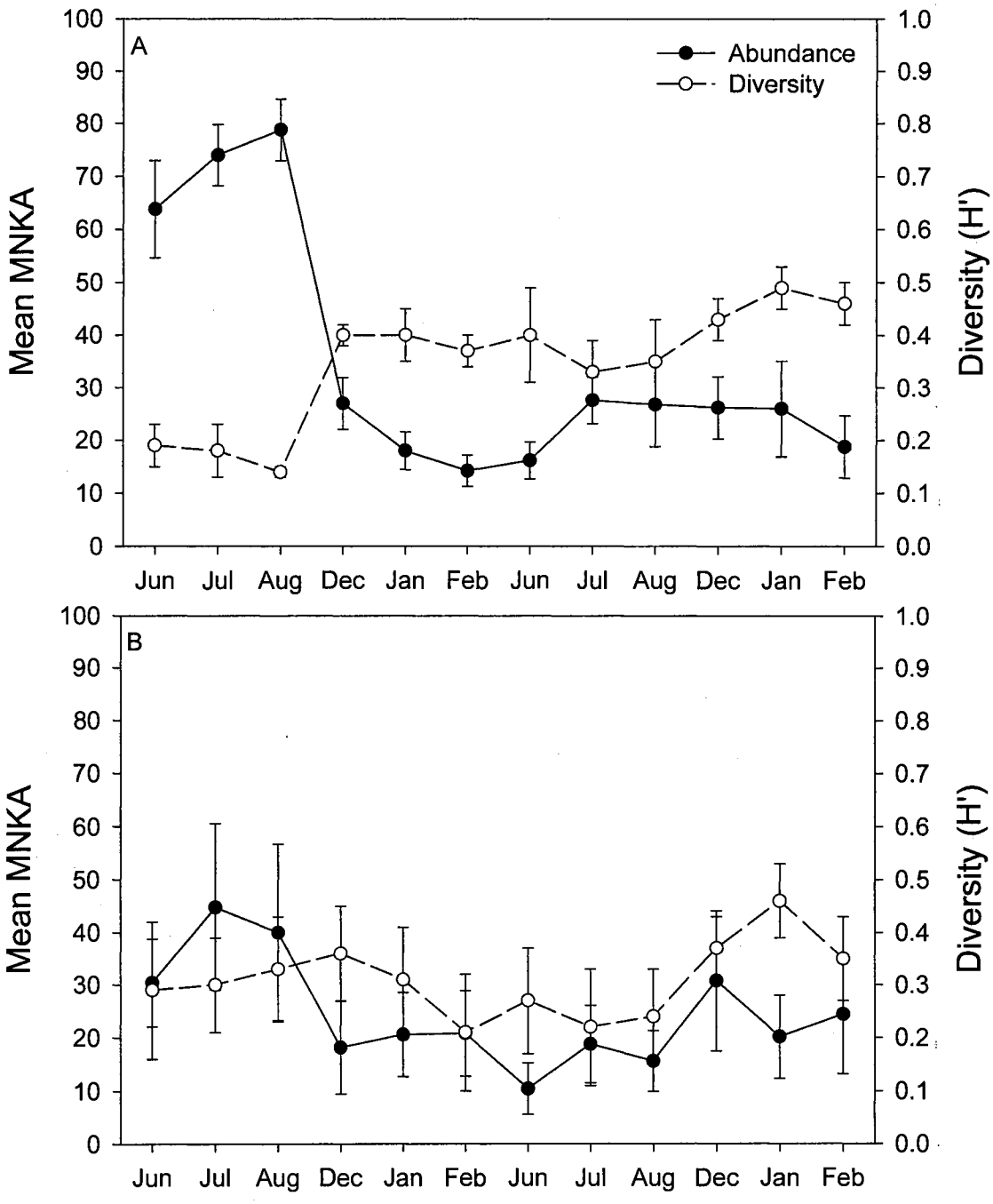
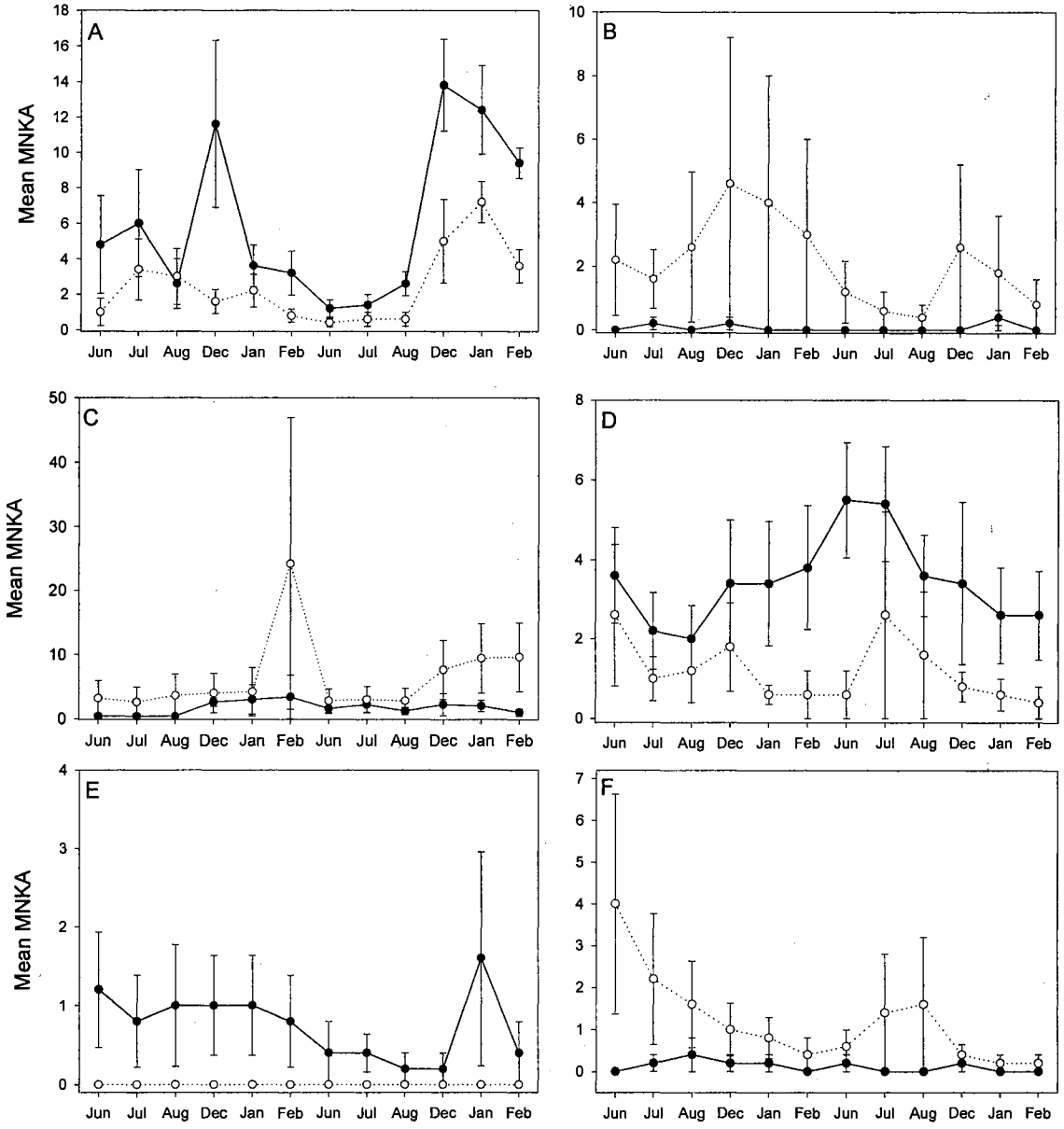

A
Abundance
Diversity
Mean MNKA
Diversity (H')
100
90
80
70
60
50
40
30
20
10
0
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Jun
Jul
Aug
Dec
Jan
Feb
Jun
Jul
Aug
Dec
Jan
Feb
B
Mean MNKA
Diversity (H')

Fig. 7. Mean minimum number known alive for 6 species of rodent inhabiting abandoned petrochemical landfarms (dashed, n = 5) and uncontaminated reference sites (solid, n = 5) in Oklahoma. Rodents species are *Reithrodontomys fulvescens* (A), *Mus musculus* (B), *Peromyscus maniculatus* (C), *P. leucopus* (D), *Microtus* spp. (E), and *Chaetodipus hispidus* (F). *Neotoma floridana*, *Reithrodontomys montanus*, and *Oryzomys palustrus* were trapped as incidentals.


A
B
C
D
E
F
Mean MNKA
Mean MNKA
Mean MNKA
Jun Jul Aug Dec Jan Feb Jun Jul Aug Dec Jan Feb

CHAPTER V

EVALUATING CONTAMINANT EXPOSURE IN COTTON RATS USING TUMOR NECROSIS FACTOR-α

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ABSTRACT

Immunological assays have been used to detect the effects of human-made contamination on the health of wildlife living on contaminated sites. Cotton rats were used to develop an *in vivo* model for the effects of petrochemical contamination on mammalian immune function. We modified existing protocols to measure the effectiveness of TNF-α produced by splenocytes harvested from cotton rats collected from petrochemical landfarms (n = 12) and uncontaminated reference sites (n = 12). Addition of actinomycin d to test samples increased lysis of test cells (WEHI 164 murine-fibrosarcoma cells) by 100 times, making the assay unusable. A range of lipopolysacharide (LPS) concentrations (0.0, 0.05, 0.5, 5.0, and 50.0 μg/ml) was tested to determine the appropriate concentration to stimulate TNF-α production in cotton rat splenocytes. Fifty μg/ml of LPS was found to be the most effective concentration for stimulating TNF-α production. Cotton rats collected from petrochemical landfarms had a 12.6% increase in lytic activity associated with TNF-α production over rats from reference sites. Stimulating splenocytes with a LPS concentration > 50.0 μg/ml, TNF-α

can be used effectively to detect changes in the immune system of mammals inhabiting contaminated sites.

INTRODUCTION

The cotton rat (*Sigmodon hispidus*) is the dominant rodent of the prairie throughout the southeastern United States (Cameron and Spencer 1981). Its widespread distribution, large size, and high densities make the cotton rat suitable for use in ecotoxicological studies. Numerous studies have used the cotton rat as a biomonitor to detect effects of anthropogenic contamination on a mammalian system. Effects of contaminants on the hematology (Robel et al. 1996), bone structure (Paranjpe et al. 1994), liver enzyme activity (Lochmiller et al. 1999), immune function (McMurry et al. 1991, McMurry et al. 1994), and chromosome structure (McBee et al. 1987) of cotton rats have been studied.

Environments are exposed to a wide variety of contaminants, each with a unique pathway of exposure. As a result, multiple methods of assessing contaminant effects in wildlife are necessary. Techniques have been developed to measure contaminant effects from molecular to organ levels. Immunocompetence has been proposed to be an important mechanism in the regulation of animal populations (Lochmiller 1996). However, because of its adaptive nature, changes in the immune system can be difficult to quantify. Contaminants adversely affect the immune system by causing immunosuppression (McMurry et al. 1993), increasing an individual's risk to infection, or by overstimulating the immune system and wasting energy (Lochmiller and Deerenberg 2000).

Assays that quantify the immune system during the primary stages of an immune response are valuable in monitoring the health of individuals experiencing chronic exposure to contamination. Acute exposure is of little interest for the development of biomarkers as the endpoint of acute exposure is typically death. In particular, the innate branch of the immune system should provide the most insight into the health of free-living individuals. Infection stimulates a cascade of events that initiate the upregulation of the immune system. The stimulation and regulation of the immune system is carried out by a series of messenger molecules, cytokines. TNF-α is one of the primary messengers responsible for initiating changes in metabolism following an immune challenge (Klassing and Johnston 1991).

Cytokines, such as tumor necrosis factor alpha (TNF-α), are responsible for regulating immune response, and have proven useful for quantifying changes in the immune system (Munoz et al. 1994). TNF-α is a cytokine produced by neutrophils, activated lymphocytes, macrophages, natural killer cells, and many other cells in response to foreign particles. TNF-α causes fever, releases stored lipids from fat cells, enhances macrophage and neutrophil chemotaxis and phagocytic activity, and initiates cell death in tumors (Bauman and Gauldie 1994).

Changes in the ability of cytokines to regulate the immune system may reduce an individual's ability to maintain itself in homeostasis. Methods for the measurement of TNF-α activity in murine systems were developed by Hogan and Vogel (1991). However, their validity in wildlife systems has not been investigated. The usefulness of this assay in wildlife petrochemical contamination will be investigated using a laboratory trial with wild-caught cotton rats. The objectives of this study were to validate methods

to quantify TNF-α activity in wild cotton rats, and to compare TNF-α activity between cotton rats inhabiting abandoned petroleum landfarms and reference sites.

METHODS

This study was conducted on 2 sites in north-central Oklahoma. One site was an abandoned petrochemical landfarm located inside a major refinery, and it was paired with an uncontaminated reference site. The reference site was located as close to the landfarm as possible to reduce variation from climate and vegetation. Both sites were located in the tallgrass prairie ecosystem and consisted of disturbed grassland habitat, with nearly pure stands of johnsongrass (*Sorghum halapense*). Contamination on the landfarm consisted of heavy metals and polyaromatic hydrocarbons (PAHs). Concentrations of metals (mg/kg) elevated above reference site soil levels on the landfarm site were As (15.2), Cd (1.1), Cr (1,652.0), Cu (954.8), F (3026.1), and Pb (1184.6). Similarly, concentrations of individual PAHs (mg/kg) elevated above reference sites were anthracene (1.37), benzo(a)anthracene (1.19), benzo(a)pyrene (1.00), benzo(b)fuoranthene (1.23), benzo(g,h,i)perylene (10.24), chrysene (2.70), indeno(1,2,3-CD)pyrene (1.40), phenanthrene (1.63), and pyrene (1.39).

Tumorcidal activity of TNF-α was quantified by exposing TNF-α-sensitive WEHI 164 murine-fibrosarcoma cells (American Type Cell Culture, CRL 1751) to culture media containing TNF-α (Hogan and Vogel 1991). A suspension of WEHI cells were cultured and adjusted to a count of 2×10^6 cells/ml. A 100-µl aliquot of 2×10^5 WEHI cells were added to each well of a 96-well flat-bottomed microtiter plate and

placed in a 6% CO_2 humidified incubator at 37°C for 24 hr to allow growth to cellular confluence.

Recombinant human TNF-α (50 μl, 50.0 μg/ml) was used as a positive control to determine if the assay worked as expected. Hogan and Vogel (1991) used actinomycin d to increase the susceptibility of WEHI cells to TNF-α, and the necessity of actinomycin d was tested by exposing recombinant human TNF-α to WEHI cells with and without actinomycin d. 2×10^5 WEHI cells and 50-μl of actinomycin d or RPMI containing 10% horse serum (RPMI-HS) were added to each well of a cell culture plate. Plates were incubated for 24 hrs, then 25 μl of [^{3}H]-thymidine (40 μCi/ml) was added to each cell, and were returned to the incubator for 4 hrs. After incubation, plates were harvested onto filter plates by a Packard Filtermate Uniharvester 96 (Packard, Meriden, CT). To each well of the filter plate, 30 μl of microscint was added and then the amount of [^{3}H]-thymidine incorporated into WEHI cells was measured by an automated plate counter as disintegrations/min (dpm).

Cotton rats were used to develop an *in vivo* model for the effects of petrochemical contamination on mammalian immune function. We used Sherman live traps (Sherman Traps Inc., Tallahassee, FL) to collect cotton rats from each site. Traps were baited with whole oats, set in the late afternoon, and checked the following morning. We removed 12 cotton rats (6 males and 6 females) weighing > 80 g from each site and transported them to Oklahoma State University for use in development of the assay. Rats were housed in standard plastic cages (47 x 27 x 20 cm) with cob shavings for bedding. Water and rat chow (Purina, St. Louis, MO) were provided *ad libitum*.

Cotton rats were killed by cervical dislocation following exposure to methoxyfluorane (Mallinckrodt Veterinary Inc., Mundelein, IL) until anesthetized. Spleens were removed aseptically, weighed, and placed in sterile petri dishes containing RPMI 1640 culture medium supplemented with 10% horse serum (RPMI-HS), 1.025% L-glutamate (200 mM), and 1.0% penicillin-streptomycin (10,000U/ml-10 mg/ml). Spleens were dissociated into individual cells by homogenization using a sterile hand glass tissue grinder with 5 ml of RPMI-HS medium. Cell suspensions were allowed to settle for 7 min and the supernatant was decanted into sterile 16- by 125-mm polypropylene screw-cap culture tubes. Cells were washed by centrifugation (8 min, 10°C, 220 x g) and suspended in 5 ml of fresh RPMI-HS medium. Erythrocytes were lysed by washing cells in 5 ml of tris-buffered ammonium chloride (0.83%, pH 7.2), followed by a wash with 5 ml of RPMI-HS. Splenocytes were counted using an automated cell counter, adjusted to a final concentration of 500,000-cells/90 µl, and maintained in RPMI-HS.

The appropriate concentration of lipopolysaccharide (LPS) from *Escherichia coli* needed to stimulate production of TNF-α was determined using 5 concentrations of LPS (0, 0.005, 0.5, 5.0 and 50.0 µg/ml). LPS concentrations used in this study were similar to those shown to initiate TNF-α production in bovine cells (Adams and Czuprynski 1990). For each cotton rat, 200 µl of adjusted splenocyte solution was added to appropriate wells of a 96-well flat-bottomed culture plate, and 50 µl of the appropriate LPS was added to each well. Splenocytes were cultured in quadruplicate and placed into a CO_2 humidified incubator at 37°C for 54 hr. After incubation, LPS-stimulated spleen cells were centrifuged for 8 min at 35 x g to set spleen cells on the bottom. Duplicates were made

of each culture plate by taking 150-µl of splenocyte supernatant and placing it into new culture plates. Duplicate plates were covered, sealed with polypropylene tape, and frozen at -70°C for later use.

We tested TNF-α activity in splenocyte samples taken from cotton rats inhabiting landfarm and reference sites with the above protocol. Microtiter plates of WEHI cells were incubated for 24 hrs, as previously described. Cotton rat splenocyte supernatants from cultures stimulated with LPS, were removed from the freezer and thawed. After incubation, 100-µl samples of cotton rat splenocyte supernatant were placed into appropriate wells of the WEHI cell plates. TNF-α supernatant was serially diluted (2-fold) across the culture plate. Column one was used as a negative control and had 100 µl of RPMI-HS added instead of the TNF-α supernatant. Plates were returned to the incubator for 18 hr. After incubation, 25 µl [H^3]-thymidine was added to each well, and returned to the incubator for 4 hrs. After incubation, cells were harvested and radioactivity measured.

Mean dpm counts were calculated for all triplicates, and all dilution factors (1/dilution) were log transformed. Values from reference sites were used to determine the appropriate concentration of LPS to be used for the assay. Data from each LPS concentration were plotted and analyzed using PROC REG (SAS 1994), and the slopes and intercepts were tested to determine which of the five LPS concentrations stimulated the most TNF-α. Differences in TNF-α production between cotton rats inhabiting landfarms and those from reference sites also were tested for each concentration of LPS stimulation using PROC REG. Differences in the resonse to TNF-α was tested for each dilution factor using t-tests with bonferroni's adjustment for multiple comparisons. All

data are presented as mean values by treatment regardless of transformation for statistical analysis; however, mean triplicate dpm values for individual cotton rats were used in statistical analyses. Statistical significance was set *a priori* at the alpha = 0.05 level, with Bonferroni's adjustment for multiple comparisons.

RESULTS

The addition of actinomycin d greatly increased the susceptibility of WEHI cells to TNF-α. However, lysis was stimulated in WEHI to such an extent that too few cells remained to carry out the remainder of the assay (Figure 1). Incorporation of [3H]-thymidine into living WEHI cells was 100 times less in the presence of actinomycin d (Figure 1a) than cells without actinomycin d added (Figure 1b). Lysis occurred to such an large proportion that the assay would not be of use. In addition, there was no observable effect from the serial dilution (slope = -7.0, $P = 0.32$). As a result, all future TNF-α assays performed in this study did not use actinomycin d.

Stimulation of lysis in WEHI cells by TNF-α was related to the concentration of LPS used to stimulate cotton rat splenocytes (Figure 2). The four lowest concentrations of LPS (0, 0.005, 0.5, and 5.0 μg/ml) did not differ from each other in their ability to stimulate lysis in WEHI cells ($F_{2,194} < 1.67$, $P > 0.12$). However, an LPS concentration of 50.0 μg/ml did produce a greater effect on lysis of WEHI cells ($F_{2,194} = 5.17$, $P = 0.007$, Figure 2f) than any of the other four LPS concentrations. Serial dilution of cotton rat serum at all levels of LPS concentration did not show the linear reduction in lytic activity observed in the serial dilution of human TNF-α standard. Because of the increased lysis observed from TNF-α supernatant collected from splenocytes stimulated

with 50.0 μg/ml LPS, comparisons of cotton rats from reference and landfarm sites will use 50.0 μg/ml LPS.

Cotton rats collected from landfarms had increased TNF-α production, however the results were not significant ($F_{2,18} = 2.34$, $P = 0.12$; Figure 3). TNF-α production was increased an average of 12.6% over cotton rats from reference sites. Serial dilution of cotton rat serum showed a dose response similar to that observed during standardization with human TNF-α standard. However, differences in the response to TNF-α was not significant between treatments at each level of dilution factor. The magnitude of the effect of TNF-α on WEHI cells was lower in cotton rats from both landfarms (slope = 8.55, $P < 0.001$) and reference sites (slope = 5.80, $P < 0.001$) did not show a reduction in lytic activity as expected from the human TNF-α standard data (slope = 15.20, $P < 0.001$).

DISCUSSION

Continued development of immunological assays is needed to provide a suite of techniques to measure the effects of contamination on wildlife. Luster et al. (1988) suggested the use of a 2-tier system for evaluating the effects of contaminants on the immune system. First-tier assays focus on changes in immune function and structure whereas second-tier assays focus on interactions among immune components. Cytokines are responsible for communication among the cells involved in an immune response and represents an assay that would fit within the second tier.

Although the standard TNF-α assay developed by Hogan and Vogel (1991) utilize actinomycin d to increase the susceptibility of cultured WEHI cells, they stated that the

use of specialized target cells (WEHI 164) are sensitive enough to TNF-α that the addition of actinomycin d is not necessary. We used the sensitive WEHI 164 clones in this assay and found that actinomycin d was not necessary.

Bacterial lipopolysacharide was used to stimulate the production of TNF-α from cotton rat splenocytes. The appropriate concentration of LPS is necessary to stimulate enough TNF-α in cotton rat splenocytes for use in the assay. Serial dilutions of TNF-α containing supernatant solution makes it necessary to generate large quantities of TNF-α. We used 4 concentrations of LPS to stimulate TNF-α production in splenocytes, however only one LPS concentration (50.0 μg/ml) stimulated enough TNF-α to create a response different from splenocytes with no LPS stimulation. As a result, a LPS concentration of at least 50.0 μg/ml should be used to stimulate cotton rat splenocytes to produce TNF-α. The appropriate LPS concentration may be much higher than the 50.0 μg/ml used in this study, and further studies should test the use of LPS concentrations of 50, 100, 200, 500, and 1000 μg/ml.

The lack of difference in TNF-α activity between treatments may be due to the inherent variability found in uncontrolled wildlife studies. In addition, a seasonal effect has been observed in the regulation of the immune system (Lochmiller et al. 1993). Cotton rats have reduced leukocytes, splenic yields, and lymphoproliferative responses during winter months (Lochmiller et al. 1994). Cotton rats were collected in the fall for this study and may represent a time when the immune system is being downregulated, perhaps resulting in a lack of responsiveness in rats collected from both landfarms and reference sites.

Cotton rats collected from both landfarms and reference sites showed considerable variation in their response to LPS stimulation. A number of factors have been found to affect immune response in individuals, increasing the amount of individual variation and reducing the ability to detect real differences due to contaminants. Because many of these factors (*e.g.*, nutrition, overcrowding, and reproductive status) cannot be controlled in wildlife, increasing the sensitivity of assays is the only way to reduce problems associated with individual variability.

Although this assay is a valuable tool for identifying the effects of contamination, it would be more useful when used in conjunction with other molecular techniques. In particular, the use of western blotting to quantify TNF-α would help to identify differences in the amount of cytokine produced as well as its activity. In addition, maintenance of WEHI cell lines has been shown to decrease sensitivity to TNF-α after several passages of the cells (Eskandari et al. 1990). Assays to measure TNF-α activity are useful as biomarkers because TNF-α exhibits a wide range of activities and affects virtually every cell type in the immune system; however, assays that use enzymes as reporter molecules, instead of [^{3}H]-thymidine, may be more precise, reliable, and inexpensive (Millett et al. 1994).

ACKNOWLEDGEMENTS

The research in this manuscript was funded by USEPA, Office of Research and Development, grant R826242-01-0. However, it has not been subjected to the Agency's required peer and policy review and therefore does not necessarily reflect the views of the Agency and no official endorsement should be inferred. Thanks to R. Carlson for help

with collection of animals; D. M. Janz and E. C. Hellgren for editorial work; and to L. Claypool and J. L. Parsons for their help with statistical analyses.

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Figure 1. Lysis of WEHI 164 murine-fibrosarcoma cells stimulated with 50 μg/ml recombinant human TNF-α standard. Actinomycin d was added (A) to increase WEHI cell susceptibility, or left out as a control (B). Incorporation of [^{3}H]-thymidine into WEHI cells was used as a measure of lysis, with increased lysis measured as a decrease in disintegrations/min. Values presented are means ± standard error.

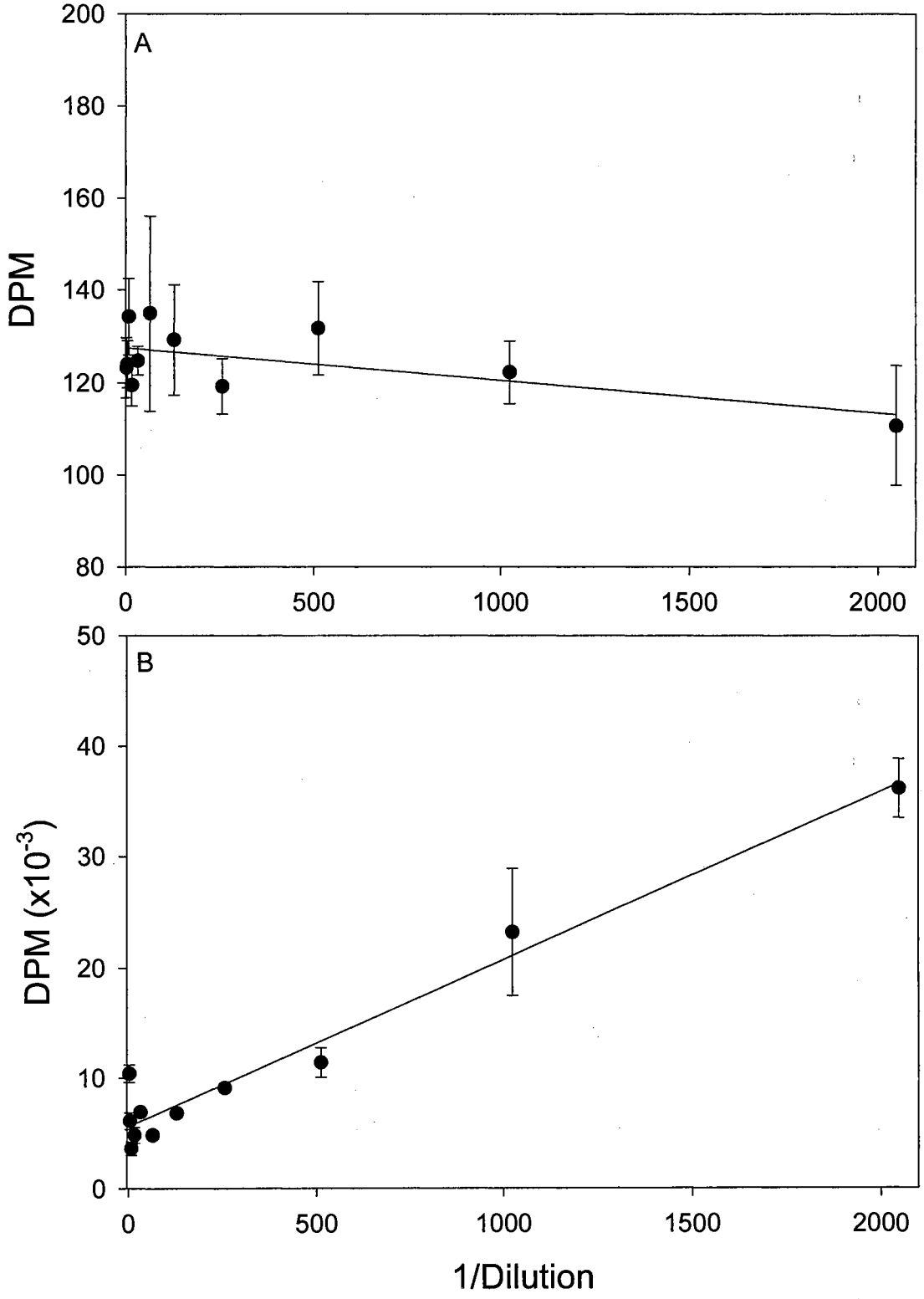

A
DPM
200
180
160
140
120
100
80
0
500
1000
1500
2000
B
DPM (x10-3)
50
40
30
20
10
0
0
500
1000
1500
2000
1/Dilution

Figure 2. Cotton rat splenocytes were stimulated to produce TNF-α using 5 concentrations of lipopolysaccharide (LPS), from *Escherichia coli*: 0.0 μg/ml (A), 0.005 μg/ml (B), 0.5 μg/ml (C), 5.0 μg/ml (D), and 50.0 μg/ml (E). All 5 LPS concentrations are presented together to show increased lysis with increased LPS concentration (F). Incorporation of [^{3}H]-thymidine into WEHI cells was used as a measure of lysis, with increased lysis measured as a decrease in disintegrations/min. Values presented are means ± standard error.

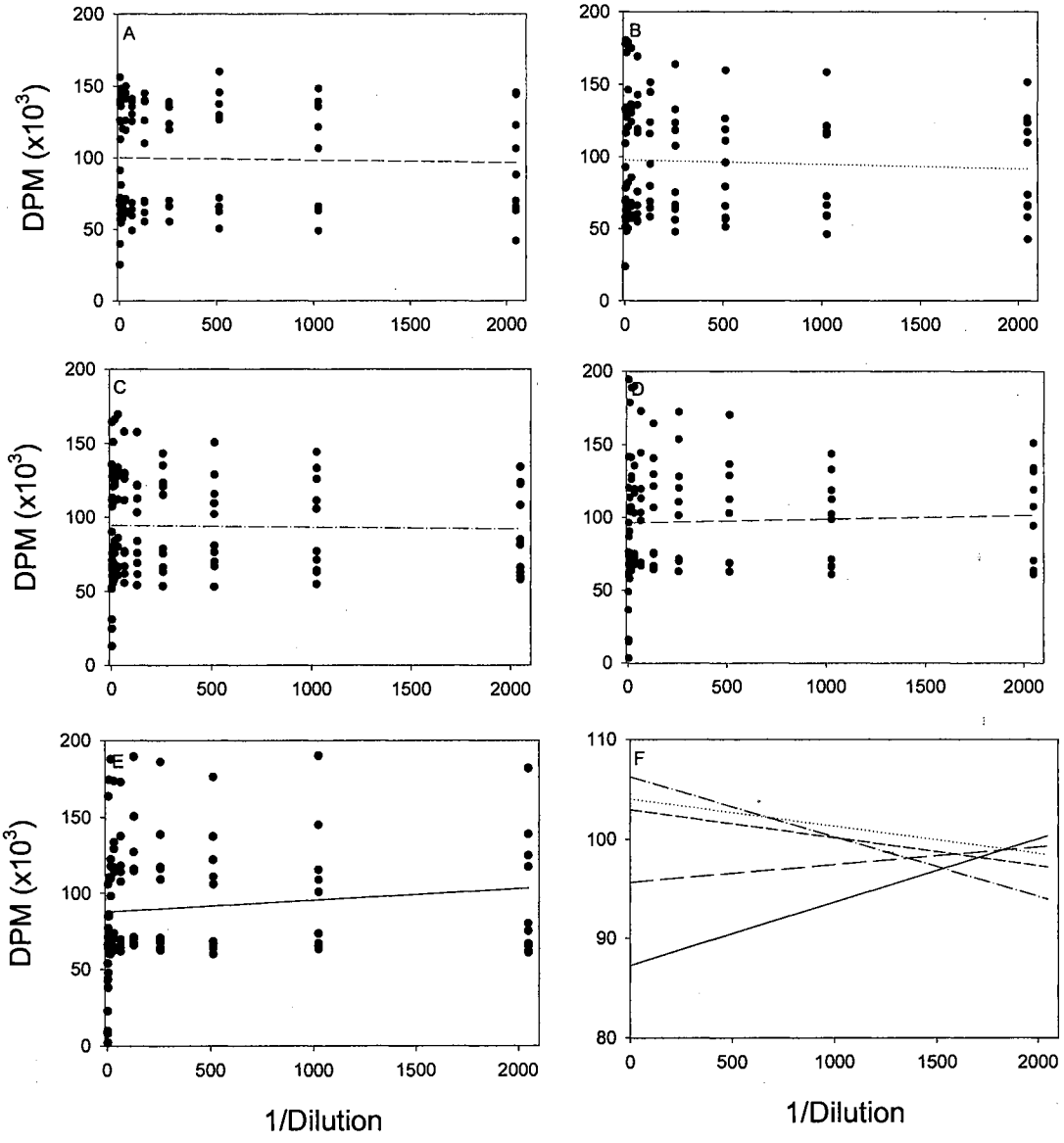
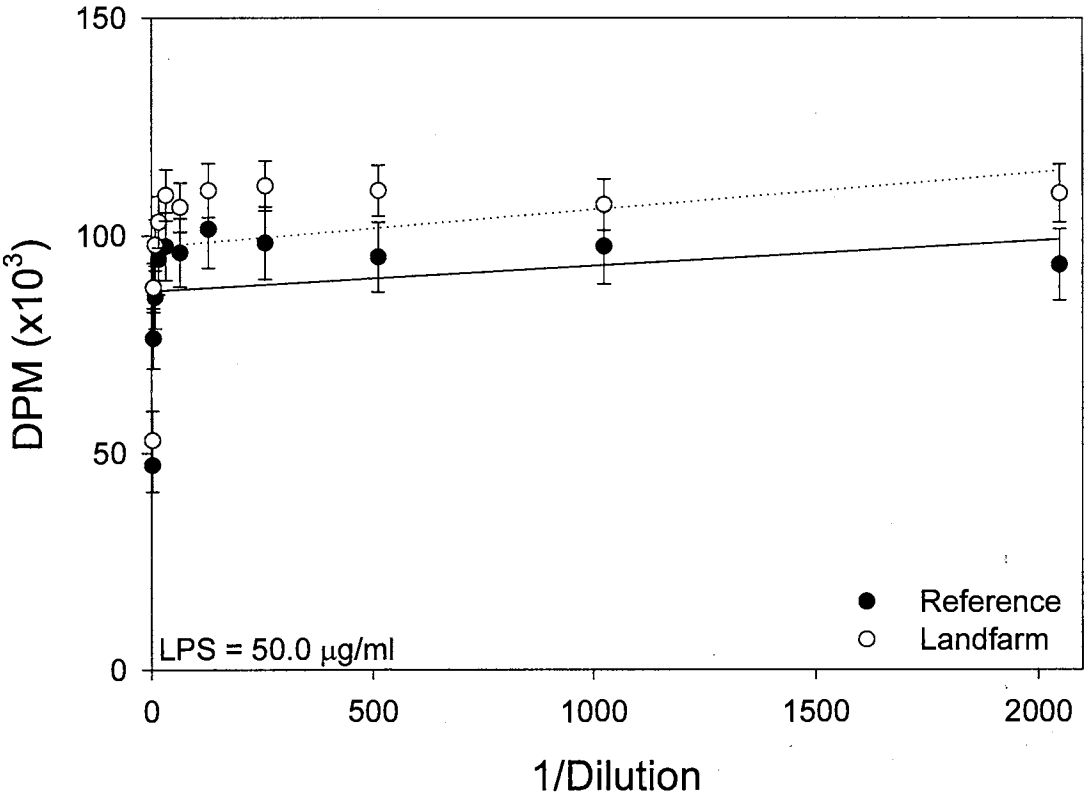

A
B
C
D
E
F
DPM (x10^3)
1/Dilution
0
500
1000
1500
2000
50
100
150
200
80
90
110

Figure 3. TNF-α production in cotton rats collected from landfarms and reference sites. All cotton rat splenocytes were stimulated with 50.0 μg/ml of lipopolysacharide (LPS). Incorporation of [^{3}H]-thymidine into WEHI cells was used as a measure of lysis, with increased lysis measured as a decrease in disintegrations/min. Values presented are means ± standard error.


150
100
50
0
DPM (x10^3)
0
500
1000
1500
2000
1/Dilution
LPS = 50.0 µg/ml
Reference
Landfarm

VITA

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