ASSESSING IMMUNOTOXICITY RISKS TO SMALL MAMMALS IN ENVIRONMENTS CONTAMINATED WITH PETROCHEMICALS

By

TIMOTHY LEE PROPST

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Wabash College

Crawfordsville, Indiana

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INTRODUCTION

The petroleum industry produces and discharges many known immunotoxicants into the environment, including petroleum-derived hydrocarbons (benzene, benzo(α)pyrene, toluene, methylcholanthrene), PCBs (polychlorinated biphenyls), and heavy metals (Descotes 1988). Oklahoma contains 10 Hazardous Waste Sites (EPA Superfund Program) with complex mixtures of petrochemical contaminants in the soil. Unfortunately, immunotoxicity of the complex mixtures of petroleum fuels, solvents, and heavy metals that contaminate soils in the environment has not been adequately investigated. Recent studies on the effects of oil ingestion on wild waterfowl suggest that complex petroleum-related pollutants can have a negative impact on immunity (Goldberg et al. 1990, Rocke et al. 1984). The difficulty involved in duplicating mixtures of contaminants found in the environment for laboratory immunotoxicity studies strongly supports the use of in situ model systems to assess these risks (Porter et al. 1984).

Several small mammal species have been used as in situ monitors of exposure to environmental contaminants (for review see Talmage and Walton 1991, McBee and Bickham 1990). In addition to tissue accumulation, studies have explored the use of biochemical, histological, population, and community endpoints (Flickinger and Nichols 1990, Rattner et al. 1993), but only limited attempts have been made to use wild rodents in assessments of immunotoxicity risks (Olson and Hinsdill 1984, Porter et al. 1984, Fairbrother et al. 1986) although rodents have long been the primary model for immunotoxicity testing in the laboratory (Dean et al. 1982, Exon et al. 1990). McMurry (1993) used a tiered (Table 1) approach to immunotoxicity assessment (Dean et al. 1982)

and observed evidence of immune suppression in wild hispid cotton rats (Sigmodon hispidus) inhabiting a petrochemical waste site in Oklahoma. McMurry's (1993) study noted that using resident animals as biomonitors has frequent limitations such as the confounding factors of age, sex, reproductive status, emigration, immigration, and predation. Additionally, not all contaminated soils will support resident populations of small mammals.

Mesocosms, long made use of in aquatic disciplines (Solomon et al. 1980, Stephenson and Kane 1984, Wayland 1991), could provide more experimental control for in situ immunotoxicity assessments. Advantages offered by mesocosms include in situ exposure to complex mixtures of contaminants on specific sites under naturally stressful conditions and experimental control of variables such as age, sex, species, physiological status, and duration of exposure. Recent studies utilizing terrestrial mammals in mesocosms have demonstrated that this approach can be successful for estimating risks to wildlife (Dickerson et al. 1994) and human (Reichrtova et al. 1988) populations. The primary objective of this study was to evaluate the efficacy of mesocosms to assess the immunotoxicity risks to feral small mammal populations associated with soils contaminated with petroleum refinery wastes. The approach taken was to expose cotton rats to contaminated soils on a petrochemical waste site where McMurry (1993) previously documented immunotoxicity in resident cotton rat populations. Mesocosms were examined for their practicality and the relative sensitivity of selected endpoints of immune system function to in situ exposure to complex mixtures of contaminants.

METHODS

Study area

This study was conducted on a Superfund Hazardous Waste Site located in Caddo County, Oklahoma. This site consisted of an abandoned oil refinery complex on 63 ha, which included a main processing plant facility, an array of unlined earthen ponds and storage pits, and a 3.4 ha soil-farm waste-treatment facility. Initiated in the early 1920's, the facility operated as a crude oil refinery until 1984.

Three contaminated (Tank Battery, API Separator, and Land Farm) and three reference study sites were selected for construction of mesocosms. The three reference sites selected were located on property that had no history of petroleum contamination and soil samples tested negative for organic contaminants (Yates 1994). The three contaminated sites contained complex mixtures of contaminants, including suspected immunotoxicants as described by McMurry (1993). The Tank Battery mesocosm was situated directly over a salvaged leaded gasoline storage tank that was demolished and removed from the site due to long-term leakage. The API Separator mesocosm was constructed on a phytotoxic area where waste sludges from the separator were disposed of in an unlined impoundment. The Land Farm mesocosm was constructed on a land treatment area where waste sludges dredged from settlement ponds were tilled into the soil for bio-degradation.

The primary soil contaminants (0-60 cm depth) on the three contaminated sites included the heavy metals chromium, lead, aluminum, arsenic, barium, zinc, and mercury. Chromium and lead were detected in soil samples from the API Separator and

Land Farm sites at levels ranging from 24 to 2,700 ppm and 14 to 304 ppm, respectively. Arsenic was detected in soil samples from the API Separator site at a level of 104 ppm. Aluminum, barium, zinc, and mercury levels in soil samples ranged form 19 to 53,800 ppm and were detected only at the API Separator site. Numerous solvents and organic petroleum products including toluene, xylenes, naphthalene, and pyrene were detected in soil samples from the API Separator site where concentrations of these and other organic compounds ranged from approximately 4 to 360 ppm (unpublished Stanley Engineering Inc. 1985; unpublished USEPA Proj. No. W68439). In addition, soil samples obtained from within 1m of each mesocosm were analyzed for heavy metal and organic contaminants and reported by Yates (1994). High pressure liquid chromatography analysis of supercritical carbon dioxide extracts of soil samples from the Land Farm mesocosm also indicated the presence of unidentified non-polar organic contaminants at this location not found at any of the other mesocosm sites (Yates 1994).

Experimental design

Mesocosms (3m x 3m) were constructed using 1-m wide metal sheeting that was buried 15 cm below ground to deter escape via burrowing. The top of the enclosure was then covered with a wire screen to prevent predation. Animals in mesocosms were provided with food (Purina Rodent Chow 5001, St. Louis, Missouri, USA) and water at all times. Shelter or cover was provided in the form of wheat straw and roofing material that was placed on the ground within each mesocosm.

As the dominant species of small mammal on the study site (McMurry 1993), the cotton rat was selected as the experimental subject for release into mesocosms. Progeny

from a captive colony of inbred (30-35 generations) cotton rats (obtained from The National Institutes of Health) were used in an effort to decrease genetic variability among individuals. Animals were individually housed in polystyrene cages with pine-shaving bedding and provided food and water ad libitum while in the laboratory. Animals were healthy, 1.5 to 3 months old, and reproductively mature at the time of release into mesocosms. Each animal was toe-clipped for identification and weighed prior to release into a mesocosm. In a series of three experimental exposure trials, animals were housed in mesocosms for either 4 weeks (acute exposure) or 8 weeks (chronic exposure). An 8-week exposure trial was conducted in both winter 1991 and in summer 1992; a 4-week exposure trial was conducted during summer 1993. At the end of each trial, animals were transported live to the laboratory where selected physiological parameters were assessed within 48 hours. Sample sizes for the 8-week exposure trials were six (winter 1991) or seven (summer 1992) individuals of each sex per mesocosm; the 4-week exposure trial consisted of 10 males only in each mesocosm (two reference sites and 3 contaminated sites were used). Variations in sample sizes were due to differences in the availability of 1.5 to 3 month-old animals in the laboratory colony at the start of a trial.

General condition and immune organs

At the time of animals were anesthetized with metofane, body weight determined, and a blood sample obtained via the retro-orbital sinus for hematological analysis and serum. Anesthetized animals were sacrificed by cervical dislocation, and weights of selected organs determined by necropsy.

Immune organ development was assessed by measuring weight and cellularity of the spleen and popliteal lymph nodes as described by McMurry (1993). Selected T-lymphocyte subpopulations in the spleen were enumerated using receptor-binding characteristics of cells to the fluorescein isothiocyanate-conjugated lectins Concanavalin A and Helix pomatia agglutinin as described for cotton rats by Davis and Lochmiller (1995).

Blood analyses

Hematological analyses included counts of white blood cells, red blood cells, and platelets, hematocrit, hemoglobin concentration, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and differential leukocyte counts using methods described by Robel et al. (1996) for the cotton rat. Serum total protein (Davis et al. 1995) and hemolytic complement activity (CH50 units; Davis and Lochmiller 1995) were determined as described previously.

Humoral immunity

A hemolytic plaque assay (Herscowitz et al. 1974) was used to measure IgM and IgG antibody responsiveness to the T-cell dependent antigen keyhole limpet hemocyanin (KLH; Cat. No. 378417, Calbiochem, CA, USA). A working solution of 1 mg KLH/mL in sterile phosphate buffered saline (PBS) was prepared. Primary immunization was accomplished 16 days prior to termination by injecting each animal subcutaneously with 200 μL (5μg KLH) of an emulsification composed of equal volumes working solution and complete Freund's adjuvant (Cat. No. F-4258, Sigma, St. Louis, MO, USA).

Secondary immunizations were administered five days prior to termination by subcutaneously injecting each rat with 100 μL KLH working solution without adjuvant.

For each animal, a splenocyte culture was prepared as previously described by Lochmiller et al. (1994) and two 200 μL aliquots of these cultures were incubated at 37° C for 2 h in Cunningham chambers with 80 μL KLH-labeled sheep red blood cells (SRBCs), 100 μL guinea pig serum, and 20 or 60 μL PBS with 5% fetal calf serum in gelatin (PBS-F-G). Hemolytic plaque formation against KLH-SRBCs was measured by manual counts of plaque-forming cells (PFC) in Cunningham chambers under a dissecting scope to determine antibody response of each animal (Jerne and Nordin 1963). One of the preparations included 40 μL of rabbit anti-cotton rat IgG serum in substitution of PBS-F-G. In this preparation, plaques indicated the activity of the rabbit immunoglobulins against the IgG of the cotton rat.

Cell-mediated immunity

Lymphoproliferative responsiveness of cultured splenocytes was measured in response to stimulation with mitogens and cytokines. Splenocyte preparations were incubated in 96-well tissue culture plates with the T-cell mitogen Concanavalin-A (Con A) at 5 μg/mL and 40 μg/mL of culture in winter 1991; and 2.5 μg/mL and 10 μg/mL of culture in summer 1992. The B-cell mitogen from pokeweed (PWM) was used at concentrations of 0.625 μg/mL and 1.25 μg/mL of culture in winter 1991 and 0.625 μg/mL and 2.5 μg/mL of culture in summer 1992. The ability of cultured splenocytes to respond to Interleukin-2 (recombinant human IL-2; 40 U/mL of culture) and proliferate was measured in winter 1991 and summer 1992. For the winter 1991 and summer 1992

trials, lymphoproliferative responsiveness in response to stimulation was assessed in vitro using the MTT (tetrazolium) reduction assay of Mossmann (1983) as adapted by McMurry et al. (1995a). was measured using the uptake of tritiated (^{3}H) thymidine as previously described for cotton rats (McMurry 1993) in summer 1993 where concentrations of Con A, PWM, and IL-2 were similar to those utilized in summer 1992. A stimulation index was calculated as a percent increase from the unstimulated control wells.

In vivo cell-mediated immunity was assessed using delayed-type hypersensitivity responses to intradermal challenges with the mitogen phytohemaglutinnin (PHA, 250 μg protein in 100 μL sterile PBS). Animals were challenged 24 h prior to termination with an intradermal injection of PHA in the rump and the resulting increase in skin-fold thickness was measured to the nearest 0.1 mm using a pressure-sensitive dial gauge (McMurry 1993). Delayed-type hypersensitivity was also measured in response to a percutaneous application of 25 μl of 3% oxazolone in ethanol (left ear) and intradermal injection of 20 μL of 1mg/mL KLH in PBS (right ear) in previously sensitized animals. Differences in ear thickness before and after challenge with these two recall antigens were measured to the nearest 0.1 mm with a pressure-sensitive dial-gauge (McMurry et al. 1994).

Nonspecific immunity

Natural killer (NK) cell activity was measured in a standard ^{51}Cr-release assay, as described previously for cotton rats (McMurry 1993). Briefly, splenocytes were incubated for 18 h in the presence of IL-2 to activate killer cells. These activated killer

cells were incubated with ^{51}Cr -labeled YAC-1 tumor cells (100:1 effector to target cell ratio) for 4 hours. Results were calculated as percent target cell lysis.

Metabolic activity of resident peritoneal macrophages was measured in vitro by the reduction of nitroblue tetrazolium dye (NBT), as indicated by absorbance @ 514 nm (McMurry et al. 1995b). Phagocytosis of fluorescein-labeled latex beads by peritoneal macrophages in vitro was measured as described by McMurry (1993). Briefly, peritoneal macrophage cultures were incubated with 1-μm fluorescein-labeled latex beads in PBS for 45 min at 37° C. Cytosmears were prepared on microscope slides and cells examined by epifluorescence microscopy for percent active macrophages (those that engulfed latex beads) and average number of beads ingested per active macrophage.

Statistical analysis

Homogeneity of variance among mesocosms for all variables was examined using Levene's test. Variables which did not meet the criteria of Levene's test were transformed (natural logarithm, square root, or rank) prior to analysis of variance (PROC GLM, PC SAS 1993). Measures of immune response in cotton rats are naturally highly variable among individuals (Lochmiller et al. 1993), therefore a $P < 0.10$ was considered for differences between mesocosms to be biologically significant. The LSD multiple comparison test (LSMEANS, PC SAS 1993) was used to determine specific site differences ($P= 0.10$). All values are reported as mean ± SE.

For the single 4-week acute exposure trial, each rat was treated as an experimental unit and the mean square of the variance among cotton rats within mesocosms was used as the error term. For the two 8-week chronic exposure trials, all replicates were pooled

so that each mesocosm served as an experimental unit. In this case the mean square of variance among mesocosms nested within treatment and trial was used as the error term in calculating the F-value for discrimination between treatment means and treatment by trial and treatment by sex interactions. Variables which indicated a significant ($P < 0.10$) interaction in either of these latter two cases were subsequently analyzed separately within the interacting groups using the mean square of variance among the reference mesocosms as the error term in calculating the F-values.

RESULTS

Animal survival

A total of 25 males was removed from the mesocosms after the 4-week acute exposure trial: Tank Battery ($\underline{n} = 7$), API Separator ($\underline{n} = 5$), Land Farm ($\underline{n} = 6$), and reference site 5 ($\underline{n} = 7$). No animals were recovered from reference site 6 due to escape through a hole in the test structure. A total of 103 animals was removed from the two chronic exposure trials, which included 59 animals from the three reference sites (winter 1991: 4 from site 1, 11 each from sites 5 and 6; summer 1992: 11 from each of the 3 sites), 15 from the Tank Battery (5 in winter 1991; 10 in summer 1992: 10), 17 from the API Separator (9 in winter 1991, 8 in summer 1992), and 12 from the Land Farm (5 in winter 1991, 7 in summer 1992). Loss of animals from mesocosms during the two 8-week trials was primarily attributed to fighting between conspecifics.

General body condition and immune organs

During the 4-week acute exposure trial, mean brain weights of cotton rats from the API Separator and the Tank Battery were significantly less ($P = 0.082$) than those of

rats from the reference mesocosm (Fig. 1). Relative liver weight of cotton rats recovered from the Land Farm mesocosm differed significantly ($P = 0.024$) from animals in the reference mesocosms (Fig. 2) during the 8-week chronic exposure trials. Differences in body (overall mean = 121.7 ± 2.3 (SE) g), adrenal gland (68 ± 16 mg), kidney (1.0 ± 0.02 g), heart (516 ± 12 mg), and lung (664 ± 18 mg) weights between treatments were not significant during either exposure (Table 2).

Differences in immune organ weights, cellularity of immune organs, and proportions of selected lymphocyte subpopulations of animals between contaminated and reference mesocosms were not significantly different ($P > 0.10$) during either the 4-week acute exposure trial or the 8-week chronic exposure trials (Table 3). Overall, animals recovered from these trials had spleen weights averaging 216 ± 12 mg consisting of 68.2 ± 5.4 x 10^6 splenocytes; mean splenic cellularity averaged 197.5 ± 16.0 x 10^3 cells/mg spleen. Popliteal lymph nodes averaged 14 ± 1 mg and contained 11.1 ± 1.1 x 10^6 mononuclear cells. Additionally, an average of 33.6 ± 2.1 % of lymphocytes stained positive for Concanavalin A and 45.0 ± 3.8% stained positive for Helix pomatia agglutinin.

Blood analyses

Mean corpuscular volume values were significantly higher ($P = 0.072$) in cotton rats from the Land Farm (62.8 ± 0.6 fL) compared to those from reference mesocosms (60.3 ± 0.3 fL) during the 8-week chronic exposure trials (Fig. 3). No other significant treatment differences in hematological parameters were observed for either of the two exposure trials. Overall, animals had a mean white blood cell count of 14.5 ± 1.1 x 10^3

cells/μL, red blood cell count of 9.2 ± 0.3 x 10^6 cells/μL, platelet count of 761 ± 15 x 10^3 platelets/μL, hemoglobin concentration of 7.5 ± 0.2 g /dL, hematocrit of 39.6 ± 3.8 %, mean corpuscular hemoglobin content of 34.3 ± 14.3 pg, and mean corpuscular hemoglobin concentration of 33.9 ± 1.6 g/dL. Differential counts averaged 62.8 ± 1.3 % neutrophils, 34.3 ± 1.3 % lymphocytes, 2.7 ± 0.2 % eosinophils, and 0.6 ± 0.1 % monocytes overall during these trials; basophils were not observed.

During the 4-week exposure trial, differences ($\underline{P} = 0.099$) in complement activity of sera were observed among mesocosms (Fig. 4). Multiple comparison analysis indicated that complement activity of cotton rats from the Tank Battery (18,893 ± 991 CH50 units/mL) was significantly lower than those from the reference (24,219 ± 3,206.3 CH50 units /mL). A significant site by trial date interaction for complement activity ($\underline{P} = 0.014$) was also observed during the 8-week exposure trials. Analysis of differences for each separate trial showed significantly lower ($\underline{P} = 0.003$) levels of activity in cotton rats from the Tank Battery (8,363 ± 706 CH50 units/mL) compared to the reference (9,971 ± 459 CH50 units/mL) during summer 1992 (Fig. 4).

Humoral immunity

Counts of IgM PFC against KLH-labeled SRBCs in animals from the Land Farm mesocosm (5157 ± 1639 PFC/spleen) during the 4-week acute exposure trial were significantly higher ($\underline{P} = 0.06$) than those from the reference (3232 ± 904 PFC/spleen) mesocosm (Fig. 5). Differences in IgM PFC during the 8-week chronic exposure trials were similar among mesocosms (overall mean = 1189 ± 209 PFC/spleen); no differences

in IgG PFC responses (overall mean = 5.20 ± 1.31 x 10^3 PFC/spleen) were observed among mesocosms (Table 4).

Cell-mediated immunity

Lymphoproliferative responsiveness of cultured splenocytes to mitogen and IL-2 stimulation was highly variable during both the 4-week acute exposure and the 8-week chronic exposure trials and no significant differences between treatment responses were indicated (Table 5). Although analysis of the stimulation indices did not indicate any significant treatment effects, cotton rats in the reference mesocosm showed significantly greater ($P < 0.10$) variation about the mean than those in the API Separator and Land Farm mesocosms for all mitogenic treatments and than those in the Tank Battery mesocosm for the 10 μg/mL Con A and IL-2 treatments after the 4-week trial.

The delayed-type hypersensitivity response assays for both the 4-week acute exposure and the 8-week chronic exposure trials indicated that in vivo cell-mediated immunity was not adversely compromised by exposure to contaminated mesocosms (Table 5). The overall mean response to PHA, oxazolone, and KLH was 32.4 ± 5.6, 14.9 ± 1.6 %, and 66.2 ± 3.7 %, respectively.

Nonspecific immunity

Tumoricidal activity of lymphokine-activated natural killer cells averaged 26.2 ± 6.2 % for both exposure trials and did not differ among mesocosms for any exposure trials (Table 5). Average yield of resident peritoneal exudate cells from cotton rats recovered during both exposure trials was 18.8 ± 1.0 x 10^6 total cells, which included 10.4 ± 0.7 x 10^6 macrophages. During the 4-week acute exposure trial, peritoneal

macrophages of cotton rats housed in the Land Farm mesocosm had significantly higher ($P = 0.026$) levels of metabolic activity as indicated by the NBT reduction assay compared to those from the reference mesocosms. An opposite effect was observed in the Tank Battery mesocosm where NBT reduction was significantly lower ($P = 0.074$) compared to reference animals (Fig. 6). In addition, phagocytic activity of peritoneal macrophages of cotton rats from the API Separator mesocosm was significantly lower ($P = 0.018$) than reference animals after the 4-week acute exposure trial (Fig. 6). No differences in macrophage activity among mesocosms were observed during the two 8-week chronic exposure trials (Table 5).

DISCUSSION

Previous studies examining how cotton rats respond to in situ exposures on various hazardous waste sites have provided mixed results. A few studies have documented chromosomal aberrations (McBee et al. 1987: Thompson et al. 1988) and cytochrome P-450 induction (Elangbam et al. 1990) in cotton rats inhabiting petrochemical-contaminated environments. At least one study has related physiological alterations such as these to actual changes in demographic attributes of cotton rat populations (McMurry 1993). However, several studies have failed to observe any substantial contaminant-induced variation in demographic, morphological, biochemical, or histopathological endpoints (Flickinger and Nichols 1990, Rattner et al. 1993) in cotton rat populations residing in contaminated environments.

Observations that animals from some hazardous waste sites show evidence for toxicity while animals from other sites do not should not be unexpected. Because in situ

studies of this type lack a suitable positive control (a population with clear signs of exposure), it is not known whether the lack of response is attributable to low toxicity, possible resistance of the exposed animals, or insensitivity of the assay. Variation in toxicity among hazardous waste sites is probably the most common reason for such discrepancies in the literature. At least for petrochemical waste sites, it is probably correct to assume that no two sites have the same complex mixtures of contaminants in the soil. For example, the three study sites selected for construction of mesocosms in this study differed considerably in the mixtures of contaminants that were present in the soil (Yates 1994, Ramanathan 1994). This variation in contaminant mixtures was reflected in the variable results of bioassays using Ceriodaphnia, Microtox©, and rice seeds with aqueous and supercritical fluid extracts of soil samples that were removed from the mesocosm (Yates 1994, Ramanathan 1994).

A concurrent study by McMurry (1993) at this same hazardous waste site observed frequent signs of immunotoxicity in the resident population of cotton rats using many of the endpoints used in this study. In particular, McMurry (1993) documented that select hematological parameters, immune organ weights, metabolic activity of macrophages, in vivo delayed-type hypersensitivity responses to PHA, and in vitro lymphoproliferative responses of cultured splenocytes to Con A were frequently altered in animals from contaminated habitats. In addition, a high incidence of dental lesions in the enamel were observed in the population , which were linked to high levels of fluoride in the soil (Paranjape et al. 1994). Collectively, these earlier studies on this study site

indicated that in situ exposure to contaminants (complex mixtures) produced detectable alterations in general body condition and immune system function in resident cotton rats.

It is here suggested that the above data provided a reasonable positive control for evaluating the efficacy of mesocosms to assess toxicity risks on this hazardous waste site. Although no differences in delayed-type hypersensitivity responsiveness were seen among mesocosms, differences in selected hematological parameters, organ weights, macrophage activity indices, and lymphoproliferative responsiveness between reference and contaminated mesocosms, similar to those reported by McMurry (1993) in the resident population, were observed. Some of the animals recovered from the contaminated mesocosms also had dental lesions similar to those observed in wild cotton rats (data not reported).

Cotton rats collected from the API Separator mesocosm during the 4-week acute exposure trial had lower absolute brain weights and lower peritoneal macrophage phagocytic activity than animals from the reference mesocosm. This mesocosm had the highest diversity of detected contaminants and the greatest soil toxicity using standardized bioassays (Yates 1994) compared to other mesocosms in this study. Two of the contaminants detected at this location were toluene and mercury. Gospe et al. (1996) demonstrated decreased brain size and weight in progeny of Sprague-Dawley rats treated with toluene during gestation. Contrino et al. (1992) showed that resident peritoneal macrophages from Lewis rats had decreased rates of erythrophagocytosis when exposed to various concentrations of $HgCl_2$.

Decreased brain weight was also seen in animals from the Tank Battery mesocosm during the 4-week acute exposure trial along with decreased peritoneal macrophage metabolic activity. Complement activity was also decreased in cotton rats from this mesocosm during the 4-week acute exposure trial and the 8-week chronic exposure trial (summer 1992). Tian and Lawrence (1995) found that in vitro lead exposure inhibited nitric oxide production of murine splenic macrophages while Ewers et al. (1982) observed low complement protein C3 levels in lead workers.

Cotton rats housed in the Land Farm mesocosm demonstrated the most differences compared to reference sites. Increased relative liver weights observed during the 8-week chronic exposure trials at this mesocosm have also been noted in other similar studies (Rattner et al. 1993, Feuston et al. 1994). Lead contamination at this site may have been responsible for the increased IgM PFC response observed during the 4-week acute exposure trial. Lawrence (1981) used the hemolytic plaque assay to determine the effect of in vitro exposure of lymphocytes to eleven individual heavy metals; only lead and nickel augmented the PFC response. Heavy metal contamination may have also been responsible for the increased metabolic activity of peritoneal macrophages in cotton rats from this site (Contrino et al. 1992). Finally, increased mean corpuscular volume, also observed in cotton rats from this mesocosm, was reported in B6C3F1 mice exposed to nitrobenzene by gavage (Burns et al. 1994) and in rats dermally administered heavy gas oil, a coal coprocessing product (Yagminas et al. 1995).

Several technical problems became apparent with the use of mesocosms for controlled exposures of small mammals on petrochemical-contaminated environments.

Foremost among these difficulties was the frequent fighting that occurred in the mixed-sex 8-week exposure trials. Although juvenile animals were introduced into the mesocosms at the start of these experiments, they became sexually active toward the end of the exposure and pregnant females were intolerant of crowding. Another difficulty which is unavoidable is the need to supplement animals with food and water. It is entirely possible that the normal stresses associated with seasonal changes in nutritional condition are important in regulating responses of animals to contaminant exposures. Lastly, the densities (animals per unit area) of animals used in our mesocosms were in excess of what resident cotton rats would experience and the stresses associated with this could have masked the immunosuppressive effects from contaminant exposure for some assays (Rabin et al. 1987). Overall, mesocosms appear to provide another useful tool for ecologists to use in toxicity risk assessments to wild animls. However, caution and careful planning are clearly warranted prior to adapting them for routine use.

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Table 1. Reference list of immunoassay methodologies adapted for use in the cotton rat to assess alterations in immune system function.

Assessment	Endpoint	Reference
Immune organs	Spleen weight and cellularity	McMurry 1993
	Popliteal lymph node weight and cellularity	
	Splenocyte subpopulations	Davis and Lochmiller 1995
	Concanavilin A positive	
	Helix pomatia agglutinin positive	
Hematology	Total serum protein	Davis et al. 1995
	White blood cell count	Robel et al. 1996
	Red blood cell count	
	Platelet count	
	Hematocrit	
	Mean corpuscular volume	
	Hemoglobin concentration	
	Mean corpuscular hemoglobin	
	Mean corpuscular hemoglobin concentration	
	Differential leukocyte counts	
Humoral immunity	Hemolytic plaque formation to keyhole limpet hemocyanin labeled sheep red blood cells	Lochmiller et al. 1994
Cell-mediated immunity	Hypersensitivity response to Phytohemagluttinin-P	McMurry 1993
	Delayed-type hypersensitivity response to Oxazolone and	McMurry et al. 1994
	Keyhole limpet hemocyanin	McMurry 1993
Nonspecific immunity	Complement activity	Davis et al. 1995
	Proliferative responses to mitogenic stimulation with Concanavilin A and Pokeweed and the cytokine Interleukin-2	McMurry et al. 1995a
	Natural killer cell activity	McMurry 1993
	Macrophage metabolic activity Reduction of nitroblue tetrazolium	McMurry et al. 1995b
	Macrophage phagocytic activity Latex bead ingestion	McMurry 1993

Table 2. Organ weights of cotton rats from three contaminated or reference mesocosms on an abandoned oil refinery in Oklahoma following acute (4-week) or chronic (8-week) exposure trials. Mean values for the three contaminated mesocosms were not significantly different from the reference mean ($P > 0.10$).

	Duration of exposure	Reference Sites			Tank Battery			API Separator			Land Farm		
Parameter (units)	(weeks)	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
Final body weight (g)	4	7	113.4	4.1	7	105.5	4.5	5	107.2	4.6	6	106.8	5.8
	8	59	120.7	3.3	15	117.1	6.8	17	126.4	4.1	12	140.1	5.1
Adrenal glands (mg)	4	4	48	5	7	51	5	5	50	4	0		
	8	59	92	18	15	64	5	17	86	7	12	94	9
Lungs (mg)	8	58	651	26	15	647	40	17	681	33	12	726	35
Kidneys (mg)	4	4	900	49	7	939	52	5	880	25	0		
	8	59	991	26	15	939	27	17	1094	26	12	1185	37
Brain (mg)	8	59	1101	7	15	1104	17	17	1119	14	12	1118	18
Heart (mg)	8	58	500	18	15	463	22	17	551	16	12	609	25
Liver (g)	4	4	4.3	0.3	7	4.6	0.2	5	4.4	0.2	0		

Table 3. Weight and cellularity of selected immune organs of cotton rats from three contaminated or reference mesocosms on an abandoned oil refinery in Oklahoma following acute (4-week) or chronic (8-week) exposure trials. Relative abundances of lymphocyte subtypes staining positive for Con A or Helix pomatia receptor binding are also presented. Mean values for the three contaminated mesocosms were not significantly different from the reference mean ($P > 0.10$).

Parameter (units)	Duration of exposure (weeks)	Reference Sites			Tank Battery			API Separator			Land Farm		
		n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
Spleen (mg)	4	7	164.4	13.2	7	139.5	9.6	5	140.3	18.7	6	161.3	17.5
	8	59	234.6	19.0	15	188.0	23.3	17	219.4	28.2	12	354.5	49.7
Total splenocytes	4	7	46.6	4.3	7	47.5	4.4	5	44.2	4.8	6	40.0	4.9
($\times 10^6$ cells)	8	59	78.2	9.3	15	48.5	7.5	17	56.1	7.7	12	109.5	26.6
Splenic cellularity	4	7	291.7	29.9	7	345.7	33.0	5	324.8	30.4	6	257.4	38.7
($\times 10^3$ cells/mg)	8	59	355.1	18.8	15	284.5	32.8	17	276.1	30.3	12	308.8	43.9
Popliteal lymph nodes	4	4	9.1	2.9	7	6.4	1.0	5	6.2	1.2	6	7.6	0.7
(mg)	8	57	14.9	2.0	14	9.8	0.9	16	19.4	3.3	12	23.1	8.5
Popliteal lymph node	4	7	2.54	0.60	7	3.06	0.58	5	3.32	0.72	6	3.37	0.68
cellularity ($\times 10^6$ cells)	8	58	11.59	1.55	14	10.38	1.02	17	18.42	3.76	12	15.98	5.70
Con A (+)	4	7	28.6	1.6	7	31.8	2.5	5	27.2	2.2	6	30.8	2.6
splenocytes (%)	8	45	32.4	1.6	11	35.7	2.4	12	33.7	2.2	11	28.4	2.2
Helix pomatia (+)	4	7	39.0	2.0	7	38.5	2.6	5	34.2	4.1	6	42.3	1.4
splenocytes (%)	8	50	41.4	1.6	12	43.2	3.6	13	47.5	2.8	12	41.3	3.5

Table 4. Hematological parameters of cotton rats from three contaminated and reference mesocosms on an abandoned oil refinery in Oklahoma following acute (4-week) or chronic (8-week) exposure trials. Number of plaque-forming cells (PFC) in response to KLH-labeled SRBCs are also presented. Mean values for the three contaminated mesocosms were not significantly different from the reference mean (P > 0.10).

Parameter (units)	Duration of exposure (weeks)	Reference Sites			Tank Battery			API Separator			Land Farm		
		n	Mean	SE	n	ean	SE	n	Mean	SE	n	Mean	SE
White blood cells	4	7	14.7	1.6	5	13.7	2.1	5	14.9	2.9	5	12.2	1.5
(x 10^3/uL)	8	57	12.9	1.0	13	13.9	1.1	15	10.6	0.8	12	14.1	1.4
Neutrophils (%)	4	7	48.4	4.9	7	38.6	4.8	5	39.8	7.6	6	58.8	5.6
	8	59	67.9	1.3	15	69.7	3.6	17	61.8	2.9	12	62.8	4.8
Lymphocytes (%)	4	7	46.4	4.9	7	56.4	5.1	5	55.2	7.7	6	35.3	5.6
	8	59	29.5	1.3	15	27.9	3.3	17	35.8	2.9	12	34.2	4.7
Eosinophils (%)	4	7	4.0	1.2	7	3.9	0.6	5	4.2	1.0	6	4.5	1.0
	8	59	2.2	0.3	15	2.5	0.5	17	2.5	0.4	12	2.8	0.5
Monocytes (%)	4	7	1.1	0.3	7	1.1	0.6	5	1.0	0.3	6	1.3	0.3
	8	59	0.6	0.1	15	0.3	0.1	17	0.2	0.1	12	0.5	0.19
Red blood cells	4	7	6.3	0.1	6	6.5	0.2	5	6.7	0.2	6	6.1	0.2
(x 10^6/uL)	8	59	5.6	0.1	15	5.7	0.2	17	5.5	0..12	12	5.4	0.2
Hemoglobin (g/dL)	4	7	12.0	0.2	6	12.4	0.3	5	12.6	0.4	6	11.7	0.3
	8	59	10.8	0.2	15	11.0	0.3	17	10.7	0.2	12	11.0	0.3
Hematocrit (%)	4	7	37.3	0.5	6	38.1	1.0	5	39.5	1.5	6	36.4	0.9
	8	59	33.5	0.5	15	34.0	1.0	17	33.3	0.7	12	33.7	1.2
Mean corpuscular volume (fL)	4	7	58.9	0.5	6	58.8	0.4	5	58.8	0.3	6	59.2	0.5
Mean corpuscular	4	7	18.9	0.2	6	19.1	0.2	5	18.8	0.2	6	19.0	0.3
hemoglobin (pg)	8	59	19.5	0.1	15	19.5	0.3	17	19.5	0.2	12	20.5	0.3
Mean corpuscular hemoglobin	4	7	32.1	0.1	6	32.4	0.2	5	32.0	0.2	6	32.0	0.3
concentration (g/dL)	8	59	32.4	0.1	15	32.4	0.2	17	32.2	0.1	12	32.7	0.3
Platelets (x 103/uL)	4	7	862	28	6	794	38	5	824	58	6	758	68
	8	57	775	21	13	700	30	17	772	42	12	721	49
Total serum protein (g/dL)	8	58	6.00	0.1	15	5.86	0.1	16	5.87	0.09	12	5.72	0.26
IgM PFC/spleen	8	49	1351	322	11	544	171	15	733	170	12	1633	689
IgG PFC (x 10^3)/spleen	8	47	44.0	16	11	26.3	8.6	13	95.0	48.6	10	62.0	42.4

Table 5. Cell-mediated and nonspecific immune function assay results of cotton rats from three contaminated and reference mesocosms on an abandoned oil refinery in Oklahoma following acute (4-week) and chronic (8-week) exposure trials. Stimulation indices of splenocyte proliferative response to Concanavalin A (Con A), pokeweed (PWM), and Interleukin-2 (IL-2), hypersensitivity response to phytohemagluttinin-P (PHA-P) and keyhole limpet hemocyanin (KLH), delayed-type hypersensitivity response to oxazolone, peritoneal macrophage metabolic and phagocytic activity measures, and natural killer (NK) cell lytic activity are included. Mean values for the three contaminated mesocosms were not significantly different from the reference mean ($P > 0.10$).

	Duration of	Reference Sites			Tank Battery			API Separator			Land Farm		
Parameter (units)	exposure (weeks)	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE
Stimulation Indices													
2.5 ug/mL Con A	4	7	58.7	20.4	7	32.9	14.7	5	18.6	6.2	6	15.4	2.4
2.5-5 ug/mL Con A	8	58	47.3	10.8	14	143.0	40.1	17	54.6	15.9	12	34.7	16.1
10 ug/mL Con A	4	7	311.5	104.4	7	139.2	50.0	5	149.1	56.4	6	104.3	28.0
10-40 ug/mL Con A	8	59	27.0	7.0	15	45.6	11.7	17	47.4	15.1	12	23.5	13.7
0.625 ug/ml PWM	4	7	514.3	197.4	7	289.6	120.4	5	262.1	53.5	6	120.1	19.8
0.625 ug/mL PWM	8	59	45.8	13.8	14	137.6	50.8	17	54.4	26.5	12	26.7	19.7
2.5 ug/ml PWM	4	7	510.0	213.5	7	281.4	120.9	5	211.4	54.6	6	109.6	26.3
1-2.5 ug/mL PWM	8	59	31.0	13.7	15	142.9	51.8	17	43.8	19.6	12	15.5	17.4
40 U/mL IL-2	4	7	120.3	49.8	7	58.0	24.4	5	46.8	16.2	6	19.4	9.4
40 U/mL IL-2	8	59	50.8	3.9	15	56.1	8.1	17	41.8	6.3	12	40.5	8.3
Hypersensitivity													
PHA-P response	4	7	166.4	16.8	7	129.1	20.6	5	168.4	25.2	6	159.8	10.4
(% increase)	8	26	186.3	10.5	5	139.6	41.3	9	129.7	21.1	5	111.5	17.8
Oxazolone response	4	7	26.0	5.5	7	37.9	11.8	5	33.3	6.5	6	17.3	3.0
(% increase)	8	58	14.9	1.9	14	18.9	5.5	17	22.6	4.7	12	20.4	4.5
KLH response	4	7	62.6	13.3	7	68.2	9.3	5	72.9	12.8	6	52.5	5.9
(% increase)	8	32	62.9	6.2	9	69.9	11.8	8	98.9	12.3	7	51.3	12.5
Macrophage Activity													
Total peritoneal	4	7	16.74	2.86	7	16.80	2.33	5	12.12	0.80	5	15.12	1.96
exudate cells (x 10^6)	8	58	19.94	1.58	15	17.85	2.74	17	24.2	2.69	12	13.93	2.82
Peritoneal exudate	4	7	8.15	1.46	7	9.09	0.98	5	6.05	0.48	5	9.14	1.64
macrophages (x 10^6)	8	58	10.84	1.27	15	10.46	1.818	17	13.57	1.79	11	8.09	1.85
Latex bead positive	4	6	44.0	5.4	7	51.9	4.7	4	50.6	7.6	5	54.2	7.7
macrophages (%)	8	48	46.7	2.0	13	43.5	3.5	17	48.5	2.4	6	42.7	7.9
NK cell activity	4	7	41.4	9.3	7	63.5	3.4	5	61.0	3.7	6	16.4	1.4
(% lysis)	8	25	27.0	2.3	5	18.5	3.8	9	19.5	1.9	5	25.0	2.9

Figure 1. Mean brain weights (± SE) of cotton rats from two petrochemical contaminated mesocosms (TnkBttry = Tank Battery and APISep = API Separator) and a reference mesocosm (Ref) in Oklahoma following a 4-week acute exposure trial. Means without common letter designations are significantly different ($P < 0.10$).

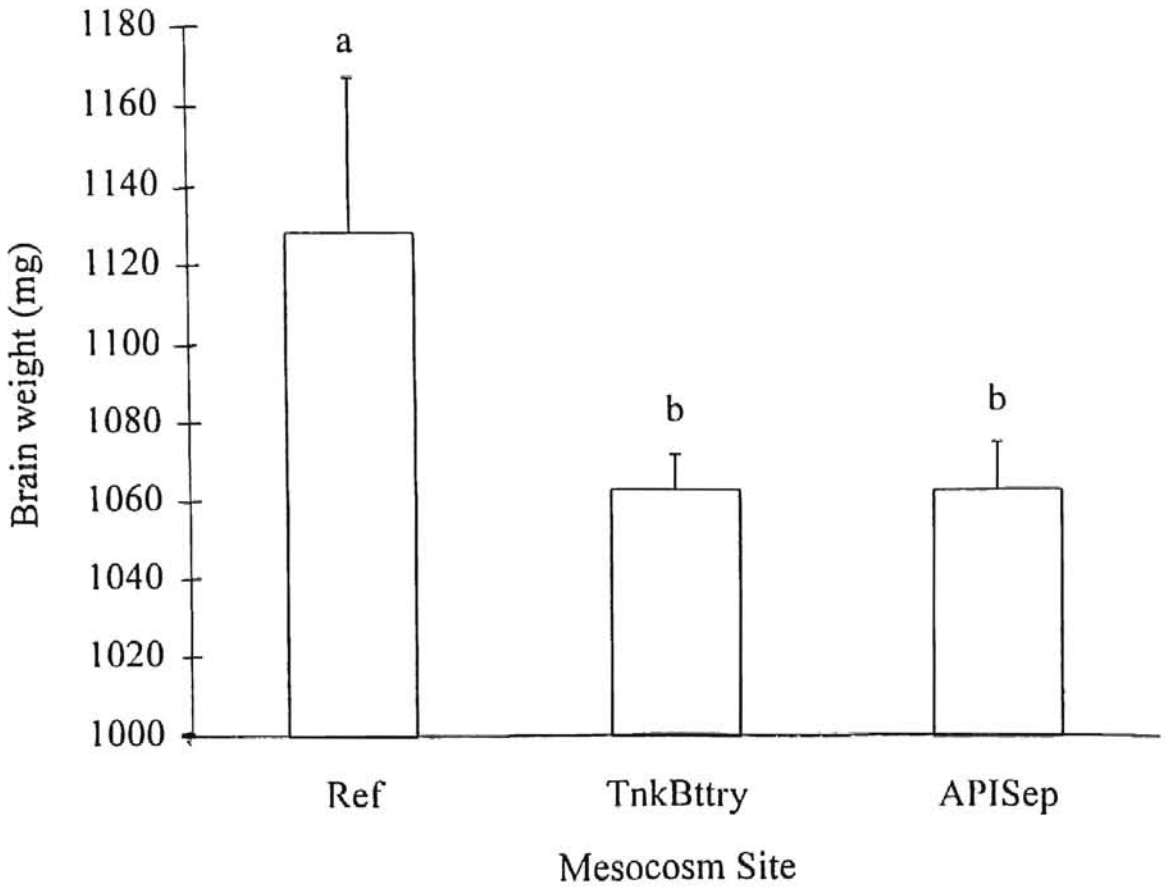


Figure 2. Mean relative liver weights (±SE) of cotton rats from three petrochemical contaminated mesocosms (TnkBttry = Tank Battery, APISep = API Separator, and LndFrm = Land Farm) and reference mesocosms (Ref) in Oklahoma following an 8-week chronic exposure trial. Means without common letter designations are significantly different ($P < 0.10$).

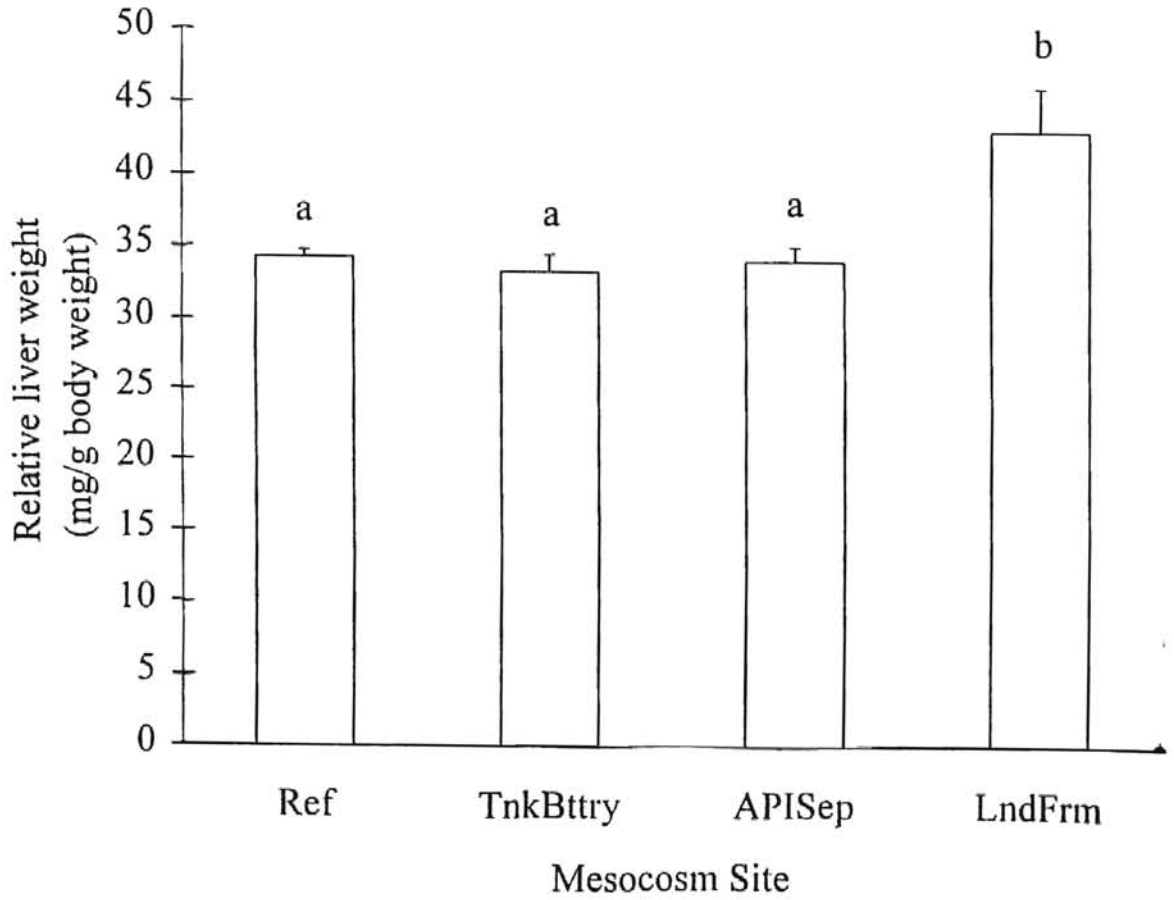


Figure 3. Average mean corpuscular volume (±SE) of cotton rats from three petrochemical contaminated mesocosms (TnkBttry = Tank Battery, APISep = API Separator, and LndFrm = Land Farm) and reference mesocosms (Ref) in Oklahoma following an 8-week chronic exposure trial. Means without common letter designations are significantly different ($P < 0.10$).

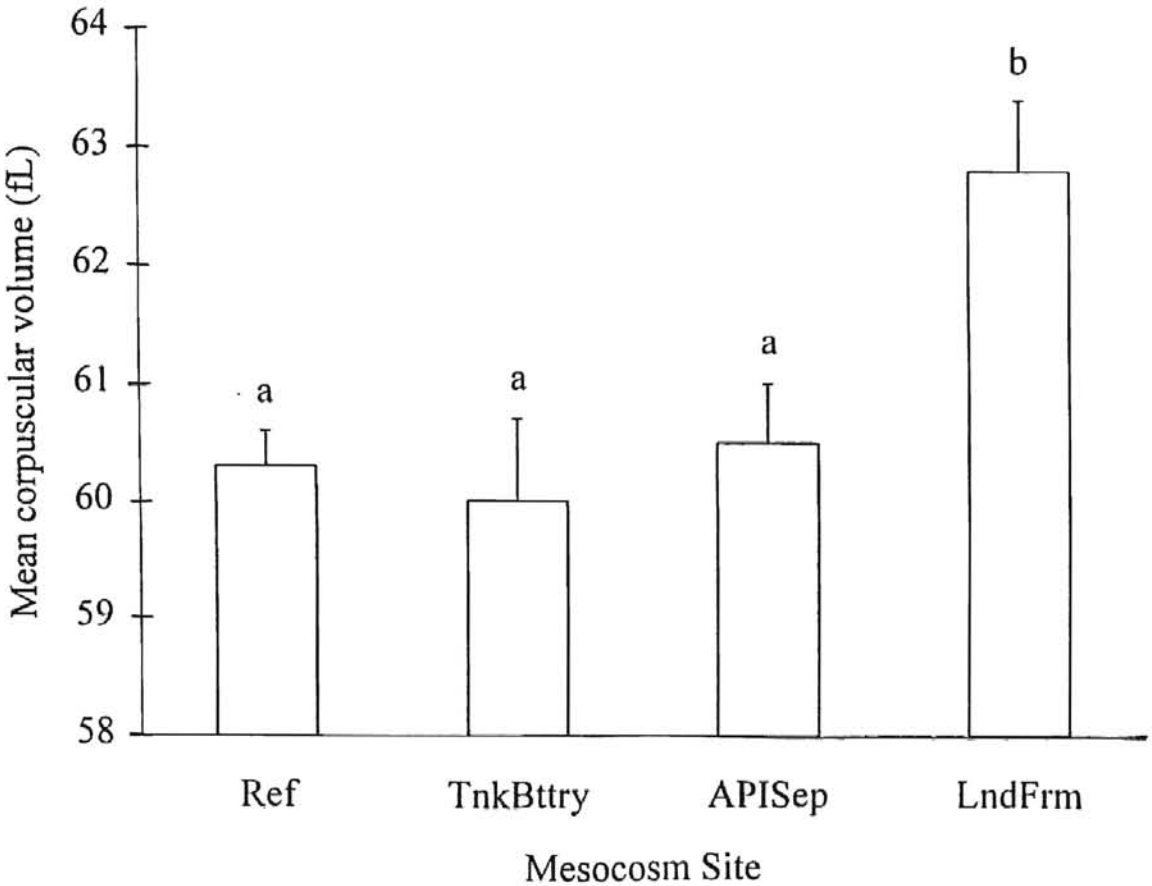


Figure 4. Mean complement activity (±SE) of cotton rats from three petrochemical contaminated mesocosms (TnkBttry = Tank Battery, APISep = API Separator, and LndFrm = Land Farm) and reference mesocosms (Ref) in Oklahoma following 4-week acute (spring 1993) and 8-week chronic (summer 1992) exposure trials. Means without common letter designations are significantly different ($P < 0.10$).

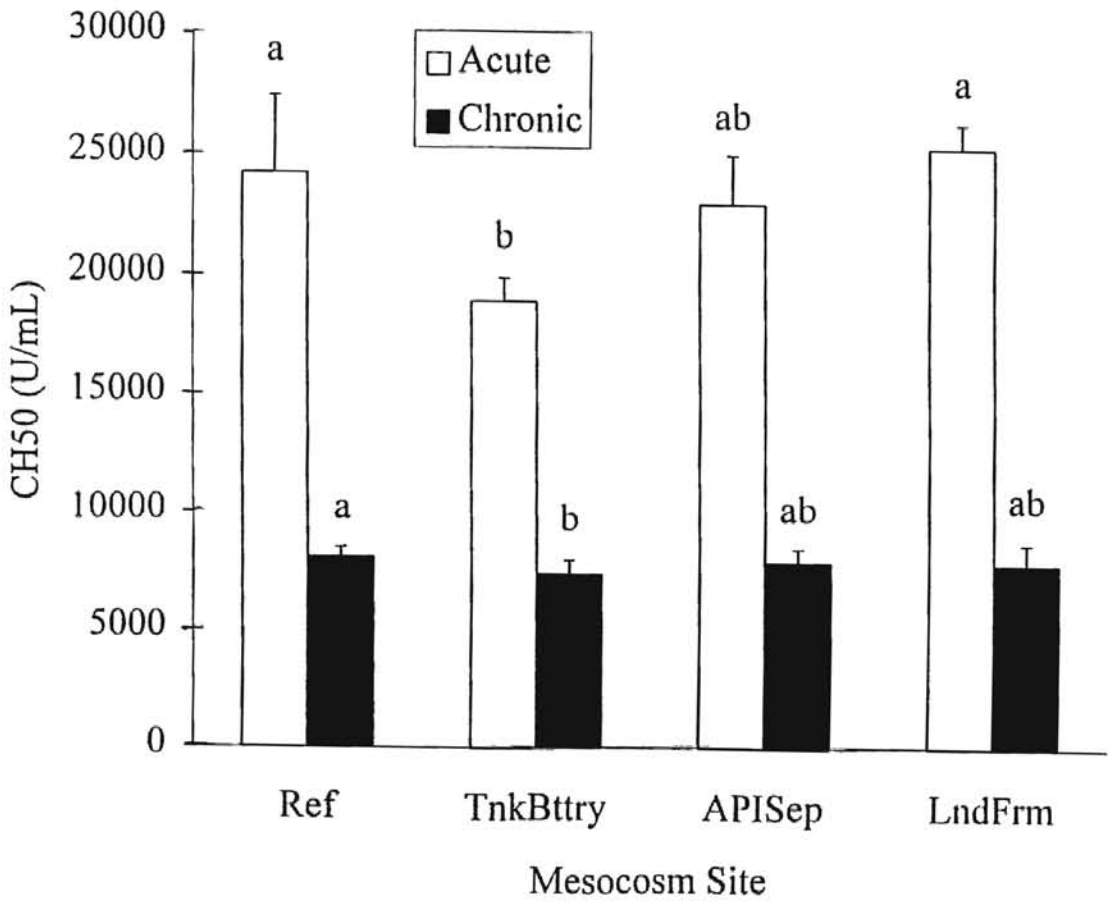


Figure 5. Mean number of plaque-forming cells (±SE) against keyhole limpet hemocyanin-labeled sheep red blood cells from spleens of cotton rats from three petrochemical contaminated mesocosms (TnkBttry = Tank Battery, APISep = API Separator, and LndFrm = Land Farm) and a reference mesocosm (Ref) in Oklahoma following a 4-week acute exposure trial. Means without common letter designations are significantly different ($P < 0.10$).

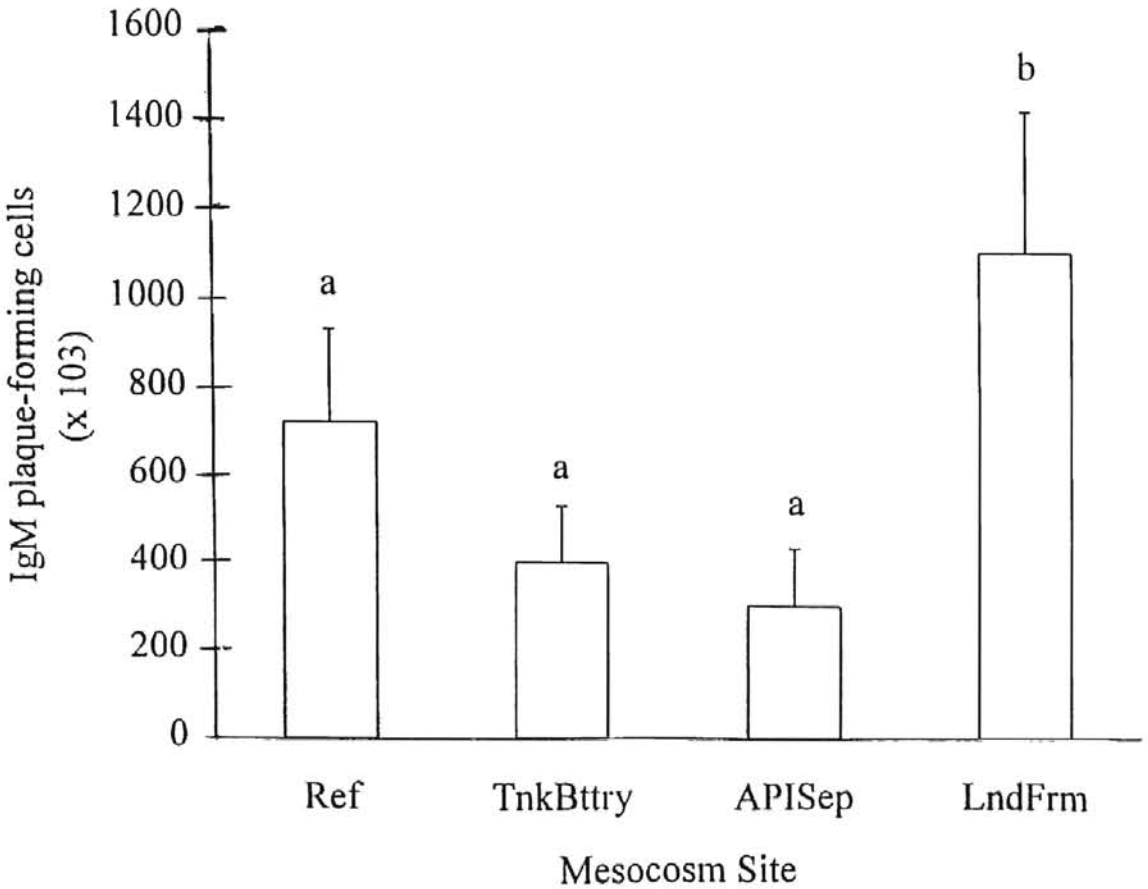
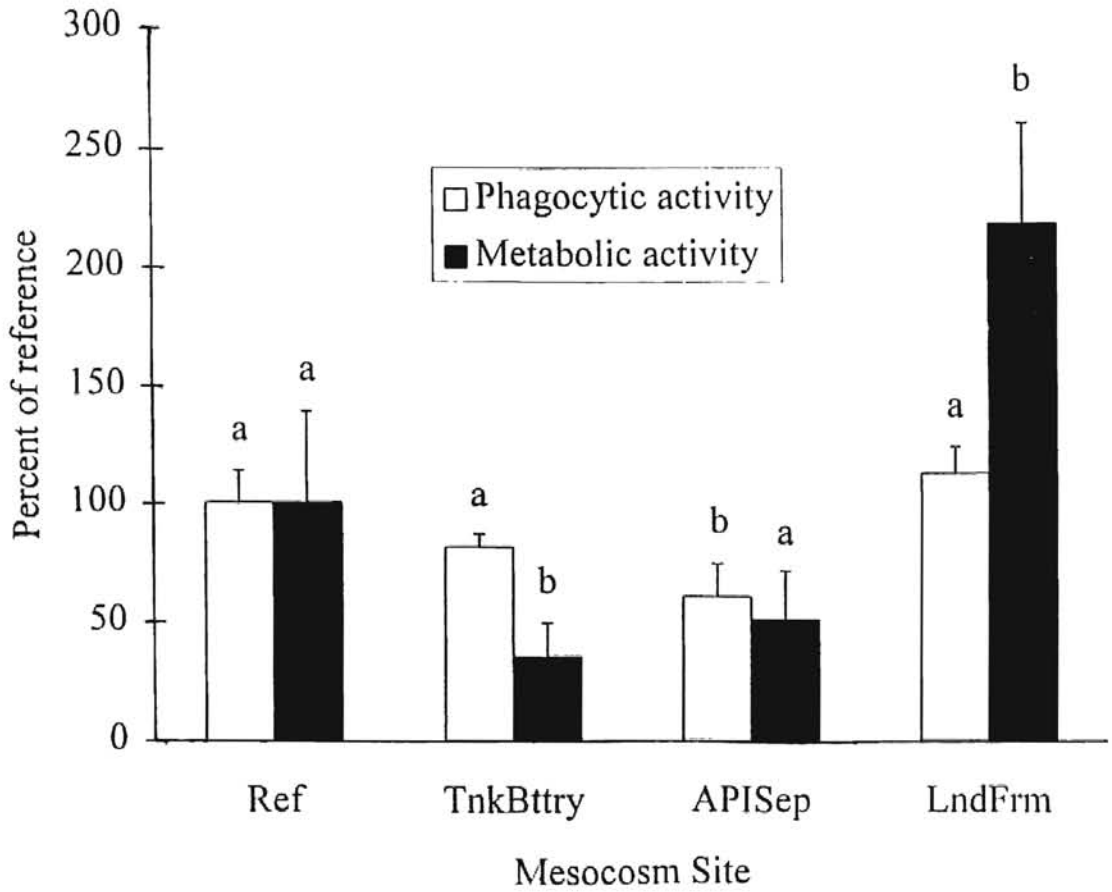


Figure 6. Metabolic and phagocytic activity of peritoneal macrophages of cotton rats from three petrochemical contaminated mesocosms (TnkBttry = Tank Battery, APISep = API Separator, and LndFrm = Land Farm) and a reference mesocosm (Ref) in Oklahoma following a 4-week acute exposure trial. Values represent mean (± SE) and are expressed as a percentage of reference responses. Means without common letter designations are significantly different ($P < 0.10$).



VITA

Timothy Lee Propst

Candidate for the Degree of

Master of Science

Thesis: ASSESSING IMMUNOTOXICITY RISKS TO SMALL MAMMALS IN ENVIRONMENTS CONTAMINATED WITH PETROCHEMICALS

Major Field: Wildlife and Fisheries Ecology

Biographical:

Personal Data: Born in Paris, Illinois, On October 27, 1968, the son of Darrell and Karen Propst.

Education: Graduated from Terre Haute North Vigo High School in Terre Haute, Indiana in June 1987; received Bachelor of Arts degree in Biology from Wabash College, Crawfordsville, Indiana in May 1991. Completed the requirements for the Master of Science degree with a major in Wildlife and Fisheries Ecology at Oklahoma State University in May 1997.

Experience: Raised in Terre Haute, Indiana; employed as stockboy and lifeguard during summers, 1985-88; employed by Eli Lilly and Co. as laboratory technician, Environmental Controls Dept., summer 1989; employed by Wabash College as research assistant, summers 1990-91; employed by Oklahoma State University, Department of Zoology, as research assistant and teaching assistant, 1991 to 1994; employed by Stover and Associates as Project Scientist, 1994-1996; employed by Stover and Associates as Assistant Project Manager-FETAX, 1996-present.