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Flow Cytometric Analysis of DNA Damage in Cotton Rats, *Sigmodon hispidus,* Inhabiting an Abandoned Colliery Strip Mine

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Mammals are a large taxonomic group and selected model species can be useful in biomonitoring, providing insight into the health of wildlife populations in contaminated habitats (Lochmiller et al. 1999; Punshon et al. 2003; Schroder and Basta 2003; Shaw-Allen and McBee 1993; Thompson et al. 1988). This study focuses on *Sigmodon hispidus* (hispid cotton rat), a small rodent that inhabits much of southeastern and south-central United States. Use of *S. hispidus* to biomonitor presence and action of pollutants at contaminated sites has been successful for several types of endpoints (Lochmiller et al. 1999; McBee and Bickham, 1988; McBee et al. 1987; McMurry et al. 1995; Thompson et al. 1988). The small home range of *S. hispidus*, *ca.* 0.35 ha, allows these animals to be easily tied to sources of contamination. This species is both nocturnal and diurnal and constructs surface and burrow nests from woven grass (Cameron and Spencer 1981). The diet of *S. hispidus* is predominately lower green stems of grasses and forbs, which may provide a route by which trace metal contaminants can enter the food web (Randolph et al. 1991; Punshon et al. 2003).

Due to extensive strip mining for coal in eastern Oklahoma during the late 1800's and early 1900's, wildlife has been exposed to metals not typically present in soils at levels above background. Orphaned mines cover *ca.* 21,000 km^2 in Oklahoma and are contaminated with trace metals including Pb, Zn, and Cd (Johnson et al. 1982). We focused on Marler Mine (MM) in Okmulgee Co., Oklahoma which was abandoned when mining ceased in 1917. Some natural revegetation has occurred at MM, but Oklahoma law did not require reclamation. Levels of Cd, Pb, and Zn (1.99, 32.80, 544.36 μg/g dry weight, respectively) remain higher there than at reference sites located at Eufaula Wildlife Management Area (EWMA), Okmulgee Co. (1.25, 27.12, 231.18 μg/g dry weight, respectively), and Lake Carl Blackwell (LCB), Payne Co. (0.10, 10.27, 56.51 μg/g dry weight, respectively), Oklahoma (Hausbeck 1994; Husby and McBee 1999). EWMA was located on unmined land within the eastern coal belt of Oklahoma and LCB was located well out of the coal belt *ca.* 250 km from MM. These reference sites were chosen based on similarity to MM in topography, plant communities, and mammalian species assemblages. All sites are within the Tall-grass prairie rolling hills/ Postoak-blackjack uplands of eastern Oklahoma (Caire 1989).

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Some metals such as Zn are necessary for normal cellular functions, but at high concentrations all metals can have detrimental effects. Cadmium and lead cause a variety of toxic effects ranging from weight loss to renal dysfunction and necrosis of testis in mammals. Damage in mitochondria, impairment of oxidative phosphorylation, and antimetabolic activities toward other metals are known effects of cadmium contamination (Landis and Yu 1998). Acute exposure to cadmium can also induce a dose dependent increase in apoptosis of liver cells in mice (Habeebu et al. 1998) and condensation of nucleoli in rats (Dudley et al. 1984). Lead interacts with nucleic acids, including reducing the ability of tRNA to bind to ribosomes (Landis and Yu 1998). Lead exposure can also cause reduction in resting metabolism (Migula et al. 1985), splenic cellularity, injury to T and B-lymphocytes, renal lesions, decreased sperm and developing follicles in small mammals (McMurry et al. 1995), chromosome breakage, and sister chromatid exchanges (Peakall 1992). Cadmium, due to similarities in structure and electron arrangement, can compete with calcium and zinc for binding sites on DNA repair enzymes and prevent the repair of normally occurring DNA damage (Satoh et al. 2002). Zinc, unlike Pb and Cd, is necessary for normal cellular functions but at high concentration can cause reduced growth rate and gastrointestinal tract inflammation (Venugopal and Luckey 1978). Zinc can also induce interchromosomal DNA fragmentation in rat cells (Haase et al. 2001). Rodents (*Oryzomys* and *Akodon*) collected from areas in Brazil contaminated with Pb, Cd, and Zn from coal washings had significant increases in percent cells with chromosomal aberrations (Bueno et al. 1992), so it is reasonable to expect increased levels of DNA damage in *S. hispidus* from MM.

Flow cytometry (FCM) is a rapid, cost-effective method for measuring presence of chromosomal lesions. FCM has been used to detect chromosomal damage in Chinese hamster ovary cells caused by atrazine (Biradar and Rayburn 1995), turtles (*Trachemys scripta*) inhabiting a radiation-contaminated site (Lamb et al. 1991), mallards dosed with methyl parathion and triethylenemelamine (Whittier and McBee 1999), and white-footed mice (*Peromyscus leucopus*) exposed to petrochemical waste (McBee and Bickham 1988). It is a rapid alternative assay to microscopic analysis in detecting whole cell clastogenicity (Biradar and Rayburn 1995). Variation in the amount of DNA present in cells for any individual is measured as the coefficient of variation (CV) around the mean of cells in the G_1 stage of the cell cycle. Unequal distribution of DNA in daughter cells is evident in larger coefficients of variation or a widening of the G_1 peak in populations of cells from animals exposed to cytotoxic agents (Bickham et al. 1992).

We used flow cytometry to assess the level of genetic damage in splenocytes from *S. hispidus* collected from MM and reference sites, EWMA and LCB. We hypothesized that *S. hispidus* from MM would have significantly larger CVs compared to CVs of reference animals and CVs from the 2 reference sites would not be significantly different.

MATERIALS AND METHODS

Animals were collected as part of an earlier study (Hausbeck 1994; Husby et al. 1999; Husby and McBee 1999) in late 1992 and early 1993 by use of Sherman live traps baited with rolled oats. Two hundred traps, with *ca.* 5 m spacing between traps, were placed along spoil piles. Tissues were collected in the field, placed in liquid nitrogen, and deposited in the Oklahoma State University Collection of Vertebrates, Frozen Tissue Collection (OSU COV) where they were stored at -80°C. Spleen tissue samples from 78 individuals (MM, $n = 26$; LCB, $n = 26$; and EWMA, $n = 26$) were randomly selected from materials deposited in OSU COV. Tissue was prepared using a modified method of Bickham et al. (1994). Tissue was ground in a citrate buffer (50µl)/trypsin (450µl) solution and incubated at room temperature for 10 min. Trypsin inhibitor (375µl) was added and solutions were incubated at room temperature for 10 min. and filtered through 37 µm monofilament cloth. Propidium iodide (PI) was used to dye DNA in isolated nuclei. Standards were prepared from calf thymocyte nuclei (CTN: Biosure). Samples were analyzed with a BD FacsCalibur Flow Cytometer at OSU Center for Veterinary Health Sciences. Excitation wavelength for PI is 488 nm and was provided by a 5-W argon ion laser. CVs were calculated using Cell Quest computer software. Three replicates of 10,000 cells each were generated for each animal. CTN standards were run initially to calibrate the machine and every 5 samples to maintain calibration and alignment of the flow cytometer, per Husby and McBee (1999).

Mean CVs were calculated from the 3 histograms for each individual and a split plot analysis of variance (ANOVA) was performed to compare mean CVs between sexes and among sites. The main unit factor of type of site (contaminated or reference) and split unit factor of sex were analyzed. Interactions examined were sex within type of site (reference pooled versus contaminated) and sex within site by type of site (sex for LCB vs. EWMA).

RESULTS AND DISCUSSION

Mean CVs from each site and sex ranged from 4.57 for females at MM to 5.40 for females at EWMA (Fig. 1). ANOVA (Table 1) revealed that, with one exception, there were no significant differences in CVs between the contaminated and reference sites. There was a significant interaction between sex and type of site (contaminated versus reference). Males from pooled reference sites had lower CVs than males from MM (5.06 versus 5.22); whereas females from MM had a significantly lower CV (4.63) compared to females from reference sites (5.23). Differences for CVs were not consistent for sexes across sites suggesting that males and females may not be responding in the same way to metals at MM, but the way in which females apparently responded was unexpected. We hypothesized that both males and females from MM would have higher CVs. With field-collected animals there is no way to control exposure time, degree of

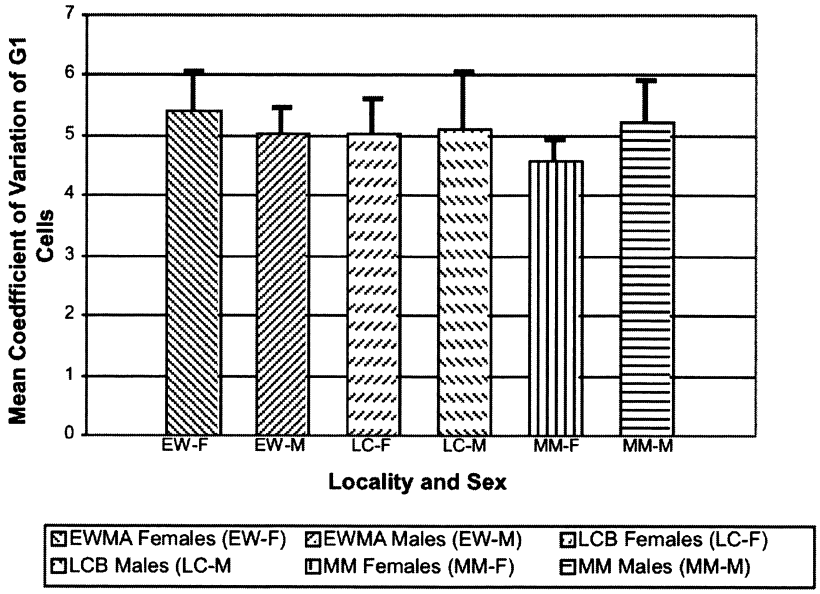

Figure 1. Mean coefficients of variation and standard deviation by sex at each site.

Table 1. ANOVA results for CVs from contaminated and reference sites.

Source	F-Value	Pr > F
EWMA vs. LCB (reference sites)	0.37	0.547
Contaminated (MM) vs. Reference (EWMA & LCB)	1.37	0.246
All males vs. all females	0.85	0.361
LCB + EWMA males vs. MM males and LCB + EWMA females vs. MM females	4.05	0.0481*
EWMA males vs. LCB males and EWMA females vs. LCB females	1.16	0.286

*Significantly different at $\alpha = 0.05$

exposure, population genetic differences, or physiological parameters such as nutritional condition or parasite load. All of these factors may have influenced our results.

Zinc is required in normal cellular processes, but at high concentrations it has detrimental effects. Schroder and Basta (2003) found that zinc did not

bioaccumulate in the bone of *S. hispidus* collected from sites in Texas contaminated with elevated amounts of metals in the soil. Hausbeck (1994) observed that elevated levels of zinc varied seasonally, lead was not bioaccumulated, and Cd was the only metal that consistently showed increased levels of accumulation in *P. leucopus* from MM when compared to animals from LCB and EWMA, but we were unable to determine tissue concentrations for *S. hispidus* from the same sites. Husby et al. (1999) and Husby and McBee (1999) compared three different measures of genetic toxicity in *P. leucopus at* these sites. They found no significant differences in frequency of chromosomal aberrations or CVs for G_1 peaks but did find that *P. leucopus* from MM had significantly longer mean DNA strand lengths compared to both reference sites.

Cadmium generally shows highest levels of accumulation in liver and kidney and can induce metallothioneins, low-molecular weight compounds involved in detoxification, in those tissues (Habeebu et al. 1998). Topolska et al. (2004) found no significant sex effect on the uptake of Cd in spleen tissues of bank voles (*Clethrionomys glareolus*). Pereira et al. (2006) reported that severe histological changes associated with acute cadmium exposure, including apoptosis and necrosis, were not present in mice and rats chronically exposed to a mixture of heavy metals, including cadmium, at an abandoned mine area.

Selection for genetically tolerant individuals from the chronically exposed MM population may be occurring, which would allow tolerant animals and their progeny to survive and thus present no detectable effects (Wickliffe et al. 2000). Additionally, new animals from adjoining areas may have migrated to this area and have not been exposed to contaminants long enough to exhibit effects (Wickliffe et al. 2000). Furthermore, other tissues may respond more dramatically than spleen to exposure to metals (Whittier and McBee 1999). Further tests using flow cytometry may need to include comparisons of cells in the S and G_2 stages of the cell cycle as well as percent cells in G_1 and G_2 to increase the statistical sensitivity of the analysis (Whittier and McBee 1999).

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REFERENCES

Bickham JW, Sawin VL, Burton DW, McBee K (1992) Flow cytometry analysis of the effects of triethylenemelamine on somatic and testicular tissue of the rat. Cytometry 13: 368-373

Bickham JW, Sawin VL, McBee K, Smolen MJ, Derr J (1994) Further flow cytometric studies of the effects of triethylenemelamine on somatic and testicular tissue of the rat. Cytometry 15: 222-229

Biradar DP, Rayburn AL (1995) Chromosomal damage induced by herbicide contamination at concentrations observed in public water supplies. J Environ Qual 24: 1222-1225

Bueno AMS, Agostini JMS, Gaidzinski K, Moreiri J, Brognoli S (1992) Frequencies of chromosomal aberrations in rodents collected in the coal-field and tobacco culture region of Criciuma South Brazil. J Toxicol Environ Health 36: 91-102

Caire W (1989) Phsyiognomic regions of Oklahoma. In: Caire W, Tyler JD, Glass BP, Mares MA, Mammals of Oklahoma, University of Oklahoma Press, Norman, Oklahoma, p 12

Cameron GN, Spencer SR (1981) Sigmodon hispidus. Mammalian Species 158:1-9

Dudley RE, Svoboda DJ, Klaassen CD (1984) Time course of cadmium-induced ultrastructural changes in rat liver. Toxicol App Pharmacol 76: 150-160

Haase H, Watjen W, Beyersmann D (2001) Zinc induced apoptosis that can be suppressed by lanthanum in C6 rat glioma cells. Biol Chem 382: 1227-1234

Habeebu SSM, Liu J, Klaassen CD (1998) Cadmium-induced apoptosis in mouse liver. Toxicol Appl Pharmacol 149: 203-209

Hausbeck JS (1994) Trace metal residues in soils and tissues of *Peromyscus leucopus* from abandoned coal stripmines. MS thesis. Oklahoma State University, Stillwater, OK, USA

Husby MP, Hausbeck JS, McBee K (1999) Chromosomal aberrancy in white-footed mice (*Peromyscus leucopus*) collected on abandoned coal strip mines, Oklahoma, USA. Environ Toxicol Chem 18: 919-925

Husby MP, McBee K (1999) Nuclear DNA content variation and double-strand DNA breakage in white-footed mice (*Peromyscus leucopus*) collected from abandoned strip mines, Oklahoma, USA. Environ Toxicol Chem 18: 926-931

Johnson FL, Gibson DJ, Risser PG (1982) Revegetation of unreclaimed coal strip mines in Oklahoma. I. Vegetation structure and soil properties. J Appl Ecol 19:453-463

Lamb T, Bickham J, Gibbons W, Smolen M, McDowell S (1991) Genetic damage in a population of slider turtles (*Trachemys scripta*) inhabiting a radioactive reservoir. Arch Environ Contam Toxicol 20: 138-142

Landis WG, Yu M-H (1998) Environmental toxicology: impacts of chemicals upon ecological systems, Sec. Ed. CRC Press LLC, Boca Raton, FL

Lochmiller RL, McMurry ST, McBee K, Rafferty DP, Lish JW, Qualls Jr CW (1999) Seasonal hepatic cytochrome P-450 induction in cotton rats (*Sigmodon hispidus*) inhabiting petrochemical waste sites. Environ Pollut 105: 191-196

McBee K, Bickham JW (1988) Petrochemical-related DNA Damage in wild rodents detected by flow cytometry. Bull Environ Contam Toxicol 40: 343-349

McBee K, Bickham JW, Brown KB, Donnelly KC (1987) Chromosomal aberrations in native small mammals (*Peromyscus leucopus* and *Sigmodon hispidus*) at a petrochemical waste disposal site: I. Standard Karyology. Arch Environ Contam Toxicol 16: 681-688

McMurry ST, Lochmiller RL, Chandra SAM, Qualls Jr CW (1995) Sensitivity of selected immunological, hematological, and reproductive parameters in the cotton rat (*Sigmodon hispidus*) to subchronic lead exposure. J Wildlife Diseases 31: 193-204

Migula P, Czapla B, Kuczynski M, Kucab D, Polowczyk J (1985) Resting metabolism rate in mice after cutaneous poisoning with zinc, cadmium, and lead. Abstract Prace Naukowe Uniwersytetu Slaskiego w Katowicach 707: 87-102

Peakall D (1992) Animal biomarkers as pollution indicators. Chapman and Hall, London

Pereira R, Pereira ML, Riberiro R, Goncalves F (2006) Tissue and hair residues and histopathology in wild rats (*Rattus rattus* L.) and Algerian mice (*Mus spretus* Lataste) from an abandoned mine area (Southeast Portugal). Environ Pollut 139: 561-575

Punshon T, Gaines KF, Jenkins RA (2003) Bioavailability and trophic transfer of sediment-bound Ni and U in a southeastern wetland system. Arch Environ Contam Toxicol 44: 30-35

Randolph JC, Cameron GN, Wrazen JA (1991) Dietary choice of a generalist grasslands herbivore S*igmodon hispidus*. J of Mammalogy 72: 300-313

Satoh M, Koyama H, Kaji T, Kito H, Tohyana C (2002) Perspectives on cadmium toxicity research. Tohoju J Exp Med 196:23-32

Schroder JL, Basta NT (2003) Ecotoxicological risks associated with land treatment of petrochemical wastes. I. Residual soil contamination and bioaccumulation by cotton rats (*Sigmodon hispidus*). J Toxicol Environ Health, Part A 66:305-325

Shaw-Allen PL, McBee K (1993) Chromosome damage in wild rodents inhabiting a site contaminated with Aroclor 1254. Environ Toxicol Chem 12: 677-684

Thompson RA, Schroder JL, Connor TH (1988) Chromosomal aberration in the cotton rat, *Sigmodon hispidus*, exposed to hazardous waste. J Environ Molec Mutagen 11:359-367

Topolska K, Sawicka-Kapusta K, Cieslik E (2004) The effect of contamination of the Krakow region on heavy metals content in the organs of bank voles (*Clethrionomys gareolus*, Schreber, 1780). Polish J Environ Stud 13:103-109

Venugopal B, Luckey TD (1978) Metal toxicity in mammals. 2: chemical toxicity of metals and metalloids. Plenum Press, New York
Whittier JB, McBee K (1999) Use of flow cytometry to detect genetic damage in mallards dosed with mutagens. Environ Toxicol Chem 18:1557-1563
Wickliffe JK, Pitts RM, Iverson S, Bickham JW (2000) Cell cycle disruption in wild rodent populations as an endpoint in detecting exposure and effect. Bull Environ Contam Toxicol 64: 448-454