GENETIC DAMAGE IN *PEROMYSCUS LEUCOPUS* COLLECTED FROM ABANDONED STRIP MINES IN EASTERN OKLAHOMA

By

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Bachelor of Science

Oklahoma State University

Stillwater, Oklahoma

1991

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 1995

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ACKNOWLEDGMENTS

My sincere thanks go out to my major advisor, Dr. Karen McBee, for her constructive guidance, for allowing me the freedom to explore my own interests and make mistakes, and for sparking my interest in mammals and evolutionary biology. I would also like to thank Dr. Marsha Black for serving on my committee, allowing me to use her laboratory, and for her guidance in setting up the electrophoretic strand break assay. Dr. Ulrich Melcher also served on this committee even though he is extremely busy with other commitments. It was his course in molecular genetics that sparked many of my interests outside of this thesis.

Lab partners John Hausbeck and Corinne McMillen provided a great deal of assistance in the field and the lab. They, along with Barbra Bowers, became very good friends over the course of completing this thesis. Their support has been invaluable. Amy Ferguson provided moral support and an extra pair of hands in the lab on several occasions. My parents, Patty and Paul Husby, provided understanding, encouragement, and advice when I needed it. Finally, I would like to thank the Department of Zoology and especially Dr. Jerry Wilhm, who accepted me to the program.

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CHAPTER 1

INTRODUCTION

This chapter is meant to explain the broad questions addressed by this thesis and to tie the remaining chapters together. Chapters two and three contain the site descriptions, specific study objectives, field and laboratory methods, results, and discussion. They will be submitted for publication in their present form.

Overview

One goal of environmental toxicology is to develop a battery of biomarker assays for assessing the impact of environmental contamination (Brusick, 1980). Due to cost, time, and equipment requirements, it is unrealistic and often redundant to perform several assays in every investigation. Instead, the most informative endpoints to be studied should be identified, and only the appropriate tests performed. To do this, it is imperative to understand several things: how specific contaminants affect specific endpoints; the relationships among endpoints measured by several different assays; and the relative sensitivities of the tests used.

This work represents one portion of a larger in situ investigation which involves the use of tissue residue analysis and various genetic and morphometric endpoints to evaluate Peromyscus leucopus (white-footed mice) that have inhabited orphaned colliary strip mines. The data presented in the following chapters represent the genetic portion of this larger investigation. This portion of the project is an attempt to measure differences in responsiveness among three assays of genetic disruption. Those three

assays are the examination of standard somatic metaphase spreads for chromosomal aberrations, measurement of total nuclear DNA content using flow cytometry, and measurement of double-stranded DNA breaks by a gel electrophoretic strand break assay.

Just as understanding the relationships of genetic endpoints is important for developing a battery of genetic tests, the interrelationships of genotoxicity tests to other toxicological endpoints is key to the development of a broad-based battery of tests. The animals in this study underwent these three assays of genetic disruption plus tissue residue analysis and a morphometric analysis. Ultimately, data from each of these classes of assays will be analyzed for statistical correlations. Such correlations among indicators at different levels of biological complexity will help in understanding not only the action of toxicants at the genetic level, but also how genetic disruptions may be translated into gross morphological disruptions.

The hypothesis to be tested here is that the amount of genetic damage measured by the chromosomal aberration assay (CAA), flow cytometry (FCM), and the electrophoretic strand break assay (SBA) will be significantly greater in animals from contaminated sites when compared to animals from uncontaminated reference sites. A second hypothesis is that these assays are significantly correlated with each other. These three assays represent three tiers of genetic damage: SBA at the molecular level, CAA at the chromosome level, and FCM at the nuclear level. In theory, exposure to a clastogen (an agent that causes chromosomal aberrations) should produce breaks in the DNA molecule which may be translated into chromosome aberrations. Alternatively, the genotoxic chemicals may inhibit the normal repair mechanisms which maintain DNA integrity. Some chromosome aberrations may cause an unequal distribution of

DNA from the parent cell to daughter cells, a situation that should be detected by FCM. By using these assays together the genetic level at which the toxicant acts may be identified.

Exposure to many kinds of metals results in some form of genotoxicity, but effects of different metal pollutants may differ from organism to organism, especially at higher taxonomic levels. Even within an organism, a given metal may differentially affect certain tissues. In addition to organismal and histological differences, the methods used to detect cellular-level responses to metal exposure may produce varying conclusions. For example, cells may respond to a mutagen differently in culture than they do as part of a living organism (Hartwig et al., 1990) and may respond differently to different culture media receiving the same treatment. This should be kept in mind when comparing studies employing cell culture to ones where animals are exposed in vivo. Likewise, an animal dosed in a laboratory may respond differently than it would in the wild (Shaw-Allen and McBee, 1993). These factors should be noted when reviewing the literature on metal genotoxicity, and may account for some of the conflicting data on the genotoxicity of specific metals. Results from one study may be very specific to the methods used and should only be extrapolated to other situations with caution.

The genotoxic effects of metals have been studied in organisms which vary widely in complexity. In mammalian systems, symptoms of high-level metal exposure are directly related to the concentration used, the duration of treatment, and the mode of uptake by the organism. Although the data are not at all conclusive, several metal salts have been shown to be clastogens in mammalian bone marrow tests (Sharma and Talukder, 1987).

Metals combine with cell organelles, macromolecules, and metabolites. They can affect the permeability of cell membranes, which can disturb energy metabolism. Proteins which bind to metals may be denatured, precipitated, allosterically altered, and may bind to nucleic acids to alter nucleoprotein conformation (Sharma and Talukder, 1987). The metals focused on in this study are cadmium, lead, zinc, and copper; each one was found in significantly higher concentrations in the soils of the strip mines compared to reference site soils (Hausbeck, 1994). Cadmium is of particular interest because it, along with the essential metal zinc, was found to be bioaccumulated in significantly higher concentrations in animals collected from the mines compared to animals collected from reference sites (Hausbeck, 1994). Lead and copper levels were not significantly higher in the mine animals (Hausbeck, 1994).

Cadmium, a non-essential element, is an extremely toxic metal (Friberg et al., 1986). It is most well know for its effect on the testes, prostate, and lungs of animals. The absence of metallothionein in those tissues may render them more susceptible compared to other tissues. Mammalian cell tests give conflicting results on the genotoxic properties of cadmium. Not only is it unclear whether cadmium has any clastogenic properties, there are also conflicting data on whether cadmium itself induces chromosomal lesions, or if its clastogenicity is manifested through co-clastogenic effects (Tang et al., 1990). Sharma and Talukder (1987) point out that early results using $CdCl_2$ in in vivo mammalian systems produced no evidence that cadmium is clastogenic. Recently, however, several investigators have reported increased numerical aberrations (2n +/- one or more chromosomes) (Selpes et al., 1992; Shimida et al., 1976) and a few have reported structural aberrations due directly or indirectly to cadmium exposure (Hartwig et al., 1990; Tang et al., 1992; Yamanda et al., 1993).

Micronucleus tests on mice injected with a variety of suspected spindle poisons produced negative results for $CdCl_2$ (Adler et al., 1991). Yamanda et al. (1993) reported that cadmium alone was not clastogenic in cultured mammalian cells, but that it was a co-clastogen when administered with mitomycin C and 4-nitroquinoline 1-oxide. This supports the notion that Cd itself does not induce DNA structural damage, but it inhibits DNA repair and replication mechanisms. Hartwig and Bayersmann (1989) found similar co-clastogenic properties of Cd when administered in conjunction with UV light. Repair inhibition and DNA synthesis inhibition have been reported to be the direct result of cadmium exposure (see Yamada et al., 1993 and references therein).

Selypes et al. (1992) found that numerical chromosome aberrations were significantly higher in the testes of mice treated with Cd in vivo. Trisomy and monosomy were found most often, but diploid, triploid, and tetraploid meiotic cells were also reported. Sbrana et al. (1993) also found a weak spindle effect in cultured human lymphocyte cells. These and other reports of numerical aberrations and aneuploidy caused by Cd exposure suggest that it may act as spindle poison when administered by itself, but not as a clastogen. The spindle effect, however, has not been found consistently. Albertini (1990) tested several known and suspected spindle poisons in yeast and found that $CdCl_2$ did not induce any chromosomal malsegregation.

Howard et al. (1991) reported that Cd causes structural chromosome aberrations in cultured Chinese hamster ovary (CHO) cells. This result is consistent with several studies of lymphocytes from cadmium- exposed industrial workers, although not all studies of human exposure have given a positive result (see references in Tang et al., 1990). Positive results from occupationally exposed humans must be carefully interpreted because most industrial processes involving cadmium also expose workers to

zinc and lead. The problem of determining the effects of several metals in combination is discussed later in this chapter.

Lead is the other nonessential metal focused on in this study. Some in vivo tests have shown that lead causes chromosome breaks, dicentric chromosomes, ring chromosomes, and translocations (Sharma and Talukder, 1987). Chronic oral administration of lead nitrate to mice decreases mitotic index. Lead acetate injected chronically and acutely in rats consistently induces high numbers of chromosome aberrations; prolonged oral exposure produces the same result but has little effect on the spindle apparatus (Sharma and Talukder, 1987). Blakey et al. (1992) found that the organolead compound triethyllead acetate was a strong clastogen when administered to CHO cells in culture. Hartwig et al. (1990) found that lead acetate administered to HeLa cells does not induce DNA strand breaks, but that it does inhibit the repair of UV-induced DNA damage. A likely mechanism for lead-induced repair inhibition is alteration of enzymes involved in the repair process (Hartwig et al., 1990), much like the mechanism proposed for cadmium (Yamanda et al., 1993). Deknudt and Deminatti (1978) reported in a study on human lymphocytes that neither lead acetate nor cadmium chloride alone were clastogenic, but that zinc chloride was. Gasiorek and Bauchinger (1981) in a similar study also found lead not to clastogenic, but reported that zinc had no effect while cadmium was a clastogen.

Zinc is of interest because it is essential in low levels and toxic at higher levels. A deficiency of zinc has severe effects on growth, reproductive performance in both sexes, fetal brain development (Casey, 1980), and the maintenance of pregnancy (Apgar, 1970). It is essential for normal DNA polymerase activity and protein synthesis (Casey, 1980) and is a requirement for the activation of several enzymes (Parisi and Vallee,

1969). In excess, zinc has a wide range of detrimental health effects. Excessive Zn in weanling pigs causes arthritis, extensive brain hemorrhages, depressed growth, and increased mortality (Brink et al., 1959). Gasaway and Buss (1972) found that food intake and body weight in mallard ducks decreases as dietary zinc increases.

Herich (1969) suggests that zinc plays a role in spindle formation and is therefore an important component of cell division. Spindle poisoning is common following metal treatment. The destruction of spindle fibers can lead to an imbalance in the separation of chromosomes, which accounts for alterations in the number of chromosomes per cell (Sharma and Talukder, 1987).

Zinc is a post-transition metal that binds directly to nucleotides and inhibits enzyme activity. Zinc acetate caused chromosome aberrations in human lymphocyte cultures, and zinc chloride caused dicentrics at low concentrations along with reduced mitotic index due to spindle disturbances (Sharma and Talukder, 1987). Like cadmium and lead, the literature on zinc gives conflicting data, and no clear conclusions about its clastogenicity can be drawn. It is generally considered less toxic than either of the nonessential metals described above because homeostatic mechanisms are already in place to regulate its concentration.

Like zinc, copper is essential, but in excess is toxic. Copper has the vital function of binding with the phosphate group on nucleotides. Its toxicity is the least understood of the four metals discussed here. The observation by Sideris et al. (1981) that high concentrations of Cu interfere with the in vitro synthesis of DNA suggests that it is a potential mutagen when found in extreme excess. It is believed to inhibit DNA synthesis, but the specific molecular mechanisms are unclear at this point (Sharma and Talukder, 1987). Swiss albino mice exposed to copper sulfate exhibited

significant increases in chromosomal aberrations compared to control mice (Agarwal et al., 1990). Cu compounds in animal cell culture studies have also been implicated as genotoxic substances. As an essential metal, Cu concentrations are regulated by homeostatic mechanisms. In humans with Wilson's disease, a mutation in a gene on chromosome 13 renders patients unable to metabolize copper, and accumulate toxic levels of the compound (Brewer et al., 1990). A similar situation occurs in canines (Brewer et al., 1990).

The current treatment of Wilson's disease is to administer low levels of zinc acetate (Brewer et al., 1990). The zinc induces production of metallothionein, which can then bind the copper ions and block its absorption (Brewer et al., 1990). Likewise, long-term exposure to zinc, cadmium, or copper at subtoxic levels causes the organism to become tolerant to subsequent high-level exposures; this is due to an induced response in metal-metabolizing proteins. The acquisition of tolerance to high doses of specific metals by prolonged exposure to low levels of that or another metal can occur in one of two ways (Roesijadi, 1992). Either prolonged exposure produces a very large pool of metallothionein, or all the pathways needed to produce the protein are already mobilized at the time of high exposure. It may be that both mechanisms contribute to the aquisition of tolerance.

These complex interactions make it difficult for investigators studying one particular metal in the environment because a specific metal is almost never found apart from other metal ores at contaminated sites or industrial facilities. One must always wonder if positive results are due to the action of several metals in combination and if negative results are the result of tolerance due to prolonged low-level exposure to that metal or another metal present. This is one of the problems with interpreting the

results of this study. The soils at the mine sites are complex mixtures containing varying levels of several heavy metals in combination. This problem can be eliminated if one confines the study to laboratory conditions where controlling several variables is relatively easy. What is lost in a lab setting, however, is the assurance that results observed in the lab can be extrapolated to "real world" situations.

Justification

In situ investigations offer information about toxic materials that is unattainable by conventional laboratory studies. Environmental conditions such as the mode of uptake, the cumulative effect of all physical and chemical variables, and seasonal effects make in situ investigations unrepeatable in laboratory settings. Potential pollutants of interest must be studied in the context of the soil composition and pH, sunlight, moisture, temperature, buffering capacity, and other conditions (Babich and Stotzky, 1983). Based solely on in vivo lab test results, claims of potential health hazards are not justifiable (Brusick, 1980). Several genetic biomarker assays have been proposed for in situ evaluations of environmental contaminants. Various tests measure different endpoints, therefore a battery of tests may be needed to match specific situations. In an effective battery, tests should overlap in agreement, yet each test should measure a unique endpoint to avoid needless redundancies (Brusick, 1980). For this reason, it is desirable to develop a better understanding of several biomarker assays.

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CHAPTER 2

CHROMOSOMAL ABERRATIONS IN PEROMYSCUS LEUCOPUS COLLECTED ON ABANDONED COAL STRIP MINES

ABSTRACT

This study was undertaken to determine the genetic consequences of long-term exposure to heavy metal pollution in small mammals. A secondary goal was to contribute toward the process of validation of chromosomal aberration analysis as an in situ biological monitoring tool. During the spring, summer, and fall of 1992, white-footed mice (Peromyscus leucopus) were collected from four metal-polluted, abandoned coal strip mines and three uncontaminated reference sites in eastern Oklahoma. Chromosomal aberrations were scored from standard bone marrow metaphase chromosome spreads. Seasonal differences were detected for aberrant cells (cells containing one or more chromosomal lesion) per individual ($P = 0.0004$), but there were no differences among sites or between sexes. Males and females were tested separately for lesions per individual, and neither sex showed a significant difference among sites, but both showed seasonal differences across sites. Finally, a chi-square analysis showed that the difference between total lesions and aberrant cells was not significantly different among sites ($P = 0.635$), indicating that lesions were distributed among cells in approximately the same way among all sites.

Keywords: cadmium, zinc, clastogen, chromosome aberrations, biomonitoring, rodents

INTRODUCTION

Heavy metal pollution from mining, smelting, and other industrial processes is an increasing environmental concern. Such pollution is often associated with strip mining, which began in Oklahoma around 1920 [1]. The removal of overburden by strip-mining results in alternating, parallel spoil piles and valleys with previously unexposed soils on the surface. These soils have different properties than the original top soil, often including higher heavy metal concentrations and altered pH.

The State of Oklahoma enacted a reclamation act in 1971, but approximately 12,000 ha of 10- to 70-yr-old surface mines remain unreclaimed in the state [1]. Several studies of abandoned strip mines have focused on revegetation and soil properties, but no work has been done on the environmental impact of strip-mine pollution on the mammals of Oklahoma. Johnson et al. [1] took soil samples at 49 strip-mine sites in the Oklahoma coal belt. On average, they found subsurface and surface soils at the abandoned mine sites to be more acidic (pH = 4.6) and contain higher levels of zinc than nearby forest soils (pH = 5.5). Lead, copper, and magnesium levels in mine soils averaged similar concentrations as those in forest soils. Hausbeck [2] took soil samples from the three reference sites and three of the four strip mines used in this study. He found significantly elevated levels of zinc at the two mines in Okmulgee Co. (Hamilton and Marler mines) when compared to the reference site in that county. The Craig Co. mine (Wayland mine) did not differ from its matched reference site or from the Okmulgee Co. reference site. All sites in Okmulgee and Craig counties differed significantly from the Payne Co. reference site, the only study site outside the coal belt. Peromyscus leucopus, including specimens from this study, living on the mines did not

show excessive hepatic or renal bioaccumulation of copper or lead [2]. Bioaccumulated zinc levels in liver and kidney were elevated at contaminated sites during certain seasons. Cadmium levels in both tissues were consistantly higher in mice from contaminated sites compared to ones from less contaminated sites.

Although there is a great deal of conflicting data [3], it is apparent that one toxic effect of zinc [4] and cadmium [5] is to increase chromosomal lesions [also see 6,7]. Studies which address the genotoxic effects of exposure to complex mixtures of metals by wild mammals are lacking. Bueno et al. [8] found that compared to conspecifics from uncontaminated reference sites, rodents (Oryzomys sp. and Akodon cursor) collected from coal-field polluted areas had significantly higher percentages of cells containing an aberrant chromosome and significantly higher numbers of total chromosome aberrations. It appears from this and other in situ investigations involving a variety of non-metal pollutants [9-12] that scoring chromosomal aberrations is an effective way to estimate the genotoxic effect of environmental contaminants on mammalian populations.

Feral rodents, especially P. leucopus, have been used effectively as bioindicators [13,14]. McBee et al. [9] and McBee and Bickham [10] found that individuals of P. leucopus and Sigmodon hispidus trapped at a petrochemical waste site had increased frequencies of chromosomal lesions and increased variation in nuclear DNA content when compared to conspecifics from pristine sites. Tice et al. [15] found P. leucopus to be highly suitable for detecting hazardous levels of genotoxic/cytotoxic pollutants because of their relative sensitivity to such pollutants and their ability to inhabit heavily impacted areas, even when optimal habitat is not available. Peromyscus leucopus typically resides in the cover of bushes or wooded areas. Diet consists primarily of nuts and seeds, but insects are also eaten [16,17]. Nests are often found in

trees, underground burrows, logs, and tree hollows. Frequent exposure to the soil through burrowing, nest building, and direct ingestion while eating and grooming, make P. leucopus a well-suited bioindicator species.

In this study, chromosomal aberrations in P. leucopus were scored to determine the clastogenic effects of strip-mined soils, which contain elevated levels of heavy metal contamination compared to background levels found at reference sites [2]. Mice collected from abandoned mines were compared to mice collected from unmined reference sites. Parameters of cytogenetic aberrancy were compared among four abandoned mine sites and three reference sites. Animals were collected during three seasons (spring, summer, and fall) in 1992 to identify differential responses within sites across seasons. The hypothesis is that mice from mine sites have a higher mean number of chromosomal lesions and cells containing a damaged chromosome than animals from reference sites.

METHODS

Peromyscus leucopus were collected during March, July, and October of 1992. All mice were trapped with Sherman live-traps baited with oats and peanut butter. Traps were set in the evening in wooded areas that typify P. leucopus habitat and animals were collected in the morning. Weight, total length, length of tail, length of hind foot, length of ear, and reproductive condition was recorded for each animal. With two exceptions, each collecting period comprised collections from seven sites. One reference site was located outside the Oklahoma coal belt in Payne Co. Mice were collected from this site during the summer and fall, but not spring. Two mine sites and one reference site were located in each county chosen for this study. Both Okmulgee and Craig counties are located in the coal belt.

In all three seasons subsamples of five adult males and five adult females from each site were chosen at random. Trapping success at the Moss mine (Craig Co.) was so poor (2 males and 4 females) in the fall that it was dropped from the analysis. Because larger samples were needed for morphometric analysis, the number of animals actually collected often greatly exceeded the subsamples chosen for chromosome analysis. Animals with grey pelage were noted as juveniles in the field and excluded from the analysis as were the ones weighing under 18 grams. Complete skeletons, minus the femurs which were used for metaphase spread preparation, were saved and deposited at The Museum, Texas Tech University. Animals were put into age classes [18], however these data were not available at the time subsamples were chosen for chromosome analysis, so the weight and pelage criteria were the only means available for excluding juveniles.

Each of the chosen mine sites was over 65 years old [1] and had naturally revegetated since abandonment. Similar florae were found at all the trapping sites. Vegetation is characteristic of oak-hickory savannah with grasslands alternating with woodlands [19]. Woodlands in this area are dominated by blackjack and post oaks (Quercus marilandica and Q. stellata), sumac (Rhus glabra), and cedar (Juniperus virginiana).

The Okmulgee Co. strip mines are referred to as Hamilton mine and Marler mine. Their corresponding reference site was located at the Eufaula Wildlife Management Area. The soils from Oklmugee Co. mines were found to contain 130 to 163 ppm Zn [1]. Hausbeck [2] found soils from Hamilton mine, Marler mine, and the reference site to have pH values of 4.08, 4.97, and 5.25 respectively. The Craig Co. mines are referred to as Moss and Wayland mines and are matched with a privately held pristine reference site. Soils from Craig Co. mines contained 40 to 46 ppm Zn [1]. No soil pH data are available for Moss mine, but the Wayland mine had a soil pH of 5.39 and the Craig Co. reference area was 6.03. One reference site outside the Oklahoma coal belt in Payne Co. had soil pH of 5.43.

The method for metaphase chromosome spread preparation followed closely that of Baker et al. [20]. All animals were sacrificed within 48 hours of capture to reduce any effects of prolonged captivity. Bone marrow was flushed from both femurs with warm hypotonic solution (0.075 M KCl), aspirated, and incubated at 37 °C for 27 minutes. Incubated cells were centrifuged out of the supernatant at 400 rpm for 90 seconds to form a pellet of cells (IEC Clinical Centrifuge). All but about 0.5 ml of supernatant remaining above the pellet was then gently removed and discarded, and cells were resuspended in the remaining 0.5 ml of KCl. Carnoy's fixative (3:1

methanol/glacial acetic acid) was then added, the suspension gently mixed, and again centrifuged. The entire supernatant was removed and replaced with fresh fixative before another resuspension. This step was repeated three more times before slide preparation. A few drops of the final cell suspension were dropped onto clean, dry, labeled slides and immediately ignited and allowed to dry.

Slides were stained in a 2% Giemsa-phosphate buffer solution for 5-7 minutes and subsequently rinsed with distilled water and allowed to dry. Prepared slides were number coded and examined in random order to ensure that the origin of the specimen was unknown while being scanned. For each collecting period, metaphase spread preparations of five males and five females from each site were randomly chosen for chromosome analysis. Fifty metaphase spreads per individual were scored for six types of lesions: chromatid breaks, chromosome breaks, ring chromosomes, dicentric chromosomes, translocation figures, and acentric fragments. Additionally, the total number of lesions out of the 50 cells per individual and the total number of aberrant cells (cells with any type of lesion) per individual were recorded.

The mean number of aberrant cells per individual and mean number of lesions per individual were compared by rank transforming the data and performing a three-way analysis of variance (ANOVA) with sex, site, and season as factors. Factors which showed significance were then compared by least significant difference (LSD) multiple comparisons. Because a fairly significant interaction ($P = 0.0809$) between sex and season was detected in mean number of lesions per individual, separate ranked ANOVA tests were performed for each season to determine which season(s) was the sourse of the sex by season interaction. Males and females were then tested separately as well. Sex never interacted significantly with site for either dependent variable. A

chi-square test was performed on sites, assuming no interaction with season or sex, for the mean difference between lesions per individual and aberrant cells per individual. The difference between mean lesions per individual and mean aberrant cells per individual reveals how aberrations are distributed among the cells of individuals. It may be considered worse to have lesions spread out evenly among cells (i.e. lesions $\approx$ aberrant cells) than to have lesions concentrated in just a few cells (lesions >>> aberrant cells) because cells with excessive damage are less likely to survive and divide.

RESULTS

Of the 480 lesions scored in 444 aberrant cells, 97.3 % were either chromatid breaks (66.88 %), acentric fragments (23.96 %), or chromosome breaks (6.46 %). These results are almost identical (67 %, 25 %, and 6 %) to those reported by Shaw-Allen [21] for P. leucopus collected from an uncontaminated reference site in Oklahoma. Ring chromosomes, dicentric chromosomes, and translocation fragments together comprised the final 2.7 %, a percentage so low that those classes were of little statistical value by themselves. The mean number of lesions in each class for each season by site combination is presented in Table 1.

The mean percent aberrant cells per individual for each site by season combination is shown in Fig. 1. The three-factor ANOVA on ranks of aberrant cells per individual revealed that season was a significant factor ($P = 0.0004$), but site, sex, and all interaction terms were not significant. Pairwise seasonal LSD comparisons, averaging over sites and sexes, showed all three seasons to be significantly different from each other, with summer having the highest mean (6.0 %) followed by fall (4.8 %) and spring (3.4 %). These values are higher than those reported by Shaw-Allen [12] for reference animals (1.727 %) or a PCB-contaminated site (1.833 %), however they are comparable to those reported by McBee [9] for P. leucopus from two reference sites (2.83 % and 3.57 %) and a petroleum waste site (10.72 %) in Texas. Because site was not a significant factor, sites were not compared by pairwise LSD tests.

Mean lesions for 50 cells per individual in males and females are presented in Fig. 2 and 3. Three two-factor ranked ANOVA tests were used to compare lesions per individual within seasons due to a marginally significant interaction ($P = 0.0809$)

between season and sex. Only the ANOVA for fall showed that sexes were significantly different. When sexes were separated and tested for seasonal differences, females had the greatest number of lesions in the summer and fall, which were not significantly different from each other, but both were significantly higher than spring. Females averaged 1.680 lesions per 50 cells in spring, 3.321 in summer, and 3.286 in fall. Males averaged 2.240 in the spring, 3.103 in the summer, and 1.931 in the fall. The only significant difference for males was between the summer and fall seasons, with neither of them significantly different from spring. Finally, the chi-square analysis of the difference between lesions per individual and aberrant cells per individual among sites, averaged over sexes and seasons, showed that no site deviated from the expected difference ($P = 0.635$).

DISCUSSION

One of the complications in doing in situ biomonitoring is that a large number of uncontrolled variables can influence the outcome, thereby making it nearly impossible to pinpoint the specific cause(s) of the results. It is much easier to control for several variables (i.e. food and water availability, temperature, concentration of the dose) in a laboratory setting. In the environment, these and other variables can vary widely between sites and may have complex interactions. Thus, the negative side of *in situ* testing is that it is difficult to tease apart the variables that are responsible for observed responses. The positive side, which in many cases outweighs the negative side, is that the results tell what happens in the "real world." For the very reason that one does control so many variables in the lab, results are often not applicable to an environmental situation.

Hausbeck [2] found significantly higher mean concentrations of bioaccumulated zinc and cadmium in the kidneys and liver of the P. leucopus from the most contaminated sites, but levels of metals varied seasonally and there was a significant season by site interaction. In general, the seasonal trends for hepatic and renal Zn concentration resemble the pattern for chromosomal lesions. The most chromosome lesions and aberrant cells were found in summer-trapped animals, followed by those trapped in fall and spring. Spring animals averaged similar or lower hepatic and renal Zn concentrations compared to unexposed lab-reared Sigmodon hispidus [2], implying that exposure to some stressor in the environment may help lower Zn concentrations. The spring animals, trapped in early March, were just coming out of winter when cold and nutritional stress is most likely. Cold and nutritional stress will induce the

production of metallothionein, which binds and sequesters heavy metals (Roesijadi, 1992). This may account for the higher zinc levels in lab reared Sigmodon compared to Peromyscus from mines, however the comparison is highly speculative considering that two different species are being compared. I use the Sigmodon data here as a reference more than for direct comparisons. It is interesting that in summer, when cold, nutritional stress, and reproductive stress were minimized, Zn levels were the highest and all sites exceeded the level found in S. hispidus. For most sites, fall was intermediate between spring and summer and only one site (Payne Co. reference) fell below the S. hispidus value.

There is some uncertainty about the ability of these two metals to cause chromosome damage in mammals. Micronucleus tests on mice injected with $CdCl_2$ did not support the idea that cadmium is clastogenic when administered by itself [21]. More evidence suggests that Cd itself does not induce DNA structural damage, but that it works as a co-clastogen by inhibiting repair and replication mechanisms [22]. Howard et al. [23] reported that cadmium causes structural chromosome aberrations in cultured Chinese hamster ovary (CHO) cells. This result is consistent with several studies of cadmium-exposed industrial workers, although not all studies of human exposure have given a positive result [24]. Zinc, unlike cadmium, is an essential metal. It is required at low levels, but is toxic at high concentrations. Sharma and Talukder [6] found that zinc acetate caused structural chromosome aberrations in cultured human lymphocytes and zinc chloride caused dicentrics.

López-González and Owen [18] found that P. leucopus from the strip mine sites were significantly smaller and showed a higher degree of directional assymetry than those from reference sites when age was accounted for. Those results suggest there

may be a disruption in the genome of exposed mice at some other genomic level, such as the level of nucleotide substitution. The difference among exposure levels at the different sites may be too low to cause inter-site differences in the number of aberrant cells or number of lesions. Exposed animals also may have an induced metabolic response which allows them to maintain subclastogenic levels of heavy metals, even when they are taking up higher levels. Metallothioneins bind and excrete both essential and nonessential metals. In humans with Wilson's disease, a genetic disorder which prevents proper copper metabolism, zinc acetate administered at low levels helps prevent over-accumulation of copper by inducing the production of metallothioneins which can bind copper and block absorption [25]. Waalkes et al. [26] found that pretreatment with zinc or low doses of cadmium reduces the tumorigenic effect of cadmium. Zinc may also lessen the effect of cadmium exposure through competition for binding sites [27]. Long-term metal exposure at subtoxic levels in the environment may act as a treatment against the accumulation of genotoxic metal levels. The possibility also exists that animals at contaminated sites have undergone selection for resistant genotypes. Laboratory-exposed marine gastropods have shown selection for tolerance at specific loci and for multilocus complexes when exposed to cadmium and zinc [25-27].

Seasonal differences in aberrant cells per individual closely follow patterns in hepatic and renal Zn concentration in mice from all sites [2], but a causative relationship cannot be assumed. Seasonal differences in hepatic and renal metal concentrations may be due to differences in the availability of metals to be taken in, or due to fluctuations in metallothionein levels in these organs. Several environmental stressors, including cold temperature, nutritional stress, and water stress will induce increased metallothionein production which would help animals bind and excrete more

metals. This could explain partially why animals in the early spring had the lowest levels of zinc and the fewest aberrant cells, followed by fall and summer. Sex differences may be explained by differences in the resources put into reproduction. The highest percentage of pregnant females were caught in the fall season, and females had a significantly higher number of lesions compared to males for that one season. The data presented here emphasize the necessity of considering season and sex in biomonitoring. Certain pollutants may affect sexes differentially, and may cause detectable effects only during certain seasons. Data which do not extend across seasons and through different stages of the reproductive cycle may only be a snapshot of the health of an animal and may not be a good reflection of the overall effect of exposure to environmental contaminants.

ACKNOWLEDGMENTS

This project was funded by U.S. E.P.A grant R-818429-01-0 to K. McBee and R.D. Owen. Thanks to C.C. McMillen, R.D. Owen, C. Lopez-Gonzalez, and S. Kasper for helping to collect specimens. We thank M. Payton for statistical consultation. D.M. Leslie, Jr. and the Oklahoma Cooperative Fish and Wildlife Research Unit graciously provided field support. This project would not have been possible without the cooperation of the landowners and the Oklahoma Department of Wildlife Conservation and personnel of the Eufaula Wildlife Management Area.

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Table 1. Means and standard deviations of lesions per 50 cells for six types of lesions. Data are for P. leucopus collected from mined sites and reference sites during three seasons.

Site	Lesion Type					
	Chromatid breaks	Acentric fragments	Chromosome breaks	Dicentric chromosomes	Ring chromosomes	Translocation figures
			Spring 1992			
Okmulgee Co.						
Marler mine	1.80 (1.81)	0.40 (0.70)	0.40 (0.52)	0.00	0.30 (0.66)	0.00
Hamilton mine	1.00 (1.33)	0.40 (0.97)	0.10 (0.32)	0.10 (0.32)	0.00	0.00
Eufaula ref.	1.50 (0.97)	0.00	0.00	0.00	0.00	0.00
Craig Co.						
Moss mine	1.80 (1.14)	0.70 (0.82)	0.30 (0.48)	0.00	0.00	0.00
Wayland mine	1.40 (1.58)	0.20 (0.42)	0.10 (0.32)	0.10 (0.32)	0.00	0.00
Craig Co. ref.	1.50 (1.18)	0.30 (0.68)	0.10 (0.32)	0.00	0.00	0.10 (0.32)
Payne Co.						
Payne Co. ref.	-	-	-	-	-	-

Table 1 cont.

			Summer 1992			
Okmulgee Co.						
Marler mine	2.10 (2.54)	1.00 (0.82)	0.40 (0.70)	0.00	0.00	0.00
Hamilton mine	2.70 (2.16)	0.90 (0.88)	0.10 (0.32)	0.00	0.10 (0.32)	0.00
Eufaula ref.	2.60 (1.78)	0.80 (0.63)	0.30 (0.48)	0.00	0.10 (0.32)	0.00
Craig Co.						
Moss mine	1.90 (1.37)	1.10 (0.88)	0.10 (0.32)	0.00	0.00	0.00
Wayland mine	1.90 (1.37)	0.50 (0.53)	0.1 (0.32)	0.00	0.00	0.00
Craig Co. ref.	1.50 (1.18)	0.20 (0.57)	0.20 (0.42)	0.00	0.10 (0.32)	0.00
Payne Co.						
Payne Co. ref.	2.56 (1.33)	0.44 (0.73)	0.22 (0.44)	0.00	0.00	0.00

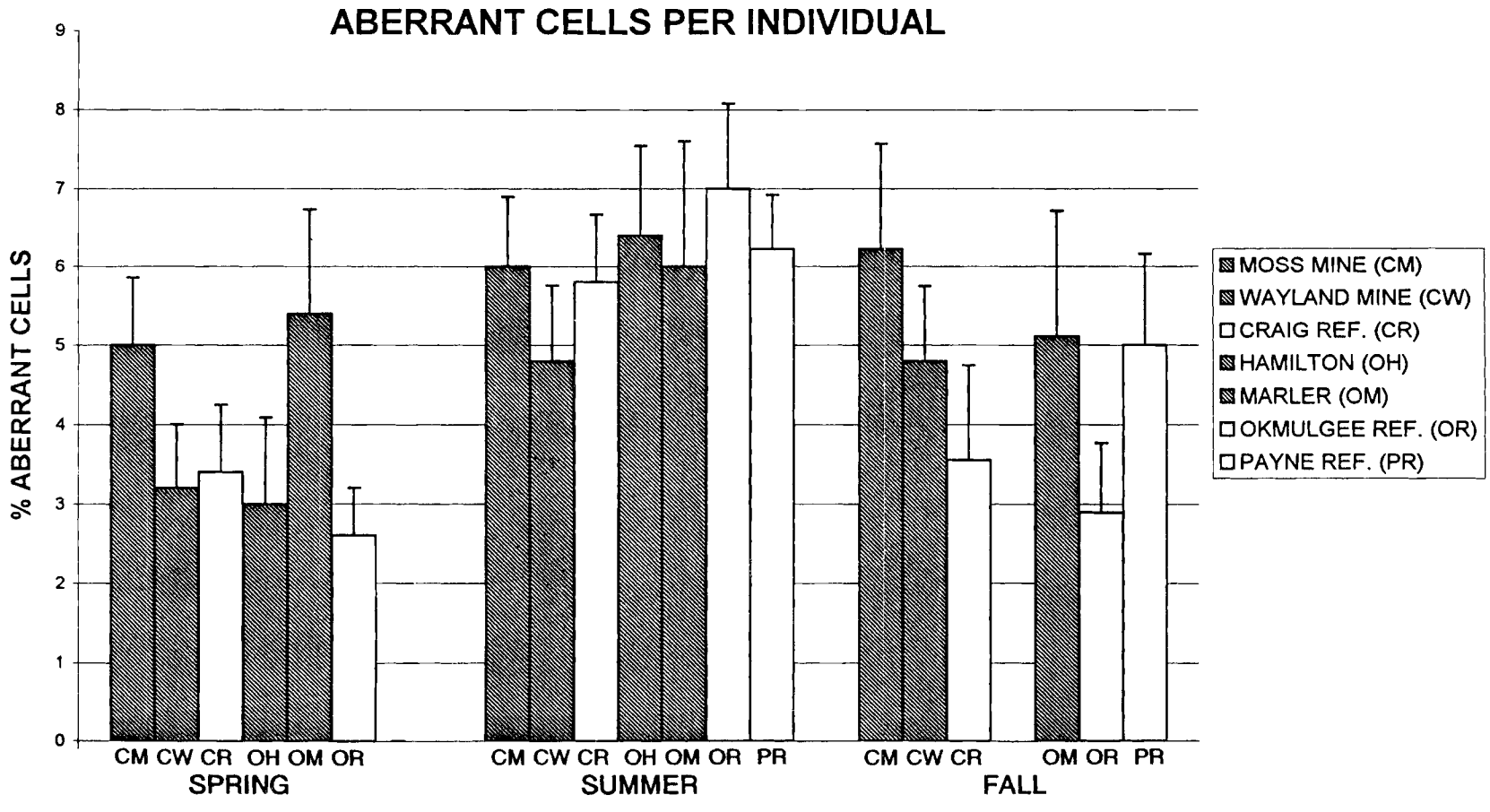
Table 1 cont.

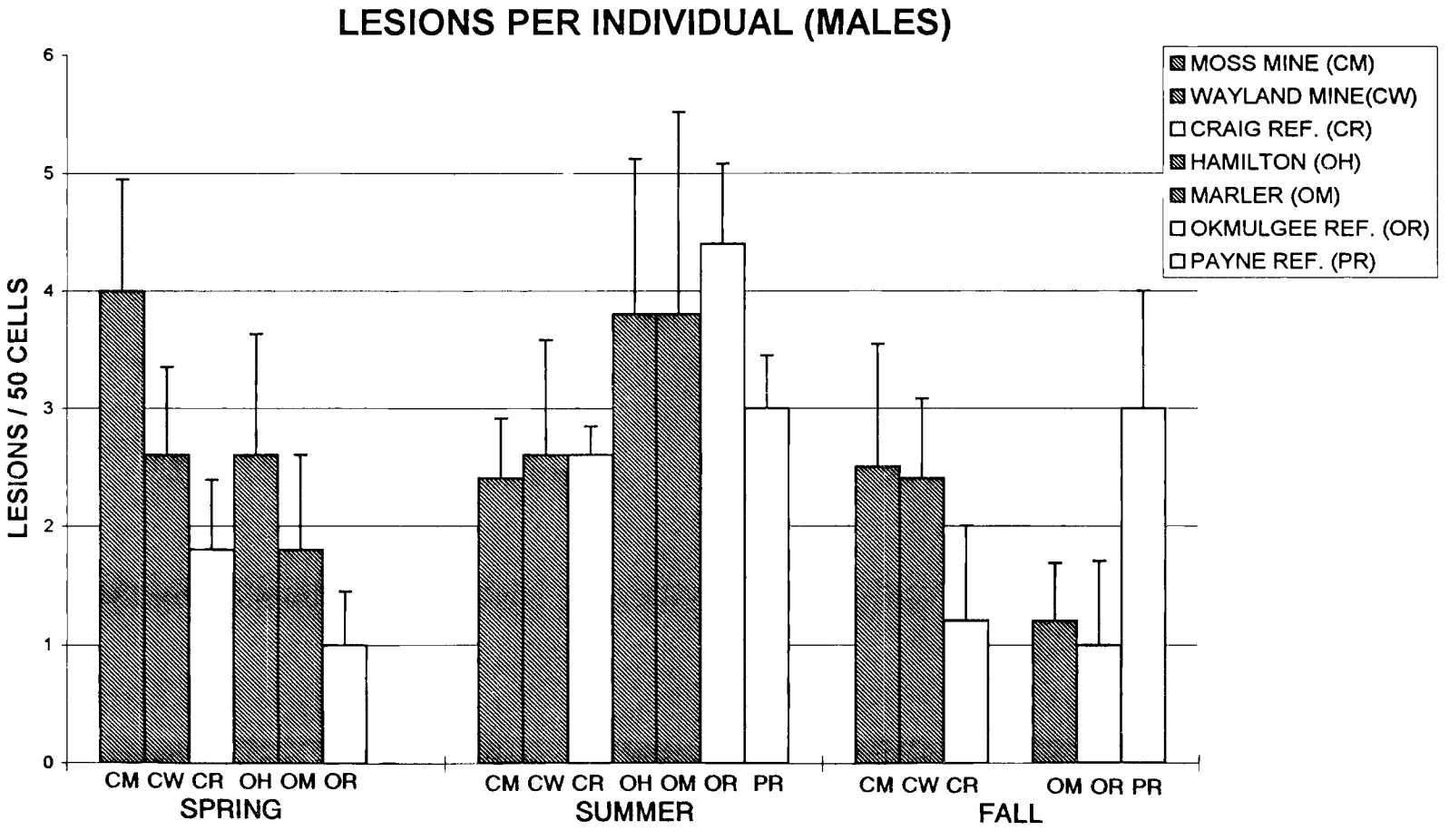
	Fall 1992					
Okmulgee Co.						
Marler mine	1.89 (2.03)	0.56 (0.73)	0.11 (0.33)	0.00	0.11 (0.33)	0.00
Hamilton mine	-	-	-	-	-	-
Eufaula ref.	0.89 (0.93)	0.44 (0.53)	0.00	0.11 (0.33)	0.00	0.00
Craig Co.						
Moss mine	2.33 (1.73)	0.89 (0.93)	0.11 (0.33)	0.00	0.00	0.00
Wayland mine	1.60 (1.17)	0.60 (0.49)	0.30 (0.68)	0.00	0.00	0.00
Craig Co. ref.	1.22 (1.20)	0.56 (0.88)	0.11 (0.33)	0.00	0.00	0.00
Payne Co.						
Payne Co. ref.	1.30 (1.34)	1.00 (0.94)	0.20 (0.63)	0.00	0.10 (0.32)	0.00

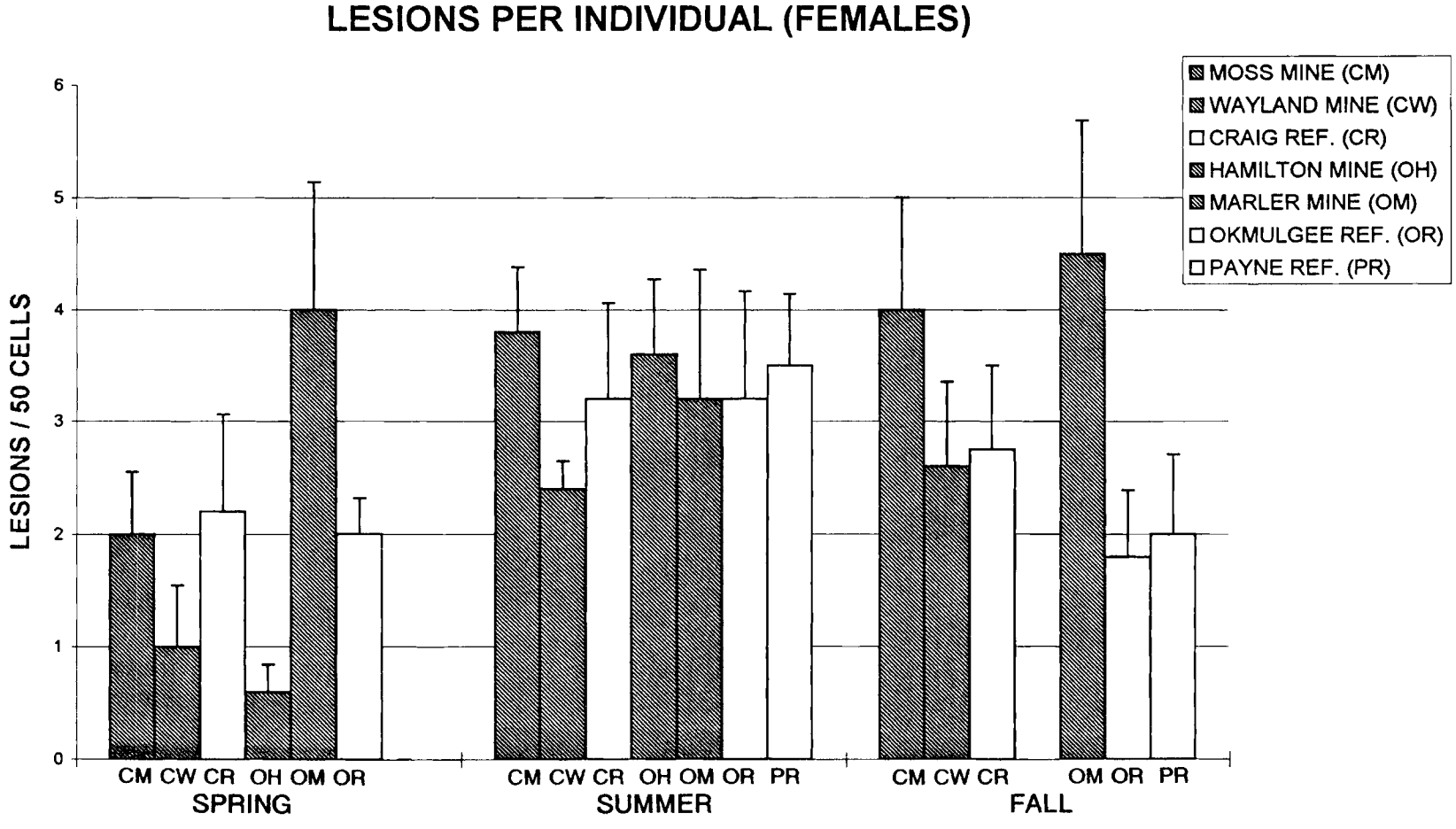
Fig. 1. Mean percent aberrant cells per individual for Peromyscus leucopus from abandoned coal strip mines and reference sites collected during three different seasons. Percents are calculated from 50 metaphase spreads. Vertical bars denote the standard error.

Fig. 2. Mean lesions per 50 metaphase spreads for female Peromyscus leucopus from abandoned coal strip mines and reference sites collected during three different seasons. Vertical bars denote the standard error.

Fig. 3. Mean lesions per 50 metaphase spreads for male Peromyscus leucopus from abandoned coal strip mines and reference sites collected during three different seasons. Vertical bars denote the standard error.


ABERRANT CELLS PER INDIVIDUAL
% ABERRANT CELLS
0
1
2
3
4
5
6
7
8
9
CM CW CR OH OM OR
SPRING
CM CW CR OH OM OR PR
SUMMER
CM CW CR
OM OR PR
FALL
MOSS MINE (CM)
WAYLAND MINE (CW)
CRAIG REF. (CR)
HAMILTON (OH)
MARLER (OM)
OKMULGEE REF. (OR)
PAYNE REF. (PR)


LESIONS PER INDIVIDUAL (MALES)
LESIONS / 50 CELLS
0
1
2
3
4
5
6
MOSS MINE (CM)
WAYLAND MINE(CW)
CRAIG REF. (CR)
HAMILTON (OH)
MARLER (OM)
OKMULGEE REF. (OR)
PAYNE REF. (PR)
CM CW CR OH OM OR
SPRING
CM CW CR OH OM OR PR
SUMMER
CM CW CR
OM OR PR
FALL


LESIONS PER INDIVIDUAL (FEMALES)
LESIONS / 50 CELLS
0
1
2
3
4
5
6
MOSS MINE (CM)
WAYLAND MINE (CW)
CRAIG REF. (CR)
HAMILTON MINE (OH)
MARLER MINE (OM)
OKMULGEE REF. (OR)
PAYNE REF. (PR)
CM CW CR OH OM OR
SPRING
CM CW CR OH OM OR PR
SUMMER
CM CW CR OM OR PR
FALL

CHAPTER 3

NUCLEAR DNA CONTENT VARIATION AND DOUBLE-STRANDED DNA BREAKAGE IN WHITE-FOOTED MICE COLLECTED FROM ABANDONED COAL STRIP MINES

ABSTRACT

White-footed mice (Peromyscus leucopus) were collected during the spring, summer, and fall of 1992 from four metal-polluted abandoned mines and three reference sites in eastern Oklahoma. Two mines in Okmulgee County were matched with a nearby reference site, as were two mines in Craig County. A third, remote reference site was added at a location outside the Oklahoma coal belt in Payne County. Intercellular DNA content variation, measured as a coefficient of variation (CV) of nuclear DNA content measured in 20,000 cells per animal, was measured among splenocytes of individuals by flow cytometry. Double-strand breakage in liver DNA from the same animals was compared using agarose gel electrophoresis. Mice trapped from mines were expected to have more intercellular DNA content variation and more DNA breakage when compared to the matched and remote reference sites. With one exception, mice from mine sites did not show any significant increase in nuclear DNA content variation during any season. Electrophoresis revealed that mice from both Okmulgee Co. mines had significantly more DNA breakage compared to the intracounty reference site in the spring. In the summer, mice from one Craig Co. mine had significantly higher levels of DNA breakage compared to the remote reference.

Key words: metals, flow cytometry, DNA strand breaks, biomonitoring, rodents

INTRODUCTION

There is increasing interest in developing in situ assays to estimate the genotoxicity of environmental contaminants [1]. Many of the most accepted assays for genetic biomonitoring involve direct observation of chromosomes or micronuclei [2]. Because such assays are widely used in laboratory studies, and more recently for in situ investigations [3,4], they are accepted as reliable and sensitive tests. They are, however, extremely time-consuming in both preparation of microscope slides and in examination of chromosome spreads. A faster and equally sensitive assay would be preferable for assessing genotoxic effects of environmental contaminants in situ.

One alternative assay receiving recent attention is analysis of nuclear DNA content variation within cells of individuals by flow cytometry [5]. Nuclear DNA in each cell is stained with a fluorescent dye before it passes through the cytometer, where it gives off a pulse of light with an intensity proportional to the amount of nuclear DNA in the cell. Variation in nuclear DNA content of cells is measured as a coefficient of variation (CV) of the mean of cells in the G1 stage of the cell cycle. Higher CVs should result from unequal amounts of DNA being distributed to daughter cells from chromosomally damaged dividing cells. Flow cytometry has been successfully used to determine genotoxic effects of several substances on dosed animals [5-7]. It has also been used, although somewhat infrequently, as an in situ test [8-11]. Additional information from flow cytometry, such as changes in the distribution of cells among phases of the cell cycle [9,12], may also prove useful in assessing the effect of contaminants on cell proliferation.

A second recently proposed biomarker assay involves determination of DNA strand breakage using agarose gel electrophoresis [13]. In this technique, DNA is extracted from some target tissue and used in electrophoresis. Samples of DNA which contain more double-strand breaks will migrate further than those with fewer breaks. Single-strand breaks can be measured by using an alkaline gel which unwinds the DNA double helix whereas double-strand breaks are detected with a nonalkaline gel. Densitometry, which converts optical densities into digital information, can be used to accurately compare DNA migration. Theodorakis et al. [13] discuss many of the advantages of the electrophoretic method over earlier methods of measuring DNA strand breakage; less DNA is needed, double- and single-strand breaks can be distinguished by altering the pH of the gel, and DNA is preserved and can be recovered using blotting techniques.

There is much to be gained by finding sensitive assays that measure a specific endpoint yet entail lesser cost, time, and equipment demands. It should be noted, however, that no matter how sensitive, these assays can't substitute for one another in terms of the endpoint to be measured. Strand breaks in DNA do not necessarily translate into chromosomal lesions or higher variation in nuclear DNA content of the cells of an individual. Indeed, the relationships among responses in different types of genotoxicity assays are largely unknown. Because different toxicants may act at different levels, it is entirely possible that assays could give conflicting results, and any relationships among assays should be applied only to the toxicant in question.

In this study, flow cytometry was used to compare variation in nuclear DNA content among spleen cells of individual Peromyscus leucopus inhabiting heavy metal contaminated strip mines and reference sites. Liver DNA was extracted from the same

mice and examined using agarose gel electrophoresis to determine levels of double-strand breakage. Results are compared to data on chromosomal aberrations in animals from those same sites. The target species, P. leucopus, is highly suitable for this type of study due to its relative sensitivity to genotoxic and cytotoxic pollutants and their relatively plastic habitat requirements which allow them to inhabit highly impacted areas where the habitat may not be optimal. Hypotheses tested in this project are that animals from strip mine sites contain more variation (higher CVs) in the nuclear DNA of their splenocytes than animals from reference sites, and that animals from strip mines have significantly more double-strand breaks than animals from reference sites. Additionally, we hypothesize that results from flow cytometric analysis and electrophoretic analysis will be consistent with chromosomal aberration data.

METHODS

Collections of P. leucopus were made in March, July, and October, 1992. Sherman live-traps baited with oats and peanut butter were used to trap animals at seven sites: a reference site in Payne Co., and two mine sites and a reference site in each of Okmulgee and Craig counties which are in the Oklahoma coal belt. The Payne Co. reference site is located outside the Oklahoma coal belt. Traps were set in wooded habitats deemed suitable for P. leucopus. Each collecting period comprises collections from seven sites with the exception of spring, when the Payne Co. site was not included.

All animals were sacrificed within 48 h of capture. Heart, kidney, liver, muscle, spleen and testes or embryos were immediately removed and frozen in liquid nitrogen in the field. In the laboratory, tissues were transferred to a -80 ^{0}C freezer for storage until analyses were completed. For each collecting period, a subsample of males and females from each site was randomly chosen for flow cytometric and DNA strand break analysis. Subsamples included animals used in the chromosomal aberration portion of this study [14].

Unless poor trapping success limited the number of animals included in the analysis, ten males and ten females were chosen randomly from the total number of animals collected from each site during each season. These subsamples were used for flow cytometry and electrophoresis; they also contain the same animals used by Hausbeck [15] to determine concentrations of bioaccumulated metals in renal and hepatic tissues. Often the total number of animals collected exceeded the subsample size used in this study. Juveniles, animals which met the criteria of having grey pelage or weighing under 18 grams, were noted in the field and excluded from this study.

Skeletons from all captured animals were prepared and deposited at The Museum, Texas Tech University.

All mine sites have naturally revegetated since abandonment, with similar florae found at all the trapping sites. The dominant trees are blackjack and post oak (Quercus marilandica and Q. stellata), sumac (Rhus glabra), and cedar (Juniperus virginiana). Abandoned mines were chosen based on soil zinc levels reported in a study by the Oklahoma Biological Survey [OBS - unpublished data]. The Okmulgee Co. strip mines are referred to as Hamilton mine (OBS - 130 ppm Zn) and Marler mine (OBS - 163 ppm Zn). Their corresponding reference site is located at the Eufaula Wildlife Management Area. Hamilton mine, Marler mine, and the corresponding reference site have soil pH values of 4.08, 4.97, and 5.25 respectively [15]. The Craig Co. mines are refered to as Moss and Wayland mines and are matched with a privately held pristine reference site. The Craig Co. mine soils contained 40 to 46 ppm Zn [OBS-unpublished data]. No soil pH data are available for Moss mine but Wayland mine had a soil pH of 5.39, the Craig Co. reference area was 6.03, and Payne Co. reference area was 5.43 [15].

Flow cytometry

Spleens were prepared according to modifications of methods described by Otto [16]. Tissues were homogenized in a few millileters of a pretreatment solution (100 ml H_2O / 4.2g citric acid / 0.5 ml Tween 20) and incubated for twenty minutes at room temperature. Samples were then centrifuged for 10 minutes at 100 x g, the supernatant was removed, and the pellets fixed with 70% cold ethanol. Cell suspensions were filtered through 37μ nylon filter cloth and stored at 4 ^{0}C for at least 24 hours in fixative. Approximately ten hours before flow cytometric analysis, the fixed cells were

centrifuged, fresh fix was added, and samples were refiltered. Five to ten drops of the sample were placed into a culture tube along with 0.5 ml of pretreatment solution and 2.5 ml DAPI staining solution (4',6-diamidino-2-phenylindone in phosphate buffer). Cells are allowed to stain for ten hours and analyzed with a Partec PAS II mercury arc lamp flow cytometer. Five DNA histograms examining 20,000 cells were generated for each sample. Calibration of the machine was maintained by using calf thymocyte nuclei after every fifth animal analyzed.

Electrophoresis

Approximately 50 μg of liver tissue from each individual was used in the DNA extraction procedure, which was modified from the chloroform/phenol procedure of Hillis and Moritz [17]. Approximately 50 mg of liver was thawed and placed into a sterile eppendorf tube. DNA extraction buffer (250μl) was added and gently mixed. To this mixture 22μl of 10% sarcosyl was added and homogenized before standing for 60 minutes on ice. An equal volume of cold, buffered phenol was added to samples which were centrifuged for 5 min. at 7000g. The aqueous layer was removed and the phenol extraction was repeated. To the final aqueous layer, an equal amount of chloroform/isoamyl alcohol (24:1) was added, mixed, and centrifuged for 5 min. at 7000g. The aqueous layer was removed, placed in a sterile eppendorf tube with 2 volumes of cold EtOH and stored at -20 °C until electrophoresis.

Prior to electrophoresis, samples were centrifuged at 13000 rpm for 10 minutes and the EtOH was decanted to leave the pellet. One ml of 70% EtOH : 30% G-50 buffer was added and the sample was again centrifuged. The aqueous layer was removed and the rest of the solution allowed to evaporate, leaving a moist pellet. To the pellet, 50μl

G-50 buffer and 1 µl RNase (10 mg/ml) was added. The samples were then mixed and allowed to stand in a refrigerator until the DNA pellet was completely dissolved. Samples were extracted in lots of 60 and electrophoresis was carried out on a 0.40 % agarose gel at 12 volts for approximately 15 h. The ethidium bromide stained gel was kept on ice for the entire 15 h. At the end of electrophoresis the stained bands were illuminated with an ultraviolet light source and photographed. The negative of the photograph was scanned with a densitometer that converts the densities of light on the negative to digital information. By using the Quantity One® (PDI) computer package, the average relative distance of DNA migration for each sample was determined based on density weighted averages. Absolute average lengths of DNA were calculated from a standard curve created from bands of known length (Hind III cut E. coli DNA).

Statistical analysis

Both data sets were rank transformed due to the results of Levene's test, which revealed that homogeneity of variance could not be assumed for season by site combinations in either data set. Three-way analyses of variance (ANOVAs) were performed with season, site, and sex as factors. Flow cytometric data showed a three-way interaction, so seasons and sexes were analyzed separately. Sex was not a significant factor for the electrophoretic data, but there was a significant season by sex interaction. These data were, therefore, analyzed with sexes pooled within seasons. For both data sets, site to site comparisons were made with pairwise LSD tests. All mine sites were compared to their intracounty reference site and the Payne County remote reference site. Spearman correlation coefficients were calculated to test any relationship among chromosomal aberrations, average DNA length, and CVs of G1

peaks. The percent of cells in the G2 and S phases were pooled for each animal. Because the vast majority of percentages fell between 0 % and 20 % they were square root and arcsin transformed [18]. Parametric ANOVA procedures were then used to compare mean percentages among seasons, sites, and sexes; pairwise comparisons among sites were made with Fisher's LSD test.

RESULTS

Trapping efforts resulted in the capture of rodent species P. leucopus, P. maniculatus, Reithrodontomys fulvescens, Microtus pinetorum, Neotoma floridana, Sigmodon hispidus, Oryzomys palustris, Zapus hudsonius (two specimens in Craig Co.), and Cheatodipus hispidus (Craig Co. only). In the field an attempt was made to distinguish between P. leucopus and P. maniculatus. Animals identified as P. maniculatus were released. Sigmodon hispidus were processed in the same manner as P. leucopus for future analysis. Several specimens of R. fulvescens were also prepared as skeletons and deposited in The Museum, Texas Tech University. Of the total number of P. leucopus collected, 53.1 % were males (all ages combined). Peromyscus are expected to be male biased [19], but the cause of the deviation from 50 % is not understood. Kaufman and Kaufman [19] report 54 % males across size classes for P. maniculatus and P. leucopus. The percentage of males was 57.0 % for the reference sites combined (n = 509), 53.3 % for the Craig Co. mines (n = 437), and 47.5 % for the Okmulgee Co. mines (n = 373). The Okmulgee Co. mines, the most contaminated [15] mines, showed the most female biased percentage, followed by the Craig Co. mines and the reference sites. It has been suggested that stress may skew populational sex ratios in favor of more females [20,21].

Analysis of variance revealed a significant difference between the ranks of the first two CVs recorded by the cytometer and the last three CVs. Because consistant values were produced in histograms produced after the second one, the first two histograms for each animal were discarded and the final three were averaged. Averages were ranked for susequent analyses. Coefficients of Variation ranged from 1.55 for

males from the Hamilton mine in the fall to 2.56 for males from the Marler mine in the summer. Males from mine sites did not have significantly higher CVs compared to males from intracounty or Payne County reference sites during any season (Figure 1). Females from one mine site, Moss mine, collected in the fall had significantly higher CVs compared to females from the matched reference site and the Payne County reference site (Figure 2). Females from mine sites did not have significantly higher CVs in any other season. Regression analysis did not show significant correlations between CV and chromosome lesions per individual (Table 1). The arcsin transformed percentages of cells in the G2/S phase showed a significant three-way season by site by sex interaction. Season and sexes were tested separately for significant differences among mine sites and their intracounty reference site. No mine site differed significantly from its matched reference site (Table 2).

Sex was not a significant factor for comparisons of mean DNA length, but season was. Analyses were, therefore, done with sexes pooled but seasons separated. Average lengths ranged from 31.38 Kilobases (Kb) for the Craig Moss mine in spring to 38.56 Kb for the Okmulgee reference site in spring. In the spring, both mines in Okmulgee County had smaller DNA fragments compared to the Okmulgee reference site. Sites in Craig County were not significantly different from each other in the spring. In the summer, DNA samples from animals collected at mines were not significantly smaller than samples from the intracounty reference sites. However, DNA fragments of animals from the Wayland mine were significantly smaller than the Payne County reference site. Two mine sites, the Moss mine and the Marler mine, actually had longer average DNA strand lengths compared to both their intracounty reference sites and the Payne County reference site. No mine sites had significantly smaller DNA fragments

compared to reference sites in the fall sample. All sites in the fall season had significantly shorter DNA fragments compared to the Payne County reference site. Regression analysis showed a significant negative correlation between average DNA size and both CV and chromosomal lesions per individual for only males in spring.

DISCUSSION

The general lack of significantly higher CVs in mine animals is consistent with the data on chromosomal aberrancy. Possible reasons for the lack of significant differences among sites with different heavy-metal contamination levels include the lack of genotoxic contamination levels, tissue specificity of metal toxicity, facilitated metabolic mechanisms, and selection for metal tolerance. Bickham et al. [5], using flow cytometry, found that different tissues within the same lab-exposed animal may respond differently to a toxicant. The production of metallothioneins, proteins which bind and excrete heavy metals, are induced by exposure to metals. The idea of selection for metal resistant genotypes has been tested extensively with marine gastropods [22-24] but not with terrestrial mammals. Metal tolernace may explain cases where mine sites have longer strand lengths compared to reference sites.

Lamb et al. [10] discuss the possibility of obtianing elevated CVs due to daily variation in the stability of the flow cytometer. Vindelov et al. [25] also address the problem of machine variation over time. Otto et al. [16] suggest that all samples to be compared to each other should be prepared, stained, and measured together for quality control purposes. After several hours of observations, we reiterate the desirability of gathering data in this way. The large sample size used in this study prevented us from processing all the samples at once. Instead, samples were prepared, stained and measured in six batches of approximately 60-70 samples. Each batch contained the most comparable entities: animals of one sex from one season were analyzed together because the main hypothesis concerned site to site comparisons. For this reason, we hesitate to read too much into significant differences among seasons or between sexes.

For flow cytometry to be useful as a biomonitoring tool, quality control standards must be devised and become universally accepted. Such standards would help to further reduce variation over time and make inter-laboratory generated data more comparable.

The electrophoretic measurement of double-stranded DNA breaks shows a tremendous amount of promise as a biomonitoring assay, especially in the non-mammalian vertebrates which have nucleated erythrocytes [13]. This stems from the fact that the DNA in blood can be extracted by putting blood directly into agarose extraction plugs, which eliminates the homogenizing and pipetting steps that introduce strand breaks. With solid tissue, it is impossible to differentiate between strand breaks introduced during extraction and those that can be attributed to a genotoxic chemical. We assume that samples were handled identically and there is no difference in the amount of manually induced strand-breakage. This is the first extensive environmental study to test this technique with mammals. Wlodek et al. [26] found it to be an effective way to measure strand breaks in irradiated Chinese hamster ovary cells in culture. Due to the clastogenic property of several metals [27], one could reasonably expect increased strand breakage in metal-exposed mice. However, no clear pattern of metal-induced strand breaks is evident in this study. In fact, many mine sites have longer mean DNA strands compared to reference sites.

In order for the electrophoretic stand break assay to become widely accepted for environmental studies, several problems must be addressed. These problems include the shearing introduced during extraction and the lack of a universally accepted set of standard DNA markers that include the DNA samples in the their range of lengths. There is much incentive to develop flow cytometry and DNA strand breakage assays due to their simplicity and speed. The first steps in their development would be to test

chemicals against more accepted assays, like the chromosomal aberration assay, in laboratory-dosed animals. It must be shown that given exposure to a certain toxicant, a certain response can be expected. With this information in hand, an assay could be used in situ where the variables are more numerous and much more difficult to control than in the laboratory.

ACKNOWLEDGMENTS

This project was funded by U.S. E.P.A grant R-818429-01-0. Thanks to all those who assisted with this project. C.C. McMillen, J.S. Hausbeck, R.D. Owen, C. Lopez-Gonzalez, and S. Kasper helped to collect specimens. C. Kaiser assisted with spleen preparations and A. Ferguson helped with DNA extraction. M. Payton provided statistical advice. D.M. Leslie, Jr. and the Oklahoma Cooperative Fish and Wildlife Research Unit provided field support. This project would not have been possible without the cooperation of the landowners and the Oklahoma Department of Wildlife Conservation and personnel of the Eufaula Wildlife Management Area.

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Table 1. Spearman correlation coefficients for chromosome lesions per individual, flow cytometric coefficients of variation, and mean DNA strand length.

Variables	Males				Females		
	Chromsome lesions	Coefficient of variation	DNA length		Chromosome lesions	Coefficient of variation	DNA length
				Spring			
Chromosome lesions	1.000				1.000		
Coefficient of variation	0.116	1.000			0.055	1.000	
DNA length	-0.450*	-0.649	1.000		0.201	0.114	1.000
				Summer			
Chromosome lesions	1.000				1.000		
Coefficient of variation	0.060	1.000			-0.392	1.000	
DNA length	0.117	-0.117	1.000		-0.249	0.018	1.000
				Fall			
Chromosome lesions	1.000				1.000		
Coefficient of variation	0.181	1.000			-0.061	1.000	
DNA length	-0.081	0.014	1.000		0.178	-0.174	1.000

* significance at ($p < 0.05$)

Table 2. Sample size, mean, and standard error of percent G2/S cells recorded by flow cytometry.

Site	Spring			Summer			Fall		
	n	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.
Okmulgee County									
					Males				
Hamilton Mine	10	5.53	1.69	11	10.98	2.87	2	11.56	0.86
Marler Mine	11	6.29	1.05	12	10.97	4.03	7	11.54	5.07
Eufaula Ref.	10	7.30	0.78	10	5.75	0.98	10	9.29	1.86
					Females				
Hamilton Mine	10	8.38	2.65	9	23.18	3.91	4	15.05	6.17
Marler Mine	11	6.43	0.67	8	23.16	5.31	5	15.01	4.97
Eufaula Ref.	10	7.86	1.45	10	15.15	2.07	5	10.76	4.61

Table 2. cont.

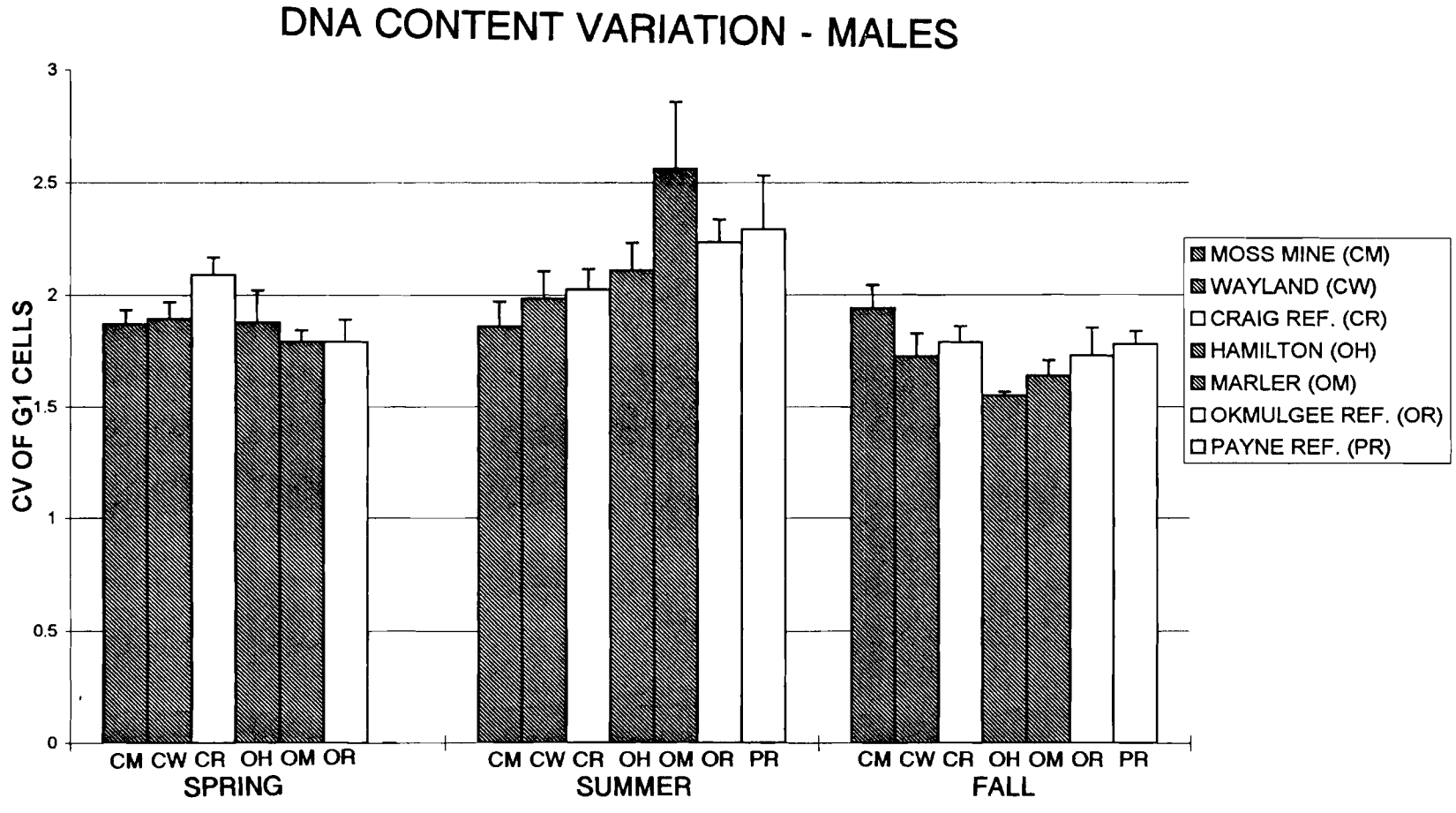
Craig County

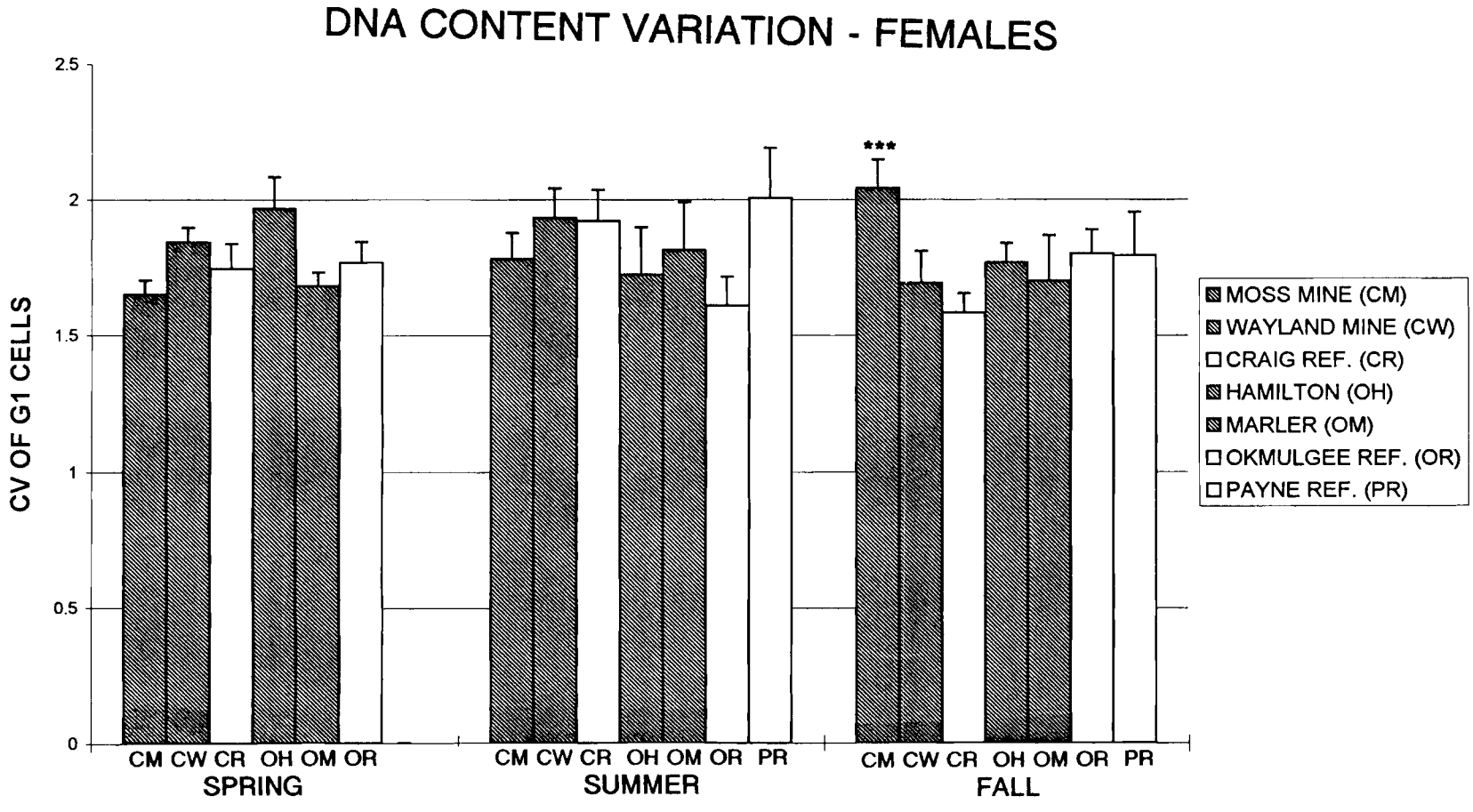
					Males				
Moss Mine	10	8.97	2.10	10	10.74	2.15	11	9.36	2.29
Wayland Mine	10	5.44	0.92	10	9.56	1.38	10	8.02	1.65
Craig Co. Ref.	8	4.67	0.49	10	7.63	2.30	10	8.38	0.94
					Females				
Moss Mine		6.48	0.55	7	12.94	3.55	7	16.35	2.81
Wayland Mine	10	7.88	1.11	7	6.75	1.04	9	11.29	1.28
Craig Co. Ref.	11	9.94	2.60	8	7.79	1.03	6	11.05	2.59

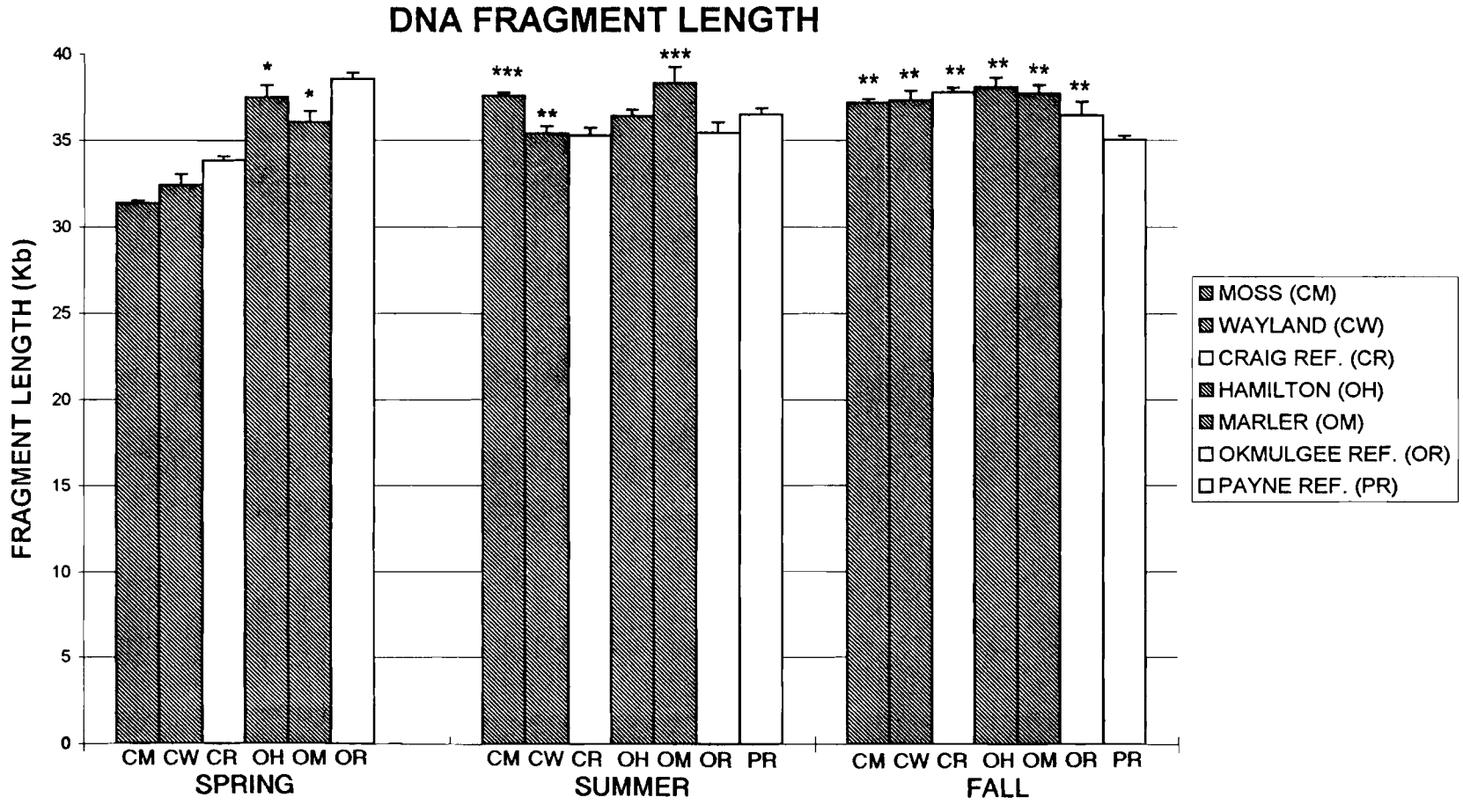
Fig. 1. Flow cytometric coefficients of variation of the nuclear DNA of g1 cells of male Peromyscus leucopus from abandoned coal strip mines and reference sites collected during three different seasons. Vertical bars denote the standard error. There were no significant differences between any mine and its intracounty reference site or the Payne Co. reference site.

Fig. 2. Flow cytometric coefficients of variation of the nuclear DNA of g1 cells in female Peromyscus leucopus from abandoned coal strip mines and reference sites collected during three different seasons. Vertical bars denote the standard error. A significant difference with both the intracounty reference site and the Payne Co. reference is denoted by three asterisks (***).

Fig. 3. Mean DNA fragment length Peromyscus leucopus from abandoned coal strip mines and reference sites collected during three different seasons. Vertical bars denote the standard error. Significant differences between animals from a particular mine and its intracounty reference site are denoted by one asterisk (*). A significant difference between a mine and the Payne County reference site is denoted by two asterisks (**). A significant difference between a mine and both the intracounty reference site and the Payne County reference site is denoted by two asterisks (***).


DNA CONTENT VARIATION - MALES
CV OF G1 CELLS
3
2.5
2
1.5
1
0.5
0
CM CW CR OH OM OR
SPRING
CM CW CR OH OM OR PR
SUMMER
CM CW CR OH OM OR PR
FALL
MOSS MINE (CM)
WAYLAND (CW)
CRAIG REF. (CR)
HAMILTON (OH)
MARLER (OM)
OKMULGEE REF. (OR)
PAYNE REF. (PR)


DNA CONTENT VARIATION - FEMALES
CV OF G1 CELLS
2.5
2
1.5
1
0.5
0
***
CM CW CR OH OM OR
SPRING
CM CW CR OH OM OR PR
SUMMER
CM CW CR OH OM OR PR
FALL
MOSS MINE (CM)
WAYLAND MINE (CW)
CRAIG REF. (CR)
HAMILTON (OH)
MARLER (OM)
OKMULGEE REF. (OR)
PAYNE REF. (PR)


DNA FRAGMENT LENGTH
FRAGMENT LENGTH (Kb)
0
5
10
15
20
25
30
35
40
CM CW CR OH OM OR
SPRING
CM CW CR OH OM OR PR
SUMMER
CM CW CR OH OM OR PR
FALL
MOSS (CM)
WAYLAND (CW)
CRAIG REF. (CR)
HAMILTON (OH)
MARLER (OM)
OKMULGEE REF. (OR)
PAYNE REF. (PR)

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