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CHROMOSOMAL ABERRANCY IN WHITE-FOOTED MICE (*PEROMYSCUS LEUCOPUS*) COLLECTED ON ABANDONED COAL STRIP MINES, OKLAHOMA, USA

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Abstract—This study was undertaken to determine the genetic consequences to small mammals of long-term exposure to heavy metal pollution. A secondary goal was to continue 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, USA. Chromosomal aberrations were scored from standard bone marrow metaphase chromosome spreads. Seasonal differences were detected for aberrant cells (cells containing one or more chromosomal lesions) 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—Metals Chromosomal aberrations Rodents Biomonitoring

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, USA, enacted a reclamation act in 1971, but approximately 12,000 ha of 10- to 70-year-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 to 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 County (Hamilton and Marler mines) when compared to the reference site in that county. The Craig County mine (Wayland mine) did not differ from its matched reference site or from the Okmulgee County reference site. All sites in Okmulgee and Craig counties differed significantly from the Payne County 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, however, elevated at contaminated sites during certain seasons. Cadmium levels in both tissues were consistently higher in mice from contaminated sites compared with 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 [6,7]. Studies that address the genotoxic effects of exposure to complex mixtures of metals by wild mammals are lacking. Bueno et al. [8] found that, compared with conspecifics from uncontaminated reference sites, rodents (*Oryzomys* and *Akodon*) collected from coalfield polluted areas had significantly higher percentages of cells containing an aberrant chromosome and significantly higher numbers of total chromosomal aberrations. It appears from this and other in situ investigations involving a variety of nonmetal 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 with 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, under-

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ground 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 with background levels found at reference sites [2]. Mice collected from abandoned mines were compared with 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. We hypothesize 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 (Sherman Traps, Tallahassee, FL, USA) 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 were 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 County. Mice were collected from this site during the summer and fall but not during 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 County) was so poor (two males and four 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 g. Complete skeletons, minus the femurs, which were used for metaphase spread preparation, were saved and deposited at The Museum, Texas Tech University, Lubbock, TX, USA. Animals were put into age classes (C. López-González and R.D. Owen, unpublished data); 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 [18]. 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 County 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 Okmulgee County mines were found to contain 130 to 163 μl/L 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 County mines are referred to as Moss and Wayland mines and are matched with a privately held pristine reference site. Soils from Craig County mines contained 40 to 46 μl/L 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 County reference area was 6.03. One reference site outside the Oklahoma coal belt in Payne County had a soil pH of 5.43.

The method for metaphase chromosome spread preparation followed closely that of Baker et al. [19]. All animals were sacrificed within 48 h 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 min. Incubated cells were centrifuged out of the supernatant at 400 rpm for 90 s to form a pellet of cells (IEC Clinical Centrifuge, Needham Heights, MS, USA). 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, with the final resuspension in a final volume of 1.5 ml fixative. 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 to 7 min 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, including 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 that showed significance were then compared by least significant difference (LSD) multiple comparisons. Because a nearly 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. 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 $\ggg$ aberrant cells) because cells with excessive damage are less likely to survive and divide.

Table 1. Means (standard deviations in parentheses) of lesions per 50 cells for six types of chromosomal lesions in *P. leucopus* collected from mined sites and reference sites during three seasons

Season and site	Lesion type					
	Chromatid breaks	Acentric fragments	Chromosome breaks	Dicentric chromosomes	Ring chromosomes	Translocation figures
Spring 1992						
Okmulgee County						
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 reference	1.50 (0.97)	0.00	0.00	0.00	0.00	0.00
Craig County						
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 County reference	1.50 (1.18)	0.30 (0.68)	0.10 (0.32)	0.00	0.00	0.10 (0.32)
Summer 1992						
Okmulgee County						
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 reference	2.60 (1.78)	0.80 (0.63)	0.30 (0.48)	0.00	0.10 (0.32)	0.00
Craig County						
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)	0.10 (0.32)	0.00	0.00	0.00
Craig County reference	1.50 (1.18)	0.20 (0.57)	0.20 (0.42)	0.00	0.10 (0.32)	0.00
Payne County						
Payne County reference	2.56 (1.33)	0.44 (0.73)	0.22 (0.44)	0.00	0.00	0.00
Fall 1992						
Okmulgee County						
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 reference	0.89 (0.93)	0.44 (0.53)	0.00	0.11 (0.33)	0.00	0.00
Craig County						
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 County reference	1.22 (1.20)	0.56 (0.88)	0.11 (0.33)	0.00	0.00	0.00
Payne County						
Payne County reference	1.30 (1.34)	1.00 (0.94)	0.20 (0.63)	0.00	0.10 (0.32)	0.00

RESULTS

Trapping efforts resulted in capture of rodent species *P. leucopus* (white-footed mouse), *P. maniculatus* (deer mouse), *Reithrodontomys fulvescens* (fulvous harvest mouse), *Microtus pinetorum* (woodland vole), *Neotoma floridana* (eastern woodrat), *S. hispidus* (hispid cotton rat), *Oryzomys palustris* (marsh rice rat), *Zapus hudsonius* (meadow jumping mouse, two specimens in Craig County), and *Chaetodipus hispidus* (hispid pocket mouse, Craig County only). Of the total number of *P. leucopus* collected, 53.1% were males (all ages combined). Populations of *Peromyscus* are expected to be male biased [20], but cause of deviation from 50% is not understood. Kaufman and Kaufman [20] report 54% males across size classes for *P. maniculatus* and *P. leucopus*. Percentage of males was 57.0% for the reference sites combined ($n = 509$), 53.3% for the Craig County mines ($n = 437$), and 47.5% for the Okmulgee County mines ($n = 373$). The Okmulgee County mines, which were the most contaminated [2] mines, showed the most female-biased percentage, followed by the Craig County mines and the reference sites. It has been suggested that stress may skew populational sex ratios in favor of more females [21,22]. Female-biased sex ratios may be the result of differential metal resistance, unequal maternal investment by stressed mothers, or possibly an increase in X-linked disorders.

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 [12] 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 Figure 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. Pair-wise 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 com-

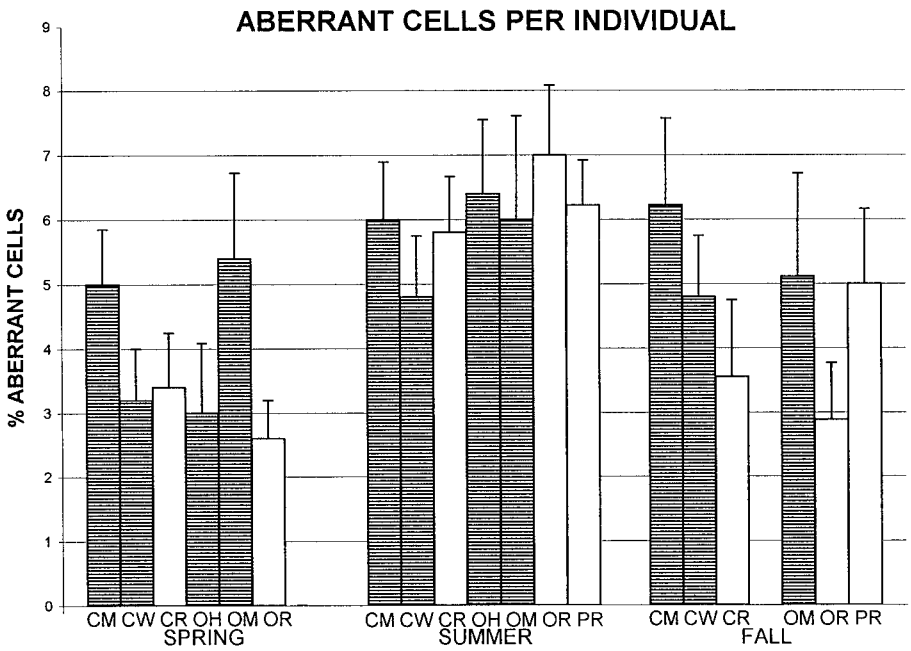

Fig. 1. Mean percent aberrant cells per individual for *Peromyscus leucopus* from abandoned coal strip mines and reference sites collected during three different seasons. Percentages are calculated from 50 metaphase spreads. Horizontal bars = contaminated sites; open boxes = reference sites. Vertical bars denote the standard error. CM = Moss mine; CW = Wayland mine; CR = Craig reference; OH = Hamilton; OM = Marler; OR = Okmulgee reference; and PR = Payne reference.

parable to those reported by McBee et al. [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 pair-wise LSD tests.

Mean lesions for 50 cells per individual in males and females are presented in Figures 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 greater numbers of lesions in the summer and fall, which were not significantly different from each other, but both were significantly higher than spring. Females averaged 1.68 lesions per 50 cells in spring, 3.32 in summer, and 3.29 in fall. Males averaged 2.24 in the spring, 3.10 in the summer,

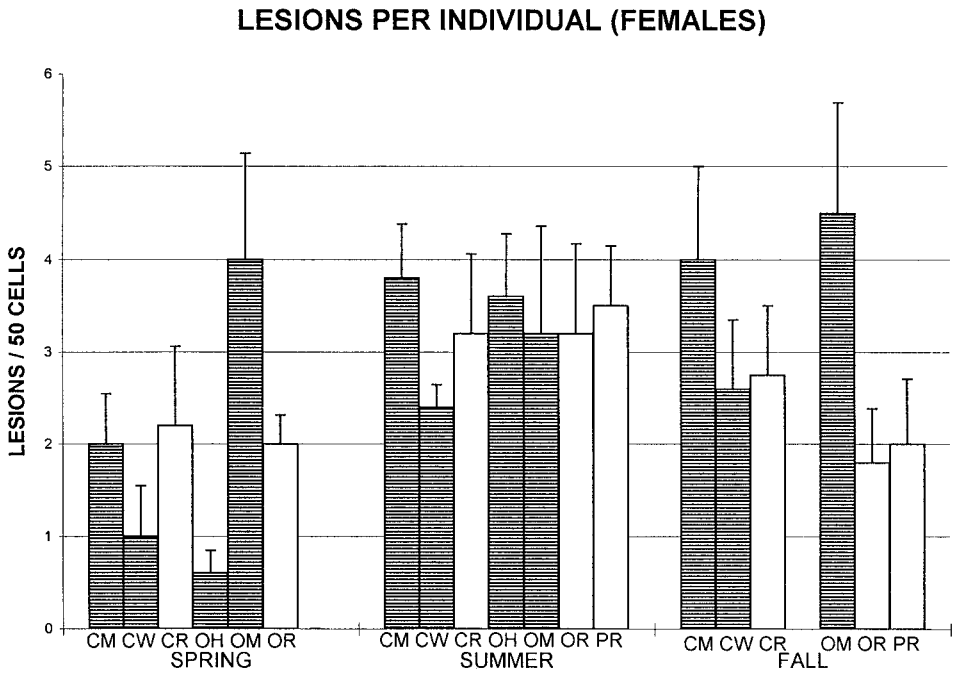

Fig. 2. Mean lesions per 50 metaphase spreads for female *Peromyscus leucopus* from abandoned coal strip mines and reference sites collected during three different seasons. Horizontal bars = contaminated sites; open boxes = reference sites. Vertical bars denote the standard error. Abbreviations defined in Figure 1.

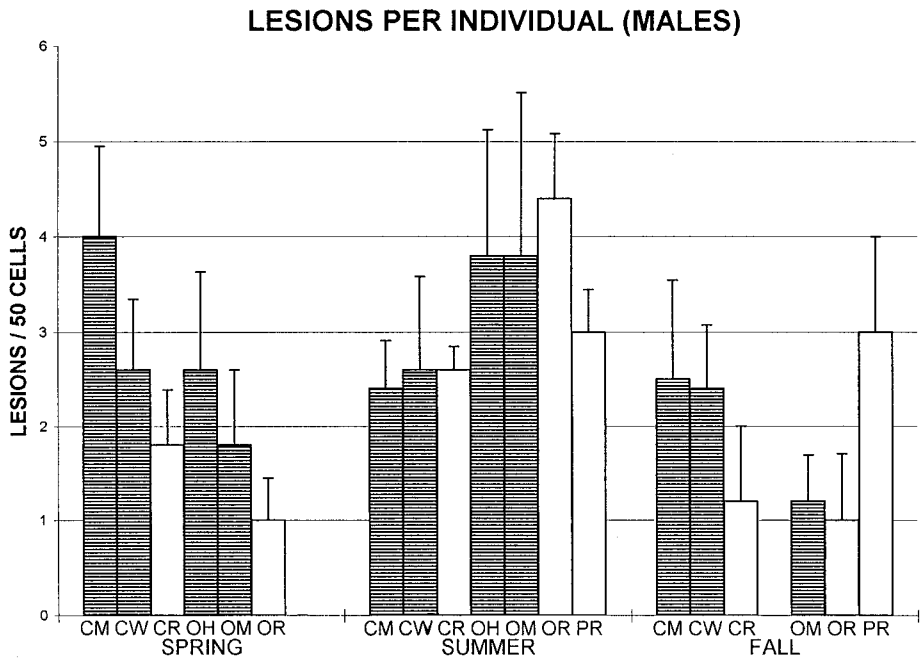

Fig. 3. Mean lesions per 50 metaphase spreads for male *Peromyscus leucopus* from abandoned coal strip mines and reference sites collected during three different season. Horizontal bars = contaminated sites; open boxes = reference sites. Vertical bars denote the standard error. Abbreviations defined in Figure 1.

and 1.93 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 with unexposed lab-reared *S. 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 [23]. 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. The use of data from *Sigmodon* here is more of a reference 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 lab-reared *S. hispidus*. For most sites, fall was intermediate between spring and summer, and only one site (Payne County 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 [24]. More evidence suggests that Cd itself does not induce DNA structural damage, but that it works as a coclastogen by inhibiting repair and replication mechanisms [25]. Howard et al. [26] 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 [27]. 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.

C. López-González and R.D. Owen (unpublished data) found that *P. leucopus* from the strip mine sites were significantly smaller and showed a higher degree of directional asymmetry than those from reference sites when age was ac-

counted for. Those results suggest a developmental disruption but do not specify a cause. Combined with the results of the present study and a companion study [2], one can rule out structural integrity of DNA and improper segregation of chromosomes as causes. The morphometric observations may still have a genetic explanation if the metals act at the nucleotide level; however, DNA alterations may not contribute to phenotypically expressed alterations if the metals act posttranscriptionally by altering proteins.

The lack of chromosomal lesions, aside from their relation to phenotypic observations, may have one of several causes. The difference among exposure levels at the different sites may be too low to cause intersite differences in the number of aberrant cells or number of lesions. Exposed animals also may have an induced metabolic response that 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 that prevents proper copper metabolism, zinc acetate administered at low levels helps prevent overaccumulation of copper by inducing the production of metallothioneins that can bind copper and block absorption [28]. Waalkes et al. [29] found that pretreatment with zinc or low doses of cadmium reduces the tumorigenic effect of cadmium by inducing nonspecific metallothionein production. Zinc may also lessen the effect of cadmium exposure through competition for binding sites [30]. 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 [31–33].

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 partially explain 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 with 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 that 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.

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