AN ECOTOXICOLOGICAL STUDY OF WHITE-FOOTED MICE (*PEROMYSCUS LEUCOPUS*) FROM TAR CREEK SUPERFUND SITE

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CHAPTER I

INTRODUCTION

The Tri-State Mining District is made up of portions of NE Oklahoma, SW Missouri, and SE Kansas. This area was mined extensively for lead (Pb) and zinc (Zn) from the 1890's until the 1970's (USEPA 2005). Three major mine sites were located in Oklahoma, the Spring and Quapaw Mine Districts, located west of the Spring River, and the Peoria District, located east of the Spring River. In 1914, the Spring and Quapaw Districts were consolidated as Picher Mining Area and mined by a single company (Weidman et al. 1932). A large portion of the land in the Picher Mining Area is owned by the Quapaw Tribe of Oklahoma (USEPA 2005). Zn and Pb ores were extracted throughout the Tri-State Mining District by use of the room-and-pillar method in which ore was dug from below ground, but large pillars of rock were left in place to provide structural support. Many of the mines were located below the water table, therefore constant pumping was required to prevent mines from filling. Sediment retention ponds or flotation ponds received water pumped from mines and also were used to separate mine tailings from desired ores (USEPA 2005).

The Tri-State Mining District was the leading producer of Zn ore in the United States during World War II. At its maximum production, the Tri-State Mining District

was responsible for approximately 75% of the Zn production in the United States (Weidman et al. 1932). Mines were abandoned in the late 1970's, leaving a large area of physical disturbance and concerns for the health of humans and wildlife in the area. Currently, 75 million tons of mine tailings, referred to as chat, and 800 acres of sediment retention ponds are present in the area. Additionally, 10,000 boreholes and 1,000 mines are present. After mining ceased, water was no longer pumped and mines began to fill. Discharge of heavily contaminated, acid mine drainage was first detected in 1979 (USEPA 2005). In the Oklahoma portion of the Tri-State Mining District, physical disturbance is estimated to be approximately 485 ha (Kirkwood 2007). In some areas, mines are located less than 100 feet below the surface, resulting in mine collapse. Elevated levels of Pb, Zn, and cadmium (Cd), a by-product of the Zn extraction process, have been detected in soil and water at the site (USEPA 2005). The site was placed on USEPA's National Priority List in 1983 as Tar Creek Superfund Site (TCSFS) and has received federal and state funding for remediation, education, and health screening since that time (USEPA 2005).

Studies documenting metals levels at TCSFS have reported highly variable results throughout the site. Pb levels measured in soil from residential and commercial areas within the site (156-2218 mg/kg) were significantly higher than reference samples (40-348 mg/kg) (USEPA 1997). Soil removed during residential yard restoration also contained Cd in excess of 100 mg/kg (USEPA 2005). Sediments from the Beaver Creek and Douthat Settling Pond areas of TCSFS contained levels of Pb from 440 to 540 mg/kg, Cd from 20 to 56 mg/kg, and Zn from 3000 to 9300 mg/kg (Moeller 2004).

Mine drainage continues to flow from mines and as run off from mine tailings piles into Tar Creek and other smaller creeks within TCSFS, which discharge into the Spring and

Neosho Rivers, and ultimately Grand Lake O' the Cherokees. Mine water also has been detected in the Boone aquifer, which overlays the Ribidoux aquifer, the main source of drinking water for the surrounding communities (USEPA 2005). The USEPA has several ongoing monitoring and restoration projects at the site, including monitoring of metal levels in sediment, surface waters, and drinking water, evaluation of residential property in surrounding municipalities, blood lead screening and health education, removal of mine tailings, and monitoring of surface waters (USEPA 2010).

The metals of concern at TCSFS, Pb, Zn, and Cd, have all been documented to have numerous effects and storage sites within the body of small mammals (Johnson et al. 1978; Goyer and Clarkson 2001; Sánchez-Chardi and Nadal 2007). Pb is the most widely used non-ferrous metal and is often released into the environment through mining and smelting activities. It has no essential role in biological systems and is most often ingested through contaminated food. Acute and chronic effects have been documented in haematopoetic, nervous, gastrointestinal, endocrine, and renal systems, and Pb has shown genotoxic effects in mammals in field and laboratory studies (Sheffield et al. 2001; Beyersmann and Hartwig 2008). Johnson et al. (1978) found highest Pb accumulation in bone, approximately 42-68% of total body burden, followed by kidney and liver, in wild-caught long-tailed field mice (*Apodemus sylvaticus*), bank voles (*Clethrionomys glareolus*), and field voles (*Microtus agrestis*) exposed to metals at abandoned Pb and Zn mines and smelter sites. Greater white-toothed shrews (*Crocidura russula*) collected from landfill sites accumulated highest levels of Pb in kidney and liver (Sánchez-Chardi and Nadal 2007). Cd also has no essential function in biological systems and is carcinogenic and genotoxic to mammals at high doses (Beyersmann and Hartwig 2008). In laboratory studies, Cd also has shown estrogenic

properties (Takiguchi and Yoshihara 2006). In wild *A. sylvaticus*, *C. glareolus*, *M. agrestis*, and *C. russula,* Cd accumulates in the kidney and liver, but absorption and retention is greatly influenced by uptake of essential metals and nutrients (Johnson et al. 1978; Sánchez-Chardi and Nadal 2007). Cd is capable of displacing several other metals, including Zn, that are essential for enzyme function (Beyersmann and Hartwig 2008). Zn is an essential metal that plays a critical role in protein synthesis and metabolism and is a constituent of numerous metalloenzymes. Johnson et al. (1978) found that Zn accumulated at highest levels in bone followed by kidney, liver, and brain in *A. sylvaticus, C. glareolus*, and *M. agrestis*.

Use of animal models, such as non-game wildlife species, is a practical way to understand potential effects of metals at sites like TCSFS. Use of wildlife species in understanding effects of exposure to metals also can be advantageous because it provides a means for investigating deleterious effects of contaminant mixtures at multiple levels of organization and at environmentally realistic concentrations (Talmage and Walton 1991; Peakall and McBee 2001). Small mammals, such as the white-footed mouse (*Peromyscus leucopus*), have been used in ecotoxicological studies and are useful because of their widespread geographic ranges, ease of collection, and short generation times (Talmage and Walton 1991; Sheffield et al. 2001). Additionally, small mammals serve roles that are vital to ecosystem function, including soil aeration, nutrient cycling, increased germination due to seed caching, predation on invasive and native invertebrates, and as prey items for other species (Sieg 1987).

The objective of this study was to investigate individual and population-level endpoints in *P. leucopus* from TCSFS and matched reference sites. I compared anogenital distance (AGD), a morphological measure sensitive to exposure to endocrine disrupting compounds

(Gray et al. 2006), between TCSFS and two reference populations to determine if a sub-lethal hormonal effect is present in *P. leucopus* at TCSFS. Additionally, I sought to determine if measurement of AGD is suitable for use in wild populations of a small mammal. I hypothesized that male mice from TCSFS would have a significantly reduced AGD when compared with reference animals. The results of this portion of my study are presented in chapter two, which is prepared for submission to *Bulletin of Environmental Toxicology and Chemistry*. I also measured frequency of structural chromosomal aberrations in *P. leucopus* from TCSFS by use of metaphase chromosome analysis. I hypothesized that *P. lecuopus* from TCSFS would have a higher incidence of structural chromosomal aberrations than mice from reference sites because of the clastogenic potential of Pb, Cd, and Zn. I used amplified fragment length polymorphism (AFLP) analysis to compare population genetic structure of metal-exposed and reference populations. I hypothesized that populations of *P. leucoupus* from TCSFS would show significant genetic differentiation from reference populations and have lower levels of heterozygosity due to indirect effects of contaminant exposure on population-level processes. Combined results of metaphase chromosome and AFLP analyses are presented in chapter three, which is in the format for *Ecotoxicology*.

LITERATURE REVIEW

Use of wildlife species as biomonitors -- Much of the policy formed by regulatory agencies like the USEPA is rooted in protection of human health and relies heavily on the results of laboratory based toxicity studies (Rattner 2009). Use of resident wildlife species for *in situ* assessments of terrestrial and aquatic environments may be more difficult to extrapolate to

other species, but may prove to be a more sensitive, realistic indicator of potential health effects on wildlife and human populations than laboratory studies. Additionally, *in situ* studies allow for inclusion of seasonal, behavioral, and physiological stressors that are difficult to duplicate in the lab (McBee and Lochmiller 1996).

Many species of small mammals, especially members of the Order Rodentia, have life history characteristics that make them practical for *in situ* assessments of contaminated environments (Sheffield et al. 2001). Rodents represent approximately 42% of all extant mammal species and 2,277 species are currently recognized (Wilson and Reeder 2005). Small mammals have short lifespans, generalized diets, small home ranges, and prefer habitats that may allow for increased exposure through ingestion and inhalation of contaminated sediments. Additionally, methods for field collection of small mammals are well-documented (Talmage and Walton 1991). Small mammals, including *P. leucopus*, have been used to measure tissue residue levels (Johnson et al. 1978; Sánchez-Chardi and Nadal 2007), genetic (Husby et al. 1999; Theodorakis et al. 2001; Topashka-Ancheva et al. 2003), enzymatic (Reynolds et al. 2006), and morphological (Santolo 2009) biomarkers, and alterations in population and community structure (Phelps and McBee 2009, 2010) at anthropogenically disturbed sites.

Natural history of Peromyscus leucopus -- *Peromyscus leucopus* ranges from southern Canada to the Yucatán peninsula and from the eastern United States to Arizona and New Mexico (Baker 1968). This species occupies a variety of habitats throughout its range, but some form of cover, such as brush or woody debris, is a common feature among all habitat types (Baker 1968; Lackey et al. 1985). *P. leucopus* is considered nocturnal, but feeds diurnally during the winter. Females generally mature from 37 to 45 days of age and the

gestation period generally lasts 22 or 23 days. Litters range from 3 to 5 pups, with larger litters found in northern portions of the range; however, animals in southern portions of the range have a year-round reproductive cycle (Layne 1968; Lackey et al. 1985). Populations of *P. leucopus* generally turn over annually due to high mortality rates, but mortality rates vary seasonally (Lackey et al. 1985).

Peromyscus leucopus has a well-characterized karyotype with a diploid number of 48 and a fundamental number of 70 to 72 (Lackey et al. 1985; Greenbaum et al. 1994). It has been used as an animal model in several studies of genotoxicity (McBee et al. 1987; Shaw-Allen and McBee 1993; Husby et al. 1999; Meier et al. 1999). Baker et al. (1983) found 2 distinct karyotypic races within *P. leucopus* that differ in presence of pericentric inversions in pairs 5, 11, and 20. The karyotypic races have a distinct zone of hybridization that has been identified in populations in Mississippi, Tennessee, and Oklahoma (Baker et al. 1983). The hybrid zone in Oklahoma has been studied using chromosomal, allozymic, mtDNA, and microsatellite analyses and all studies found asymmetrical gene flow from northeast to southwest across the hybrid zone (Baker et al. 1983; Stangl and Baker 1984; Nelson et al. 1987; Schmidt 1999). Stangl and Baker (1984) found that the karyotypic races did not correspond to recognized subspecies of *P. leucopus* and suggested that the chromosomal races are morphologically distinct phenotypes.

Wildlife ecotoxicology at Tar Creek Superfund Site -- Several studies have used resident wildlife species to evaluate effects of heavy metal contamination in the Tri-State Mining District. Beyer et al. (2004) measured tissue residue levels, enzymatic activity, and presence of histopathological lesions in populations of 13 species of birds collected throughout the Tri-State Mining District. Significantly elevated levels of Pb and Cd were found in all

species, but elevated Zn levels were present only in mallards (*Anas platyrhynchos*). Activity of aminolevulinic acid dehydratase (ALAD), an enzyme necessary for heme synthesis, is negatively correlated with Pb exposure. ALAD activity decreased by > 50% in American Robins (*Turdus migratorius*), Northern Cardinals (*Cardinalis cardinalis*), and *A. platyrhynchos* collected within the Tri-State Mining District when compared with animals from reference sites. Despite tissue levels of Cd and Pb that are typically indicative of Pb and Cd poisoning, no histopathological lesions were observed (Beyer et al. 2004). A single incident of Zn toxicosis in a trumpeter swan (*Cygnus buccinator*) collected in a mill pond near Picher, OK, was reported by Carpenter et al. (2004). Elevated Zn concentrations (11.2 ppm) were detected in serum, liver, kidney, and pancreas, and histopathological lesions were detected in pancreas and kidney (Carpenter et al. 2004). Conder and Lanno (1999) found significantly elevated levels of Pb in mandibles of white-tailed deer (*Odocoileus virginianus*) collected from TCSFS when compared with individuals from reference sites. Zn levels did not differ among sites and Cd levels were below detection limits for all sites (Conder and Lanno 1999). Schmitt et al. (2006) measured metal levels in crayfish (*Orconectes* spp.) and six fish species commonly consumed by local residents, common carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*), flathead catfish (*Pylodictis olivaris*), largemouth bass (*Micropterus salmoides*), spotted bass (*M. punctulatus*), and white crappie (*Pomoxis annularis*), collected from the Spring and Neosho Rivers and several tributaries within TCSFS. Elevated levels of Pb, Cd, and Zn were detected in all species from within TCSFS when compared with fish from reference sites. Metal levels were higher in animals collected from the Spring River than those from the Neosho River and fish collected in tributaries had higher tissue metal concentrations than fish in both rivers. The highest metal levels were

detected in *Orconectes* spp. and *C. carpio*. Schmitt et al. (2006) suggested that metal levels in crayfish and fish from TCSFS may cause adverse health effects in humans and other wildlife species if consumed.

Roark and Brown (1996) found significant differentiation in allozymes of three enzymes in populations of mosquitofish (*Gambusia affinis*), bluntnose minnow (*Pimephales notalus*), and blackstripe topminnow (*Fundulus notatus*) inhabiting a creek containing elevated levels of Pb and Cd when compared with a reference creek. Populations of *P. notalus* and *F. notatus* had higher proportions of heterozygous genotypes in the contaminated creek, but no difference was detected in the *G. affinis* population. *In vitro* experiments indicated that two of the enzymes showed allozyme inhibition when exposed to Zn, but no inhibition was caused by Pb (Roark and Brown 1996). Hays and McBee (2007) used flow cytometry to measure genetic effects of metal exposure on red-eared slider turtles (*Trachemys scripta*). Turtles collected from TCSFS had a significantly higher incidence of aneuploidy, an indicator of non-disjunction during mitosis, than animals from reference sites. Blood Pb levels did not differ significantly among sites, but elevated blood Cd was detected in *T. scripta* from TCSFS (Hays and McBee 2007).

Only a few studies of small mammals have been conducted at TCSFS. Phelps and McBee (2009, 2010) investigated the structure and composition of small mammal communities at two sites within TCSFS compared to reference sites. Small mammal communities within TCSFS had significantly reduced species diversity, including richness and evenness, when compared with reference sites. Both sites within TCSFS were dominated by *P. leucopus*, which represented approximately 60% of the individuals collected at both sites (Phelps and McBee 2009). Population size, survival and recapture estimates,

reproductive success, and mean minimum longevity did not differ among populations (Phelps and McBee 2010). Coolon et al. (2010) studied the composition and diversity of intestinal microbial communities in *P. leucopus* and the deer mouse (*Peromyscus maniculatus*) from TCSFS. There was no difference in microbial diversity between contaminated and reference sites; however, bacterial assemblages did differ with more resistant taxa present in mice from contaminated sites. Additionally, this study found that both species collected from metal-contaminated sites had reduced body size and lower fat content (Coolon et al. 2010).

Toxicity of metals -- Anthropogenic activities, such as mining, smelting, and burning of fossil fuels, play a large role in the increased, worldwide distribution of metals into air, soil, water, and food (Goyer and Clarkson 2001). In nature, metals exist in inorganic forms as ions or salts and as organic forms in combination with organic compounds (Caussy et al. 2003). Thirty metals are known to have toxic effects on neurological, hematological, reproductive and endocrine systems and can be carcinogenic, clastogenic, teratogenic, or genotoxic (Goyer and Clarkson 2001). Toxicity of metals depends on interaction with essential metals, formation of metal-protein complexes, age, stage of development, and immune status of the host, and chemical form or speciation of the metal (Goyer and Clarkson 2001). Uptake of Pb and Cd can be influenced by presence of essential compounds, including calcium, iron, and Vitamins C and D. Additionally, there is an inverse relationship between dietary protein content and toxicity of Pb and Cd (Goyer and Clarkson 2001). Metallothioneins, a class of over 40 metal-binding proteins, have a wide range of functions, including essential metal homeostasis and protection against metal toxicity. The common protein, albumin, aids in movement and disposition of metals in the body (Goyer and Clarkson 2001). The bioavailability of inorganic and organic metals is strongly dependant upon oxidation state. Pb

exists in oxidation states II and IV; however, in most inorganic compounds it exists in the II oxidation state. Zn and Cd are both found in divalent states in nature. All three of these metals can form a variety of organic and inorganic complexes (Bradl et al. 2005). The complexes or species in which metals exist have different properties that can influence bioavailability and fate (Caussy et al. 2003).

Metals as endocrine disruptors -- Endocrine disrupting compounds are capable of altering the function of hormonal systems by mimicking naturally occurring hormones, blocking hormone receptors, or inhibiting hormone metabolism (Tyler et al. 1998; Diamanti-Kandarakis et al. 2009). Endocrine disruptors can be synthetic or naturally occurring compounds. At least five metals, i.e. mercury, arsenic, uranium, Pb, and Cd, have demonstrated endocrine disrupting properties (Tyler et al. 1998; Dyer 2007). Study of endocrine disruptors is relatively new. Since the 1990's emphasis has been placed on studies of potential deleterious effects of endocrine disrupting compounds on laboratory animals, wildlife, and humans (Dyer 2007; Hotchkiss et al. 2008).

Srivastava et al. (2004) found that low doses of Pb decreased ovarian mRNA and protein levels in rats (*Rattus norvegicus*) and that these reductions could be reversed by gonadotropins. This study concluded that Pb elicits endocrine effects via the hypothalamic-pituitary-ovarian axis by altering release of luteinizing hormone by the ovaries (Srivastava et al. 2004). Pb does not activate the estrogen receptor or act as an estrogen mimic (Choe et al. 2003; Dyer 2007). Cd acts as an estrogen mimic and is capable of forming a high-affinity complex with estrogen receptors (Takiguchi and Yoshihara 2006). Johnson et al. (2003) found that ovarectomized *R. norvegicus* dosed with 0.5 and 5 µg/kg Cd showed increased

uterine weight, earlier onset vaginal opening, and increased mammary development relative to untreated individuals.

Metals as genotoxicants -- Many xenobiotics are clastogenic and cause alterations in chromosomal structure (Shugart 1999; Bryant 2007). Clastogenic mutagens can act in germ cells or somatic cells via: formation of DNA adducts, induction of oxidative stress, alteration of signal transduction pathways, interference with DNA repair systems, or alteration in DNA replication (Shugart 1999; Beyersmann and Hartwig 2008). Germ cell mutations can lead to lowered fecundity and can be passed on as heritable genetic mutations. Somatic mutations can result in tumor formation, induction and progression of cancer, and formation of tumor metastases. Genetic alterations in somatic cells are some of the easiest to detect because many types of somatic cells can be analyzed and there are numerous biomarkers, including flow cytometry, COMET analysis, micronucleus analysis, and metaphase chromosome analysis, that have been developed to detect structural alterations (McBee and Lochmiller 1996; Bickham et al. 2000) A much broader scope of physiological effects including metaplasias, heart disease, and cataracts also can be induced by somatic mutations (Hartman 1983).

Numerical and structural chromosome aberrations are useful biomarkers with which to measure disruption of cytogenetic structure in natural populations (Shugart 1999; Dmitriev and Zakharov 2001). Structural aberrations, including chromosome and chromatid breaks, ring chromosomes, acentric fragments, and dicentric chromosomes, are often present at very low frequencies; however, increased frequencies and persistence of genetic insults can lead to further response at other levels of organization (Shugart 1999; Bryant 2007). Chromosome aberrations occur spontaneously at low frequencies, but their frequency may be increased by

exposure to physical and chemical stressors (Bryant 2007). Tanaka et al. (2008) found spontaneous chromatid-type aberrations to occur in 0.069% of cells in mice (*Mus musculus*).

Although several metals, including Pb, Cd, and Zn, have been documented to increase incidence of structural chromosomal aberrations, the mechanisms by which they cause chromosomal lesions are unknown (Hartwig 1994; Shugart 1999). Cd and Pb may cause chromosomal lesions by altering the redox state within cells and releasing free radicals that can damage DNA (Hartwig et al. 2002; Beyersmann and Hartwig 2008). Cd can reduce the amount of antioxidative enzymes such as glutathione reductase, glutathione peroxidase, catalase, and superoxide dismutase in cells, potentially leading to DNA damage by reactive oxygen species (Beyersmann and Hartwig 2008). Pb compounds given at toxic doses also cause DNA strand breaks and may enhance effects of other mutagens at lower doses (Roy and Rossman 1992).

Metals and population genetic structure -- In addition to assessment of genetic integrity within individuals, it is also imperative that we understand how anthropogenic disturbance alters genetic structure at the population level. Recent emphasis has been placed on population and ecosystem-level effects due to contaminant exposure. Habitat destruction and fragmentation can lead to reduction in population size, increased isolation, and reduced gene flow (Bickham et al. 2000; Medina et al. 2007). Bickham et al. (2000) proposed that somatic effects, seen as structural chromosomal aberrations, can lead to stress or adverse health effects, which can result in mortality or decreased reproduction leading to population bottlenecks that ultimately result in reduced genetic diversity at the population level.

Earthworm (*Lumbricus rubellus*) populations exposed to sewage sludge containing Cd, Pb, Zn, and copper (Cu) had significantly different allele and genotype frequencies and

higher multi-locus heterozygosity than reference populations. Additionally, unique alleles were detected in worms from exposed populations that were not present in reference populations (Peles et al. 2003). Martins et al. (2009) used AFLP analysis to assess populations of historically exposed *Daphnia longispina* from a site contaminated with mine drainage containing Pb, Cd, and Zn. Exposed populations showed no significant reduction in genetic diversity; however, populations from the mine-disturbed habitat showed significantly different population genetic structure than reference populations (Martins et al. 2009).

Anogenital distance and endocrine disruptors -- Anogenital distance, the distance between the genital papilla and anus, has been used in several laboratory studies to measure effects of endocrine disrupting compounds (Gray et al. 2006). In mammals, AGD is determined by fetal androgen exposure and is sensitive to litter composition, uterine position, and *in utero* exposure to some endocrine disrupting compounds (Cantoni et al. 1999; Hotchkiss and Vandenbergh 2005). Laboratory studies have documented that exposure to compounds with estrogenic activity results in a decreased AGD, relative to body weight, in male *M. musculus* and *R. norvegicus* (Gray et al. 2006); however, only one study has measured AGD in wild rodents (Santolo 2009).

Gray et al. (2000) found that male *R. norvegicus* treated with benzylbutyl and diethylhexyl phthalates (0.75 g/kg/d) had significantly reduced AGD when compared with control animals. No effect was seen in female *R. norvegicus.* Both compounds reduced the sexual dimorphism in AGD by > 50% (Gray et al. 2000). Santolo (2009) measured AGD in *P. maniculatus*, *M. musculus*, and western harvest mice (*Reithrodontomys megalotis*) exposed to selenium via agricultural drainwater. In all three species, mice from the contaminated site had significantly higher levels of selenium in liver tissue, reduced body

condition, and enlarged livers. Male *P. maniculatus* showed reduced AGD, relative to body length, when compared with reference animals (Santolo 2009).

Metaphase chromosome analysis -- Metaphase chromosome analysis allows for visual identification of many classes of chromosomal damage, including chromosome and chromatid breaks, ring chromosomes, and acentric fragments. This method has been used successfully in field and laboratory studies of wildlife species (McBee and Bickham 1987; Topashka-Ancheva et al. 2003, Alimba et al. 2006). Topashka-Ancheva et al. (2003) used metaphase chromosome analysis to assess chromosomal aberrations in the Macedonian mouse (*Mus macedonicus*), sibling vole (*Microtus epiroticus*), yellow-necked field mouse (*Apodemus flavicollis*), and *C. glareolus* collected from an industrial site contaminated with Cu, Pb, Cd, and Zn. Individuals of all species from sites with high levels of Pb and Cd had the highest percentage of chromosomal aberrations (Topashka-Ancheva et al. 2003). Alimba et al. (2006) found dose dependent increases in the presence of structural chromosomal aberrations and significantly lower mitotic indices in bone marrow cells of *R. norvegicus* dosed with leachates containing heavy metals from municipal landfills.

AFLP as an ecotoxicological tool -- Historically, allozymic data or microsatellites have been used to assess genetic structure of populations; however, these methods require previous sequence data for the organism of interest. Analysis of AFLP allows for screening of a larger portion of the genome and requires no previous knowledge of the genome of the study organism (Vos et al. 1995; Bagley et al. 2001). Only small amounts (ca. 50 ng) of nuclear DNA are required for AFLP analysis. AFLP has been used to determine population genetic structure, presence of hybridization and inbreeding, and reconstruction of phylogenies in plants, bacteria, and fungi (Bensch and Âkesson 2005).

Dasmahapatra et al. (2008) used AFLP to determine genetic diversity and inbreeding in wild and captive-bred old field mice (*Peromyscus polionotus subgriseus*). Inbreeding coefficients measured by AFLP and microsatellite analysis were highly correlated and matched with pedigree-based coefficients. They suggested that AFLP is a valuable tool in measuring genetic differentiation in wild populations and produces similar results to microsatellite analysis, which is often more labor intensive (Dasmahapatra et al. 2008).

AFLP has not been used widely to investigate genetic structure in wild populations of vertebrates; however, AFLP has the potential to aid in providing information on genetic structure of populations in highly disturbed habitats (Bensch and Âkesson 2005).Takami et al. (2004) used AFLP to examine genetic diversity and population structure of rural and urban populations of cabbage white (*Pieris rapae*) and gray-veined butterflies (*Pieris melete*) to determine if human activities in urban habitats alter population genetic structure. No difference in genetic diversity was detected between rural and urban populations when individuals were pooled across sampling periods; however, seasonal and temporal subdivisions were detected in populations of both species (Takami et al. 2004).

SUMMARY

Several studies have employed ecotoxicological methods to assess wild populations at TCSFS; however, many of these studies investigated only a single endpoint. My study used three endpoints, measurement of AGD, metaphase chromosome analysis, and AFLP analysis to study *P. leucopus* from TCSFS. The uptake of Cd, Pb, and Zn has been well-documented at TCSFS (Beyer et al. 2004; Carpenter et al. 2004; Moeller 2004; Hays and McBee 2007).

Measurement of AGD has been used primarily in laboratory studies of endocrine disrupting compounds. Cd has been documented to have estrogenic properties including increased mammary development and uterine weight in females and reduced AGD in males (Johnson et al. 2003; Couto-Moraes et al. 2010). Only one study, Santolo (2009), has used measurement of AGD in wild populations of rodents exposed to xenobiotics. Xenobiotics can cause structural chrom0somal aberrations via numerous mechanisms (Bryant 2007). Chromosomal aberrations have been measured in wild populations exposed to metals and other xenobiotics (McBee et al. 1987; Husby et al. 1999; Alimba et al. 2006) and methods for preparation and scoring of metaphase chromosome spreads are well tested and standardized (Preston et al., 1987; Baker and Qumsyieh 1988). Population genetic structure may be altered in populations exposed to contaminants due to population bottlenecks; reduced survivorship, fecundity, or recruitment; induced mutations; or selection for tolerant genotypes (Bickham et al. 2000). AFLP analysis has not been broadly used in vertebrate taxa exposed to xenobiotics, but has been shown to produce measures of genetic differentiation and inbreeding similar to those measured by microsatellites (Dasmahapatra et al. 2008).

REFERENCES

Alimba CG, Bakare AA, Latunji CA (2006) Municipal landfill lechates induced chromosome aberrations in rat bone marrow cells. African J Biotechnol 5:2053-2057

Bagley ML, Anderson SL, May B (2001) Choice of methodology for assessing genetic impacts of environmental stressors: polymorphism and reproducibility of RAPD and AFLP fingerprints. Ecotoxicol 10:239-244

Baker RH (1968) Habitats and distribution. In: King JA (ed) Biology of *Peromyscus* (Rodentia). American Society of Mammalogists, Lawrence, pp 98-126

Baker RJ, Robbins LW, Stangl Jr. FB, Birney EC (1983) Chromosomal evidence for a major subdivision in *Peromyscus leucopus*. J Mammal 64:356-359

Baker RJ, Qumsiyeh MB (1988) Methods in chiropteran mitotic chromosomal studies. In: Kunz T (ed) Ecological and behavioral methods for the study of bats. Smithsonian Institution Press, Washington D.C., pp 425-435

Bensch S, Âkesson M (2005) Ten years of AFLP in ecology and evolution: why so few animals? Mol Ecol 14:2899-2914

Beyer WN, Dalgam J, Dudding S, French JB, Mateo R, Miesner J, Sileo L, Spann J (2004) Zinc and lead poisoning in wild birds in the Tri-State mining district (Oklahoma, Kansas, and Missouri). Arch Environ Contam Toxicol 48:108-117

Beyersmann D, Hartwig A (2008) Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. Arch Toxicol 82:493-512

Bickham JW, Sandhu S, Hebert PDN, Chikhi L, Athwal R (2000) Effects of chemical contaminants on genetic diversity in natural populations: implications for biomonitoring and ecotoxicology. Mutat Res 463:33-51

Bradl H, Kim C, Kramar U, Stüben D (2005) Interactions of heavy metals. In: Bradl HB (ed) Heavy metals in the environment: origin, interaction and remediation. Elsevier, Amsterdam, pp 28-164

Bryant PE (2007) Origin of chromosomal aberrations: mechanisms. In: Obe G, Vijayalaxmi (eds) Chromosomal alterations: methods, results, and importance in human health. Springer, New York, pp 177-199

Cantoni D, Glaizot O, Brown RE (1999) Effects of sex composition of the litter on anogenital distance in California mice (*Peromyscus californicus*). Canadian J Zool 77:124-131

Carpenter JW, Andrews GA, Beyer WN (2004) Zinc toxicosis in a free-flying trumpeter swan (*Cygnus buccinator*). J Wild Dis 40:769-774

Caussy D, Gochfeld M, Gurzau E, Neagu C, Ruedel H (2003) Lessons from case studies of metals: investigating exposure, bioavailability, and risk. Ecotoxicol Environ Saf 56:45-51

Choe SY, Kim SJ, Kim HG, Lee JH, Choi Y, Lee H, Kim Y (2003) Evaluation of estrogenicity of major heavy metals. Sci Tot Environ 312:15-21

Conder JM, Lanno RP (1999) Heavy metal concentrations in mandibles of white-tailed deer living in the Picher Mining District. Bull Environ Contam Toxicol 63:80-86

Coolon JD, Jones KL, Narayanan S, Wisley SM (2010) Microbial ecological response of the intestinal flora of *Peromyscus maniculatus* and *P. leucopus* to heavy metal contamination. Mol Ecol 19:67-80

Couto-Moraes R, Felicio LF, Bernardi MM (2010) Post-partum testosterone administration does not reverse the effects of perinatal exposure to cadmium on rat offspring development. J Appl Toxicol 30:233-241

Dasmahapatra KK, Lacy RC, Amos W (2008) Estimating levels of inbreeding using AFLP markers. Heredity 100:286-295

Diamanti-Kandarakis E, Bourguignon J, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC (2009) Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endo Rev 30:293-342

Dmitriev SG, Zakharov VM (2001) Estimate of cytogenetic homeostasis in natural

populations of some small murid rodents. Russian J Devel Biol 32:373-380

Dyer CA (2007) Heavy metals as endocrine-disrupting chemicals. In: Gore AC (ed) Endocrine-disrupting chemicals: from basic research to clinical practice. Humana Press Inc., Totowa, pp 111-133

Goyer RA, Clarkson TW (2001) Toxic effects of metals. In: Klassen CD (ed) Casarett and Doull's toxicology: the basic science of poisons. McGraw-Hill, New York, pp 811-867

Gray Jr. LE, Ostby J, Furr J, Price M, Veeramachaneni DN, Parks L (2000) Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. Toxicol Sci 58:350-365

Gray Jr. LE, Wilson VS, Stoker T, Lambright C, Furr J, Noriega N, Howdeshell K, Ankley GT, Guillette L (2006) Adverse effects of environmental antiandrogens and androgens on reproductive development in mammals. Intl J Androl 29:96-104

Greenbaum IF, et al. (1994) Cytogenetic nomenclature of deer mice, *Peromyscus* (Rodentia): revision and review of the standardized karyotype. Cytogen Cell Genet 66:181-195

Hartman PE (1983) Mutagens: some possible health impacts beyond carcinogenesis. Environ Mutagen 5:139-152

Hartwig A (1994) Role of DNA repair inhibition in lead- and cadmium- induced genotoxicity: a review. Environ Health Persp 102:45-50

Hartwig A, Asmuss M, Ehleben I, Herzer U, Kostelac D, Pelzer A, Schwerdtle T, Bürkle A (2002) Interference by toxic metal ions with DNA repair processes and cell cycle control: molecular mechanisms. Environ Health Persp 110:797-799

Hays KA, McBee K (2007) Flow cytometric analysis of red-eared slider turtles (*Trachemys scripta*) from Tar Creek Superfund Site. Ecotoxicol 16:353-361

Hotchkiss AK, Vandenbergh JG (2005) The anogenital distance index of mice (*Mus musculus domesticus*): an analysis. J American Assoc Lab An Sci 44:46-48

Hotchkiss AK, Rider CV, Blystone CR, Wilson VS, Hartig PC, Ankley GT, Foster PM, Gray CL, Gray LE (2008) Fifteen years after "Wingspread" – environmental endocrine disrupters and human and wildlife health: where we are today and where we need to go. Toxicol Sci 105:235-259

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

Johnson MS, Roberts RD, Hutton M, Inskip MJ (1978) Distribution of lead, zinc, and cadmium in small mammals from polluted environments. Oikos 30:153-159

Johnson MD, Kenney N, Stoica A, Hilakivi-Clarke L, Singh B, Chepka G, Clarke R, Sholler PF, Lirio AA, Foss C, Reiter R, Trock B, Paik S, Martin MB (2003) Cadmium mimics the *in vivo* effects of estrogen in the uterus and mammary glands. Nat Med 9:1081-1084

Kirkwood NG (2007) CHAT: approaches to long-term planning for the Tar Creek Superfund site, Ottawa County, Oklahoma. In: Macey GP, Cannon JZ (eds) Reclaiming the land: rethinking Superfund institutions, methods and practices. Springer, New York, pp. 267-292

Lackey JA, Huckaby DG, Ormiston BG (1985) Peromyscus leucopus. Mammal Spec 247:1-10

Layne JN (1968) Ontogeny. In: King JA (ed) Biology of *Peromyscus* (Rodentia). American Society of Mammalogists, Lawrence, pp 148-254

Martins N, Bollinger C, Harper RM, Ribeiro R (2009) Effects of acid mine drainage on the genetic diversity and structure of a natural population of *Daphnia longispina*. Aquat Toxicol 92:104-112

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

McBee K, Lochmiller RL (1996) Wildlife toxicology in biomonitoring and bioremediation: implications for human health. In: Bloom AD, de Serres F (eds) Ecotoxicity and human health: a biological approach to environmental remediation. CRC Press, Boca Raton, pp. 163-181

Medina MH, Correa JA, Barata C (2007) Micro-evolution due to pollution: possible consequences for ecosystem responses to toxic stress. Chemosphere 67:2105-2114

Meier JR, Wernsing P, Torsella J (1999) Feasibility of micronucleus methods for monitoring genetic damage in two feral species of small mammals. Environ Mol Mutagen 33:219-225

Moeller LA (2004) Effects of metal contamination on developing red-eared slider turtles (*Trachemys scripta*) and implications for the species as a biomonitor. Thesis, Oklahoma State University

Nelson K, Baker RJ, Honeycutt RL (1987) Mitochondrial DNA and protein differentiation

between hybridizing cytotypes of the white-footed mouse, *Peromyscus leucopus*. Evol 41:864-872

Peakall DB, McBee K (2001) Biomarkers for contaminant exposure and effects in mammals. In: Shore RF, Rattner BA (eds) Ecotoxicology of wild mammals. John Wiley & Sons, New York, pp 551-576

Peles JD, Towler WI, Guttman SI (2003) Population genetic structure of earthworms (*Lumbricus rubellus*) in soils contaminated by heavy metals. Ecotoxicol 12:379-386

Phelps KL, McBee K (2009) Ecological characteristics of small mammal communities at a Superfund site. American Mid Nat 161:57-68

Phelps KL, McBee K (2010) Population parameters of *Peromyscus leucopus* (white-footed mice) inhabiting a heavy metal contaminated Superfund site. Southwest Nat 55:363-373

Preston RJ, Dean BJ, Galloway S, Holden H, McFee AF, Shelby M (1987) Mammalian *in vivo* cytogenetic assays. Analysis of chromosome aberrations in bone marrow cells. Mut Res 189:157-165

Rattner BA (2009) History of wildlife toxicology. Ecotoxicol 18:773-783

Reynolds KD, Schwarz MS, McFarland CA, McBride T, Adair B, Strauss RE, Cobb GP, Hooper MJ, McMurry ST (2006) Northern pocket gophers (*Thomomys talpoides*) as biomonitors of environmental metal contamination. Environ Toxicol Chem 25:458-469

Roark S, Brown K (1996) Effects of metal contamination from mine tailings on allozyme distributions of populations of Great Plains fishes. Environ Toxicol Chem 15:921-927

Roy NK, Rossman TG (1992) Mutagenesis and comutagenesis by lead compounds. Mut Res

298:97-103

Sánchez-Chardi A, Nadal J (2007) Bioaccumulation of metals and effects of landfill pollution in small mammals. Part I. the greater white toothed shrew (*Crocidura russula*). Chemosphere 68:703-711

Santolo GM (2009) Small mammals collected from a site with elevated selenium concentrations and three reference sites. Arch Environ Contam Toxicol 57:741-754

Schmidt CA (1999) Variation and congruence of microsatellite markers for *Peromyscus leucopus*. J Mammal 80:522-529

Schmitt CJ, Brumbaugh WG, Linder GL, Hick JE (2006) A screening-level assessment of lead, cadmium, and zinc in fish and crayfish from northeastern Oklahoma, USA. Environ Geochem Health 28:445-471

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

Sheffield SR, Sawicka-Kapusta K, Cohen JB, Rattner BA (2001) Rodentia and Lagomorpha. In: Shore RF, Rattner BA (eds) Ecotoxicology of wild mammals. John Wiley & Sons, New York, pp 215-314

Shugart LR (1999) Structural damage to DNA in response to toxicant exposure. In: Forbes VE (ed) Genetics and ecotoxicology. Taylor and Francis Publishers, Philadelphia, pp 151-167

Sieg CH (1987) Small mammals: pests or vital components of the ecosystem. In: Proceedings, Great Plains wildlife damage control workshop, Rapid City, pp 88-92

Srivastava V, Dearth RJ, Hiney JK, Ramirez LM, Bratton GR, Dees WL (2004) The effects

of low-level Pb on steroidogenic acute regulatory protein (StAR) in the prepubertal rat ovary. Toxicol Sci 77:35-40

Stangl, Jr. FB, Baker RJ (1984) A chromosomal subdivision in *Peromyscus leucopus*: implications for the subspecies concept as applied to mammals. J Mammal 64:356-359

Takami Y, Koshio C, Ishii M, Fujii H, Hidaka T, Shimizu I (2004) Genetic diversity and structure of urban populations of *Pieris* butterflies assessed using amplified fragment length polymorphism. Mol Ecol 13:245-258

Takiguchi M, Yoshihara S (2006) New aspects of cadmium as endocrine disruptor. Environ Sci 13:107-116

Talmage SS, Walton BT (1991) Small mammals as monitors of environmental contaminants. Rev Environ Contam Toxicol 119:47-145

Tanaka K, Kohda A, Toyokawa T, Ichinohe K, Oghiso Y (2008) Chromosome aberration frequencies and chromosome instability after long-term exposure to low-dose-rate gamma-irradiation. Mut Res 657:19-25

Theodorakis CW, Bickham JW, Lamb T, Medica PA, Lynne TB (2001) Integration of genotoxicity and population genetic analyses in kangaroo rats (*Dipodomys merriami*) exposed to radionuclide contamination at the Nevada Test Site, USA. Environ Toxicol Chem 20:317-326

Topashka-Ancheva M, Metcheva R, Teodorova S (2003) A comparative analysis of the heavy metal loading of small mammals in different regions of Bulgaria II: chromosomal aberrations and blood pathology. Ecotoxicol Environ Saf 54:188-193

Tyler CR, Jobling S, Sumpter JP (1998) Endocrine disruption in wildlife: a critical review of

the evidence. Crit Rev Toxicol 28:319-361

USEPA, United States Environmental Protection Agency (1997) Record of decision: residential areas, Operable Unit 2, Tar Creek Superfund site, Ottawa County, Oklahoma. USEPA, Region 6, Dallas, Texas

USEPA, United States Environmental Protection Agency (2005) Five-year review report: third five-year review report for the Tar Creek Superfund site, Ottawa County, Oklahoma. USEPA, Region 6, Dallas, Texas

USEPA, United States Environmental Protection Agency (2010) Tar Creek (Ottawa County) Oklahoma: current status. USEPA, Region 6, Dallas, Texas

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucl Acid Res 23:4407-4414

Weidman S, Williams CF, Anderson CO (1932) Miami-Picher lead-zinc district. University of Oklahoma Press, Norman

Wilson DE, Reeder DM (2005) Mammal species of the world: a taxonomic and geographic reference, 3rd edn. Johns Hopkins University Press, Baltimore

CHAPTER II

ANOGENITAL DISTANCE IN WHITE-FOOTED MICE (*PEROMYSCUS LEUCOPUS*) FROM A METAL CONTAMINATED SUPERFUND SITE

Abstract -- Although not typically measured in field studies of small mammals, anogenital distance has been measured in laboratory studies of rodents exposed to endocrine disrupting compounds. Cadmium, an endocrine disrupting compound with estrogenic properties, is one of the main contaminants at Tar Creek Superfund Site. I measured anogenital distance of 117 white-footed mice (*Peromyscus lecuopus*) from Tar Creek Superfund Site and two unmined, reference sites. I found significant sexual dimorphism in anogenital distance and a significant site*reproductive status effect was seen in males and females; however, no indication of contaminant effects was seen in this study.

Anogenital distance (AGD), the distance between the genital papilla and anus, is determined by *in utero* hormone exposure. In mammals, AGD is a morphological indicator of fetal androgen action and is sensitive to litter composition, uterine position,

and *in utero* exposure to some endocrine disrupting compounds (Cantoni et al. 1999; Hotchkiss and Vandenbergh 2005). In rodents, AGD is sexually dimorphic with males having longer AGD than females. A positive correlation between body mass and AGD is seen in both sexes (Gallavan et al. 1999). High levels of within-population variation in AGD in laboratory populations of rodents have been documented (McIntyre et al. 2001; Manno 2008), but AGD is not a standard measurement taken in evaluation of wild populations of rodents so there is little knowledge regarding variation in AGD in wild populations. It has been measured in only one field study of deer mice (*Peromyscus maniculatus*), western harvest mice (*Reithrodontomys megalotis*), and house mice (*Mus musculus*) (Santolo 2009).

Tar Creek Superfund Site (TCSFS) is a 10,360-ha portion of the Tri-State Mining District located in Ottawa Co., Oklahoma, that was heavily mined for lead (Pb) and zinc (Zn) from the 1890's until the 1970's. TCSFS is contaminated with Pb, Zn, and Cd and was placed on the United States Environmental Protection Agency's National Priority List in 1983. Although not mined as a commodity at TCSFS, Cd was released during the Zn mining process (USEPA 2005). Soil from two sites within TCSFS contained total Cd levels ranging from 20 to 56 mg/kg and soil removed during restoration of residential properties contained Cd in excess of 100 mg/kg (Moeller 2004; USEPA 2005). Only minor remediation has occurred since mining ceased, leaving a large area of disturbed habitat and 75 million tons of mine tailings (USEPA 2005). I measured AGD in wild-caught, male and female white-footed mice (*Peromyscus leucopus*) from TCSFS and

matched reference sites to investigate changes in AGD in animals potentially exposed to an endocrine disrupting compound *in situ*.

Endocrine disrupting compounds are synthetic or natural substances that can alter hormonal or homeostatic systems. Generally, endocrine disruptors elicit effects by mimicking naturally occurring hormones, blocking hormone receptors, or inhibiting hormone metabolism; however, specific action occurs on numerous receptors and pathways, including nuclear receptors, non-nuclear steroid hormone receptors, non-steroid receptors, orphan receptors, and enzymatic pathways involved in steroid biosynthesis or metabolism (Tyler et al. 1998; Diamanti-Kandarakis et al. 2009). A number of xenobiotics have been documented to have endocrine disrupting properties, including organochlorine pesticides, polychlorinated biphenyls, phthalates, and some metals (Tyler et al. 1998). AGD is controlled by androgen action during fetal development and has been used as a bioassay of exposure to estrogenic and antiandrogenic endocrine disruptors (Tyler et al. 1998).

McIntyre et al. (2001) found that male rats (*Rattus norvegicus*) exposed *in utero* to flutamide, an antiandrogenic treatment for prostate cancer, showed significantly reduced AGD at postnatal day 1 and reduced AGD was detected through adulthood in those individuals. The same study also found that flutamide-induced reductions in AGD were highly correlated with increased incidence of reproductive tract malformations (McIntyre et al. 2001). Male *R. norvegicus* exposed to low doses (0.1 to 1 µg/kg/d) of 2, 3, 7, 8-tetrachlorodibenzodioxin during sexual differentiation showed alterations in the

reproductive system consistent with demasculinization. At puberty, exposed males had reduced AGD, sperm count, epididymis weight, and seminiferous tubule diameter (Mably et al. 1992). Santolo (2009) studied wild populations of *P. manicultaus, R. megalotis*, and *M. musculus* exposed to selenium. Non-reproductive male *P. maniculatus* exposed to selenium had smaller AGD than reference animals, but this relationship was not seen in reproductive males or in *M. musculs* and *R. megalotis*. Exposed populations of all three species also had significantly elevated levels of liver selenium, enlarged livers, and reduced body condition (Santolo 2009).

Several laboratory-based studies have documented the estrogenic potential of cadmium (Cd), (Takiguchi and Yoshihara 2006); however Cd's mechanism of action is still not completely understood. Stoica et al. (2000) found that Cd blocks the binding of estradiol to estrogen receptor-α and can activate the receptor by activating the hormone binding domain. Ovarectomized *R. norvegicus* dosed with Cd had increased mammary development and uterine weight relative to control animals, suggesting the activation of estrogen receptors by Cd (Johnson et al. 2003). Couto-Moraes et al. (2010) found that *R. norvegicus* exposed to 10 and 20 mg/kg Cd *in utero* and through 7 days of lactation had reduced AGD and body length when compared with control animals on postnatal day 1. Treatment with testosterone reversed these effects in the 10 mg/kg dose group by postnatal day 21, but reversed only body length effects in the 20 mg/kg dose group (Couto-Moraes et al. 2010).

I hypothesized that male *P. leucopus* from TCSFS would have significantly smaller AGD than males from reference sites that AGD in female *P. leucopus* would not differ among the sites. Additionally, I compared the level of variation in AGD in wild populations of *P. leucopus* to determine the similarity of AGD measurements reported in laboratory studies and by Santolo (2009).

Materials and Methods

I measured AGD in 117 adult *P. leucopus* (TCSFS, male = 28, female = 36; SNWR, male = 14; female = 11; OWMA, male = 19, female = 9). I collected *P. leucopus* from a 16-ha portion of TCSFS owned by the Quapaw Tribe of Oklahoma. An old mine and crushing operation is present at the site, which is dominated by mine tailings. Vegetation at the site is oak/hickory forest with undergrowth of poison ivy and greenbriar. SNWR is approximately 165 km south of TCSFS. The site is approximately 8,498 ha and is used for hunting and cooperative agriculture on a seasonal basis. Trapping efforts were focused around refuge headquarters. OWMA is located approximately 110 km west of TCSFS. It is managed by the Oklahoma Department of Wildlife Conservation and used for hunting and recreation on a seasonal basis. Both reference sites were selected because of similarities in plant communities and because they were far enough away from TCSFS to reduce the possibility of downstream or airborne contamination from TCSFS.

Small mammals were trapped with Sherman live traps baited with rolled oats and scratch grain and placed in edge habitat, wooded areas with deadfall, and visible small mammal runs. Trapping was conducted at TCSFS in Spring, Summer, and Fall 2009, SNWR in Spring and Summer 2009, and OWMA in Fall 2009 and Winter 2010. SNWR was abandoned and OWMA added as a reference site after the Summer 2009 trapping period due to poor trap success at SNWR. Trap effort was unequal at the sites because trapping ceased after 20 *P. leucopus*/site/season were collected. Upon capture, individuals were placed in a mesh bag and mass was determined to the nearest gram with a Pesola spring scale. Hindfoot length and AGD were measured to the nearest mm with a ruler. Pregnant or lactating females and scrotal males were categorized as reproductive and all other individuals were categorized as non-reproductive. All individuals were inspected for the presence of external parasites and any morphological abnormalities. All field methods were approved by the Oklahoma State University Institutional Animal Care and Use Committee (ACUP No. AS 066).

Anogenital distance was converted to an anogenital index (AGI) by dividing AGD (mm) by mass (g). AGI is a means to correct for body size when measuring AGD in rodents (Gallavan et al. 1999; Hotchkiss and Vandenbergh 2005). Levene's test was used to confirm homogeneity of variance. To confirm that AGD is a sexually dimorphic trait in *P. leucopus*, mean AGI was compared by use of analysis of variance (ANOVA) for males and females at each site. Pearson product-moment correlation coefficients were determined for comparisons between AGD and mass in males and females. The remaining analyses were performed on males and females separately. AGI was compared

using 2-way ANOVA with site of collection and reproductive status as factors. The effect of season of collection was compared only within sites because all sites were not trapped in all seasons.

Results and Discussion

Mean AGD values for reproductive and non-reproductive males and females pooled across season are presented in Figure 2.1. As expected, strong sexual dimorphism was present in AGD with males having a significantly larger AGD than females at all sites (TCSFS, $F_{1,63} = 395.44$, $P < 0.0001$; SNWR, $F_{1,23} = 89.13$, $P < 0.0001$; OWMA, $F_{1,26} = 46.55$, $P < 0.0001$). Mass and AGD were significantly positively correlated in males ($r_{59} = 0.56$, $P < 0.0001$) and females ($r_{54} = 0.42$, $P = 0.001$). Manno (2008) found similar correlations between AGD and mass in male (r = 0.46) and female (r = 0.69) *M. musculus*. Some studies (Gallavan et al. 1999; Santolo 2009) have used total body length to correct for size in calculation of AGI, but in mark-recapture studies of small mammals body length can be difficult to measure in live, unsedated animals.

There was a significant interaction of site and reproductive status on AGI in male *P. leucopus* ($F_{2,55} = 4.88$, $P = 0.01$); therefore, main effects were not interpreted in this analysis. *Post hoc* analysis of least squared means revealed several significant differences among site/reproductive status combinations. Reproductive males from SNWR were not included in *post-hoc* analyses because they did not meet minimum sample size requirements. Non-reproductive males from OWMA had significantly smaller AGI than

reproductive males from OWMA ($P = 0.01$) and non-reproductive males from SNWR and TCSFS ($P < 0.0001$ for both). Female *P. leucopus* also showed a significant site and reproductive status interaction ($F_{2,55} = 6.64$, $P = 0.003$) in mean AGI. Non-reproductive females from OWMA had a significantly smaller AGI than non-reproductive females from both SNWR ($P = 0.0007$) and TCSFS ($P = 0.007$).

Seasonal comparisons (Table 2.1) were not made for females from OWMA and SNWR because there were fewer than 5 individuals collected in some seasons. There was not a significant difference in AGI among seasons in females ($F_{2,33} = 1.01$, $P = 0.37$) or males ($F_{2,25} = 1.42$, $P = 0.26$) collected at TCSFS. At SNWR, males collected in spring had a significantly larger mean AGI than males collected in summer ($F_{1,13} = 6.25$, $P = 0.03$). Similarly, males collected from OWMA in fall 2009 had a significantly larger mean AGI ($F_{1,17} = 7.09$, $P = 0.02$) than males collected in winter 2010.

Significant interactions between site and reproductive status provide an indication that AGD is variable among sites of collection and that reproductive status, especially in males, should be considered when comparing AGD. I found that AGI was smaller in reproductive males than non-reproductive males at SNWR and TCSFS, but this difference was not significant at either site. Males from OWMA showed the opposite trend with non-reproductive males having significantly smaller AGI than reproductive males. The relationship between reproductive status and AGI at SNWR and TCSFS is similar to the findings of Santolo (2009) for *P. maniculatus.* He found that reproductive male *P. maniculatus* had smaller AGD, when adjusted for body length, than non-

reproductive males at all sites studied. This relationship was not seen in *M. musculus* or *R. megalotis* (Santolo 2009). AGI in reproductive and non-reproductive females was not significantly different at TCSFS, SNWR, or OWMA. Similarly, Santolo (2009) found no within site differences in AGI between reproductive and non-reproductive females in any of the three species studied.

Because climate can vary across a species' range, I suggest that reproductive status may be more appropriate to include in analyses than season of collection and will allow for comparison of AGD across wide geographic ranges. However, Zehr et al. (2001) found that female *R. norvegicus* with short AGD had different pubertal onset than females with longer AGD, suggesting that reproductive status and AGD may co-vary. Santolo (2009) recommended that only non-reproductive males should be included in field studies of AGD to remove potential variation of AGD due to reproductive status; however, in my study the sample size was insufficient to focus only on non-reproductive males. Several of the significant differences detected among males were between individuals from OWMA and the other sites. Non-reproductive males from OWMA had much smaller AGDs than other males potentially because of the low AGD values measured during winter 2010 (Table 2.1). Animals were not collected from the other sites during winter 2010 so no comparison among sites was possible. I would expect that the males collected during winter would not be reproductively active in this portion of the range and represent a large portion of the non-reproductive males from that site (Lackey et al. 1985). Differences in AGD between reproductive and non-reproductive males may be

due to skin stretching when testes are descended or changes in mass associated with reproductive status.

The data presented herein provide no indication of significant reduction in AGD in *P. leucopus* from TCSFS. Moeller (2004) found levels of total Cd in sediment at TCSFS ranging from 20 to 56 mg/kg, but found only 1-4 mg/kg to be bioavailable. Increased mammary development and uterine weight were induced in female *R. norvegicus* at 5-10 µg/kg Cd; however, irreversible reduction of AGD was detected only in males receiving repeated oral doses of 20 mg/kg/d Cd (Johnson et al. 2003; Couto-Moraes et al. 2010). The bioavailable levels of Cd reported in sediment at TCSFS are higher than doses used in induction of estrogenicity by Cd, but well below levels of Cd capable of reducing AGD in *R. norvegicus* (Johnson et al. 2003; Couto-Moraes et al. 2010). Also, many metals, including Cd, Pb, and Zn, are capable of synergistic or antagonistic interactions that may alter their bioavailability (Bradl et al. 2005). I did not measure tissue residue levels for *P. leucopus* at TCSFS. Incorporation of tissue residue analysis and measurement of AGD would provide a broader understanding of Cd accumulation and potential effects at this site. The lack of significant difference in AGI among the 3 sites in this study also may be a result of the small sample sizes and high within population variation. Results reported by Santolo (2009) for male mice were based on large sample sizes (*P. maniculatus*, $n = 212$; *R. megalotis*, $n = 60$; *M. musculus*, $n = 52$); however, the amount of within population variation reported for *P. maniculatus* (AGD: 5.1-14.5 mm) was similar to variation measured in *P.leucopus* in this study (AGD: 6-16 mm) (Santolo 2009).

Measurement of AGD in laboratory studies of endocrine disruption or hormone effects is well documented (Cantoni et al. 1999; Gallavan et al. 1999; Couto-Moraes et al. 2010); however, AGD is not routinely measured in field studies of small mammals. Due to the endocrine disrupting properties of some xenobiotics, AGD has the potential to be a valuable, easy to measure endpoint in wild populations of small mammals. Laboratory studies typically utilize individuals from genetically homozygous laboratory colonies matched for age, sex, and weight; however, wild-caught animals vary in these factors. *M. musculus*, a commonly used laboratory species, has different anogenital morphology, with the anus at the base of the tail, than *P. leucopus*, potentially confounding comparisons between these data and published laboratory studies (Manno 2008). Laboratory studies of endocrine disrupting compounds may not accurately represent the effects that these compounds can have in a wild population and extrapolation from laboratory to field settings must be done with caution.

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References

Bradl H, Kim C, Kramar U, Stüben D (2005) Interactions of heavy metals. In: Bradl HB (ed) Heavy metals in the environment: origin, interaction and remediation. Elsevier, Amsterdam, pp 28-164

Cantoni D, Glaizot O, Brown RE (1999) Effects of sex composition of the litter on anogenital distance in California mice (*Peromyscus californicus*). Canadian J Zool 77:124-131

Couto-Moraes R, Felicio LF, Bernardi MM (2010) Post-partum testosterone administration does not reverse the effects of perinatal exposure to cadmium on rat offspring development. J Appl Toxicol 30:233-241

Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC (2009) Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr Rev 30:293-342

Gallavan Jr. RH, Holson JF, Stump DG, Knapp JF, Reynolds VL (1999) Interpreting the toxicologic significance of alterations in anogenital distance: potential for confounding effects of progeny body weights. Repro Toxicol 13:383-390

Hotchkiss AK, Vandenbergh JG (2005) The anogenital distance index of mice (*Mus musculus domesticus*): an analysis. J American Assoc Lab An Sci 44:46-48

Johnson MD, Kenney N, Stoica A, Hilakivi-Clarke L, Singh B, Chepka G, Clarke R, Sholler PF, Lirio AA, Foss C, Reiter R, Trock B, Paik S, Martin MB (2003) Cadmium mimics the *in vivo* effects of estrogen in the uterus and mammary glands. Nat Med 9:1081-1084

Lackey JA, Huckaby DG, Ormiston BG (1985) Peromyscus leucopus. Mamm Spec 247:1-10

Mably TA, Moore RW, Petersen RE (1992) *In utero* and lactational exposure of male rats to 2, 3, 7, 8-tetradichlorodibenzo-*p*-dioxin I. effects on androgenic status. Toxicol Appl Pharmacol 114:97-107

Manno III FAM (2008) Measurement of digit lengths and the anogenital distance in mice. Phys Behav 93:364-368

McIntyre BS, Barlow NJ, Foster PMD (2001) Androgen-mediated development in male rat offspring exposed to flutamide *in utero*: permanence and correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues. Toxicol Sci 62:236-249

Moeller LA (2004) Effects of metal contamination on developing red-eared slider turtles (*Trachemys scripta*) and implications for the species as a biomonitor. MS Thesis, Oklahoma State University

Santolo GM (2009) Small mammals collected from a site with elevated selenium concentrations and three reference sites. Arch Environ Contam Toxicol 57:741-754

Stoica A, Katzenellenbogen BS, Martin MB (2000) Activation of estrogen receptor-α by the heavy metal cadmium. Molec Endocrinol 14:545-553

Takiguchi M, Yoshihara S (2006) New aspects of cadmium as endocrine disruptor. Environ Sci 13:107-116

Tyler CR, Jobling S, Sumpter JP (1998) Endocrine disruption in wildlife: a critical review of the evidence. Crit Rev Toxicol 28:319-361

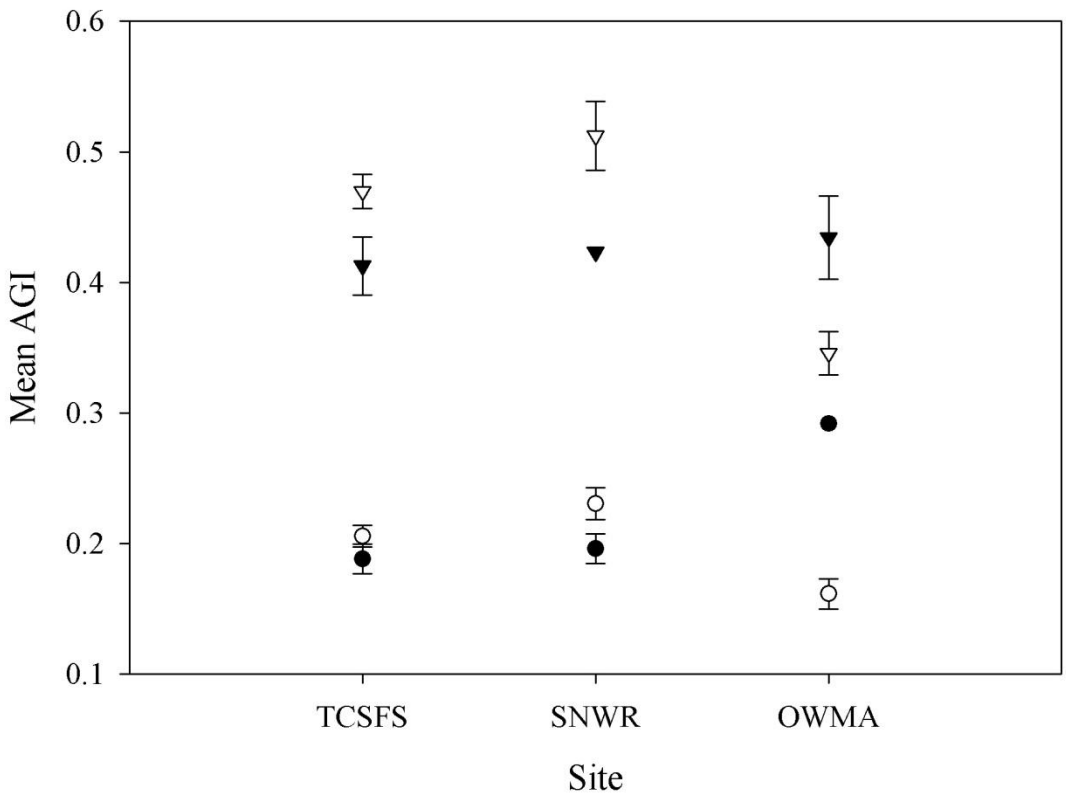
USEPA, United States Environmental Protection Agency (2005) Five-year review report: third five-year review report for the Tar Creek Superfund Site, Ottawa County, Oklahoma. USEPA, Region 6, Dallas, Texas

Zehr J, Gans SE, McClintock MK (2001) Variation in reproductive traits is associated with short anogenital distance in female rats. Dev Psychobiol 38:229-238

Table 2.1 Mean anogenital index (AGI) of *Peromyscus leucopus* evaluated at Tar Creek Superfund Site (TCSFS), Sequoyah National Wildlife Refuge (SNWR), and Osage Wildlife Management Area (OWMA). Means were compared across rows where all $n \geq 5$. Means followed by the same uppercase letter are not significantly different ($p \geq 0.05$).

	Spring			Summer			Fall			Winter		
		%			%			%			%	
Site/Sex	*n*	Repro.	Mean	*n*	Repro.	Mean	*n*	Repro.	Mean	*n*	Repro.	Mean
Female												
TCSFS	20	35.00	0.21^{A}	7	28.57	0.19^{A}	9	55.56	0.19^{A}	---	---	---
SNWR	9	33.33	0.22	2	0	0.24	---	---	---	---	---	---
OWMA	---	---	---	---	---	---	7	14.23	0.19	2	0	0.14
Male												
TCSFS	13	0	0.48^{A}	7	14.23	0.43^{A}	8	25.00	0.47^{A}	---	---	---
SNWR	7	0	0.56^{A}	7	14.23	0.45^{B}	---	---	----	---	---	---
OWMA	---	---	---	---	---	---	7	100	0.43^{A}	11	0	0.35^{B}

Figure 2.1 Mean anogenital index (AGI) ± 1 S.E. for male and female *Peromyscus leucopus* (pooled across seasons) collected from Tar Creek Superfund Site (TCSFS), Sequoyah National Wildlife Refuge (SNWR), and Osage Wildlife Management Area (OWMA). Closed triangles and circles represent reproductive males and females, respectively, and open triangles and circles represent non-reproductive males and females, respectively. Points with no error bars had no within population variation.


0.6
0.5
0.4
0.3
0.2
0.1
Mean AGI
TCSFS
SNWR
OWMA
Site

CHAPTER III

CHROMOSOMAL ABERRATIONS AND POPULATION GENETIC STRUCTURE IN WHITE-FOOTED MICE (*PEROMYSCUS LEUCOPUS*) FROM TAR CREEK SUPERFUND SITE

Abstract – This study investigated the potentially deleterious effects of heavy metals on individual and population level genetic endpoints in white-footed mice (*Peromyscus leucopus*) from Tar Creek Superfund Site (TCSFS). Metaphase chromosome analysis was performed on 89 *P. leucopus* from TCSFS and two unmined, reference sites. Amplified fragment length polymorphism analysis was used to investigate differences in population genetic structure between two populations from within TCSFS and six reference sites representing broad geographic distribution in Oklahoma. No significant difference in the frequency of lesions per cell, aberrant cells per individual or mitotic index was detected among the three sites. AFLP analysis revealed distinct clustering of populations from eastern and western Oklahoma. The two TCSFS populations did show significant differentiation from reference populations; however, the Beaver Creek population showed deviation from more populations than the population from Douthat Settling Pond.

INTRODUCTION

Maintenance of genetic integrity at the individual and population level is critical for appropriate ecological function; however, individual and population level endpoints are not often considered simultaneously in studies of wildlife species exposed to contaminants (Bickham et al. 2000). Laboratory studies have been used to assess the genotoxic potential of metals at the individual level, but population-level effects are difficult to measure in a laboratory setting (McBee and Lochmiller 1996). Individual effects may be magnified in laboratory dosing studies due to use of genetically similar individuals that are often exposed to unrealistic and uniform levels of test compounds and these systems cannot adequately take into account multiple stressors that organisms experience *in situ* (Linzey and Grant 1994). Damage caused by genotoxicants occurs at the molecular or cellular level, but emergent effects at higher levels of organization can occur and may be unclear based solely on the mechanism of toxicity. Contaminants may also affect reproductive success, survivorship, recruitment, and organismal health, leading to effects at the population level (Bickham et al. 2000). Incorporation of individual and population-level endpoints in small mammal populations exposed to genotoxicants *in situ* will allow for more accurate understanding of potential deleterious genetic effects of heavy metal exposure.

The Tri-State Mining District encompasses portions of Missouri, Kansas, and Oklahoma, and was an area of heavy mining in the early 1900's, with portions of the district being mined as late as 1970. Lead (Pb) and zinc (Zn) were extracted using a variety of mining strategies, including strip, room-and-pillar, cave, and surface mining (Weidman et al. 1932). During peak mining years the Tri-State Mining District produced

1.3 million tons of Pb and 5.2 million tons of Zn. From 1918 through 1945 this area led the world in Zn production. Due to the lack of reclamation requirements at the time mining ceased, much of the Tri-State Mining District remains heavily degraded (USEPA 2005). Tar Creek Superfund Site (TCSFS) is a 10,360-ha portion of the Tri-State Mining District located in Ottawa County, in extreme NE Oklahoma. Currently, 75 million tons of mine tailings and 324 ha of wet and dry sediment retention ponds are present on the site (USEPA 2005). In addition to physical alterations to the landscape, TCSFS is contaminated with Pb, Zn, and cadmium (Cd), all of which are documented to have genotoxic potential (Sheffield et al. 2001; Beyersmann and Hartwig 2008). Potential health effects of these metals led to the placement of TCSFS on the United States Environmental Protection Agency's National Priority List in 1983 (USEPA 2005).

Structural chromosomal damage is a useful biomarker for assessing effects of environmental contamination at the individual level and may provide a necessary link to understand alterations of population genetic structure (Shugart 1999; Bickham et al. 2000; Peakall and McBee 2001). Genotoxicants can cause genetic damage by direct formation of adducts with DNA, but many act via indirect mechanisms, including damage irreparable by normal enzymatic repair systems, inhibition of enzymatic repair systems, alterations in DNA replication, or production of reactive oxygen species (Shugart 1999; Bryant 2007). Numerical and structural chromosome aberrations are useful biomarkers with which to measure disruption of cytogenetic structure in natural populations. Structural chromosomal aberrations can lead to decreased fertility, induced birth defects, and carcinogenesis, and include strand breaks, exchange figures, and deletions (Shugart 1999; Bryant 2007). These aberrations can occur spontaneously and

their frequency may be increased by exposure to physical and chemical stressors (Bryant 2007). Bickham et al. (2000) proposed that somatic effects, seen as structural chromosomal aberrations, can lead to stress or adverse health effects on individuals. This can result in mortality or decreased reproduction that can cause population bottlenecks and ultimately lead to reduced genetic diversity at the population level.

Lead, Cd, and Zn can act as genotoxicants, but the exact mechanisms by which they cause chromosomal lesions are unknown (Hartwig 1994; Beyersmann and Hartwig 2008). Some studies show that Pb and Cd are mutagenic only at high doses, but cause indirect genotoxic effects at lower doses (Hartwig 1994). Pb and Cd can interfere with DNA repair mechanisms, specifically base and nucleotide excision repair, and alter the redox state within cells, releasing free radicals that can damage DNA (Hartwig et al. 2002; Beyersmann and Hartwig 2008). Because Zn is an essential metal, its genotoxic potential has received less attention than Pb and Cd; however, Hikiba et al. (2005) found that ZnO significantly increased chromatid breaks in Syrian hamster (*Mesocricetus auratus*) embryo cells in culture.

Populations exposed to contaminants can be affected in several ways, including induction of mutations, population bottlenecks, and selection. They may have increased genetic diversity due to new mutations directly induced by a mutagen or decreased genetic diversity from bottlenecks or selection for resistant alleles (Bickham et al. 2000). Amplified fragment length polymorphism (AFLP) analysis is a useful toxicological tool to determine differences in levels of heterozygosity and population genetic structure between contaminated and uncontaminated populations. Allozyme or microsatellite analyses have previously been used to investigate effects of contaminants on population

genetic structure in wild populations (Belfiore and Anderson 2001). However, studies of invertebrates (Martins et al. 2009), fungi (Muller et al. 2004), plants (Labra et al. 2003), and fish (Whitehead et al. 2003; McMillan et al. 2006) have applied AFLP methods in studies of populations exposed to contaminants. AFLP allows for screening of a larger portion of the genome than other molecular methods, requires no previous knowledge of DNA sequence in the organism of interest, and requires only small amounts of nuclear DNA (Vos et al. 1995; Bagley et al. 2001). The purpose of my study was to determine if white-footed mice (*Peromyscus leucopus*) exposed to Pb, Cd, and Zn at TCSFS showed increased incidence of structural chromosome aberrations as measured by metaphase chromosome analysis (of bone marrow cells), and altered population genetic structure as determined by AFLP, when compared with wild populations of *P. leucopus* from uncontaminated areas in Oklahoma.

METHODS

Study sites -- I collected *P. leucopus* from three sites for inclusion in metaphase chromosome analysis (Fig. 3.1). The Beaver Creek site (BC) is a 16-ha portion of TCSFS owned and managed by the Quapaw Tribe of Oklahoma and Bureau of Indian Affairs. An old mine and crushing operation is present at BC and the landscape at the site is dominated by mine tailings. Vegetation at the site is dominated by blackjack oak (*Quercus marilandica*), post oak (*Q. stellata*), and American elm (*Ulmus americana*) forest with undergrowth of poison ivy (*Toxicodendron radicans*) and greenbriar (*Simlax bon-anox*). Reference sites were selected because of availability, similarities in plant

communities, and sufficient distance from TCSFS to reduce the possibility of heavy metal contamination from water or airborne particles from TCSFS. Sequoyah National Wildlife Refuge (SNWR) was originally selected as a reference site and is located approximately 165 km south of TCSFS. The site is approximately 8,498 ha and is located in Muskogee, Haskell, and Sequoyah counties, OK. It is used for hunting and cooperative agriculture on a seasonal basis. The refuge habitat is bottomland hardwood, river bluffs, and shrub-scrub grassland. Trapping efforts were concentrated in edge habitat near refuge headquarters. Due to poor trap success at this site, it was abandoned after the first 2 trapping sessions. It was replaced with Osage Wildlife Management Area (OWMA), which is located approximately 110 km west of TCSFS in Osage Co. and is managed by the Oklahoma Department of Wildlife Conservation. This site is undeveloped and is used for hunting and recreation on a seasonal basis. Habitat at OWMA is a mixture of cross-timbers, bottomland hardwood forest and tallgrass prairie. Trapping was focused in the Rock Creek Management Unit. Mice were collected from an additional site within TCSFS, Douthat Settling Pond (DSP), in Spring 2010 for inclusion in population level genetic analysis. Douthat Settling Pond is a desiccated sediment retention pond approximately 8 km from BC. Habitat at the site is similar to that of BC; however, the site is directly adjacent to Tar Creek and is prone to flooding during periods of heavy rainfall.

Field methods – I used Sherman live traps baited with rolled oats and scratch grain to trap *P. lecuopus* at all sites. Trapping effort was made at TCSFS in Spring (Trap nights, n = 1005), Summer (n = 600), and Fall (n = 500) 2009, SNWR in Spring (n = 2260) and Summer (n = 2000) 2009, OWMA in Fall (n = 1480) 2009 and Winter (n = 400) 2010,

and DSP in Spring (n = 2400) 2010. Trap effort was unequal at the sites because trapping ceased once 20 *P. leucopus*/site/season (10 males, 10 females) were collected from BC, SNWR, and OWMA. At DSP, effort was made to collect 5 male and 5 female *P. leucopus* for AFLP analysis. All methods were approved by Oklahoma State University Institutional Animal Care and Use Committee (ACUP No. AS 066).

Metaphase chromosome analysis -- The method of metaphase chromosome spread preparation followed Baker and Qumsiyeh (1988). Upon return to the lab, animals were injected subcutaneously with a solution of equal parts yeast and sucrose in distilled water (0.1 ml/10g body weight) as a mitotic stimulant. Animals were housed for 24 h post-injection in cages with commercial bedding, food, and water. After 24 h, animals were killed and the long bones of both hind limbs removed. Bone marrow was flushed from both tibias and femurs with a 0.075 M KCl solution warmed to 37 ^{o}C. The suspension was aspirated vigorously with a pipette and then incubated for 27 min at 37 ^{o}C. Following incubation, the cell suspension was centrifuged at 800 xg for 90 s. Most of the solution was removed and the cell pellet was gently resuspended in the remaining volume (2-3 ml) of hypotonic solution. The cell suspension was fixed with 5-6 ml Carnoy's fix (3 parts methanol: 1 part acetic acid) and gently mixed to resuspend. The fixed suspension was then centrifuged for 90 s and fixed 3 more times following the above procedure. Fixed cell suspensions were dropped onto clean, dry, number-coded microscope slides, ignited, and air-dried. Once dry, slides were stained in a 1% Geimsa stain solution. Tissues (spleen, muscle, heart, kidney, liver, and blood) were collected from individuals at the time of sacrifice. Tissues and voucher specimens are deposited in the Oklahoma State University Collection of Vertebrates.

Fifty metaphase chromosomal spreads with clear, well-stained chromosomal morphology and minimal overlaps were scored for each individual. *P. leucopus* has a well characterized karyotype with a diploid number of 48 (Greenbaum et al. 1994). Only chromosomal spreads containing 46-50 chromosomes (2n ± 2) were included in analysis. Each spread was scored for the presence of aberrations as classified by Preston et al. (1987). Achromatic gaps were not included as aberrations because their cytogenetic significance is questionable (Preston et al. 1987). Additionally, the mitotic index (# mitotic cells/ 1000 cells) was calculated for each individual. All recorded structural chromosomal aberrations and several randomly selected normal spreads for each individual were verified by a second investigator and documented by digital photography.

Amplified fragment length polymorphism analysis -- Eight populations of *P. leucopus* from throughout Oklahoma were included in analysis of population genetic structure. Populations from a broader geographic area were included in this analysis to account for geographic factors influencing population genetic structure. Muscle samples from *P. lecucopus* at BC, DSP, SNWR, and OWMA and skeletal muscle or toe clips provided by the OSU Collection of Vertebrates were used for AFLP analysis. Localities (Fig. 3.1) along with sample sizes are as follows: BC (n = 16), DSP (n = 11), SNWR (n = 4), OWMA (n = 7), Randlett in Cotton Co. (COTT, n = 6), Ft. Supply Wildlife Management Area, Woodward Co. (WOOD, n = 6), Oolagah Wildlife Management Area, Nowata Co. (OOL; n = 9), and Lake Carl Blackwell, Payne Co. (LCB; n = 9).

DNeasy Tissue Kits (Qiagen Inc., Valencia, California) were used to extract total DNA from tissues. The AFLP protocol was modified from Vos et al. (1995) and analyses were performed at Tarleton State University (Stephenville, TX). Approximately 200 ng

total genomic DNA was digested for 3 h at 37^{o}C with 20 units each of *Ase*I and *Eco*RI and appropriate volumes of restriction buffer. Ligation reactions were performed on the restriction products by the addition of 75 pmol each *Eco*RI and *Ase*I adapters, 3 units T4 DNA ligase, 12 µl water, and appropriate volumes of buffer. Ligation mixtures were incubated for 10 h at 16 ^{o}C. Ligation products were diluted by addition of 160 µl 10mM Tris. Pre-selective polymerase chain reactions amplified a subset of ligated fragments. Amplifications were carried out in 50 µl volumes containing 10 µl diluted ligation product, 5X Buffer, 25 mM $MgCl_2$, 10 mM deoxynucleoside triphosphates (dNTPs), 10 µM of each pre-selective primer, and 2.5 units Taq DNA polymerase. Amplification conditions included an initial incubation at 72 ^{o}C for 60 s followed by 20 cycles of 94 ^{o}C for 50 s, 56 ^{o}C for 60 s, and 72 ^{o}C for 120 s. Five microliters of preselective reaction product were diluted in 90 µL 10 mM Tris and used to conduct selective polymerase chain reactions using five selective primer pairs (CAC-TCT, CAC-TAG, CAC-TGT, CAT-TAA, CAT-TGT; Table 3.1). Each selective reaction was performed in a total volume of 25 µL containing 5 µL diluted preselective product, Buffer 5X, 25 mM $MgCl_2$, 10 mM dNTPs, 10 µM of each selective primer, and 2.5 units of Taq DNA polymerase. The thermal profile for selective reactions was: 24 cycles of 94 ^{o}C for 50 s, 65–56.6 ^{o}C (0.7 ^{o}C reduction for 2nd through 13th cycle) for 60 s, and 72 ^{o}C for 120 s.

The *Eco*RI primers used in selective reactions were fluorescently labeled for detection by a BeckmanCoulter CEQ8000 Genetic Analysis System (Beckman Coulter, Inc., Fullerton, California). Beckman Coulter Fragment Analysis software (Version 9.0) was used to analyze fragments based on an internal size standard. Data generated by fragment analysis software were a matrix of 0's and 1's with 0 indicating absence of a

fragment and 1 indicating presence of a fragment. Automated scoring of fragments was confirmed by manual scoring. Only fragments that could be definitively scored were included in analyses. Replicate AFLP analysis was performed on a randomly selected subset of 20 individuals. Although rare, discrepancies can be detected between replicates. If discrepancies were detected between AFLP runs, that fragment was excluded from analysis for all individuals. Only loci that were polymorphic in at least 5% of individuals were used for statistical analysis.

Statistical methods -- The Wilcoxon-Mann-Whitney test was used to compare number of lesions per cell and number of aberrant cells per individual among sites. Mitotic index was compared among sites using ANOVA.

Statistical analysis of AFLP fragments was performed using GenAlEx, version 6.3 (Peakall and Smouse 2006). Principal coordinates analysis (PCoA) was used to measure divergence among populations. A Mantel test was performed to determine if detected differences in population genetic structure among populations were based solely on geographic distance. Analysis of molecular variance (AMOVA) was used to calculate Φ_{PT} and determine if there was a significant deviation from panmixia. Pairwise Φ_{PT} comparisons were made between sites to determine if significant differences in population genetic structure were present with pair wise probabilities calculated based on 9999 permutations.

RESULTS

Metaphase spreads were scored for 89 *P. leucopus* collected from BC (*n* = 45), SNWR (*n* = 21), and OWMA (*n* = 23) (Table 3.2). The number of lesions per cell did not differ significantly among sites ($X_2^2 = 1.22$, $P = 0.55$). The highest mean number of lesions per cell was present in animals from SNWR followed by BC and OWMA. There was no significant difference among sites in the number of aberrant cells per individual ($X_2^2 = 0.78$; $P = 0.68$) and percent aberrant cells per individual ranged from 1.82 to 2.04% among the three sites. Only 4 types of aberrations were observed, acentric fragments, chromatid breaks, chromosome breaks, and ring chromosomes (Figs. 3.2, 3.3). Chromatid breaks and acentric fragments represented the most common types of aberrations detected in all three populations, representing 48.98% and 43.88% of aberrations, respectively. Chromosome breaks represented 7.40 and 8.00% of aberrations at both SNWR and BC, respectively, but none were observed in animals from OWMA. One ring chromosome was observed in an individual collected from SNWR. No translocation figures or dicentric chromosomes were observed. Mitotic index varied within all three populations (Table 3.2); however, there was no significant difference in mitotic index among the three sites ($F_{2,86} = 2.29$, $P = 0.11$).

A total of 151 AFLP fragments were identified, with 58.9 % (89 fragments) being scored as polymorphic. The percentage of polymorphic loci at the 8 sampling sites averaged 53.37% (SE = 2.67%) and varied from 40.45% at SNWR to 62.92% at LCB (Table 3.3). All of the observed bands were locally common and occurred in > 5% of individuals. No private bands, fragments occurring only in a single population, were

observed. DSP and BC each presented one locally common band that was present in less than 25% of all populations (Table 3.3). Average mean unbiased heterozygosity was 0.201 (SE = 0.008) across the eight populations and ranged from 0.178 at OOL to 0.23 at LCB (Table 3.3).

The first two axes of the PCoA explained 68.60% of the variation present in the AFLP data set with individuals clustered into two distinct, non-overlapping groups (Fig. 3.4). Group 1 contained all individuals from COTT, WOOD, and LCB populations. Group 2 contained all individuals from BC, DSP, OOL, OWMA, and SNWR populations A Mantel test confirmed isolation by distance ($R^2 = 0.51$, $P = 0.001$). AMOVA showed that 68% of variation detected by AFLP was the result of within population variation; however, there was significant deviation from panmixia ($\Phi_{PT} = 0.32$, $P = 0.01$). Pair wise Φ_{PT} values (Table 3.4) parallel the results of the PCoA for group 1 with no significant differences in pair wise Φ_{PT} values ($P \geq 0.05$) between COTT, WOOD, or LCB. The BC population had significantly different population genetic structure than all populations except DSP ($P = 0.20$ for DSP; $P \leq 0.05$ for all other populations). DSP differed significantly from the 3 most western populations (COTT, WOOD, and LCB) that clustered together as Group 1 and from SNWR in Group 2. However, DSP did not differ significantly from OWMA and OOL. Pairwise Φ_{PT} comparisons differed significantly between SNWR and all other sites.

DISCUSSION

Sites disturbed by mining, like TCSFS, provide unique challenges with contamination by multiple compounds usually over large spatial and temporal scales. Bickham et al. (2000) clearly documented the link between individual level genetic effects and population-level effects; however, studies do not often consider genetic effects at multiple levels of organization simultaneously. Studies have documented the ability of small mammals to accumulate heavy metals. Previous research at TCSFS documented the accumulation of heavy metals in several taxa (Conder and Lanno 1999; Beyer et al. 2004, Moeller 2004; Schmitt et al. 2006). Hausbeck (1995) found significantly higher mean concentrations of Zn and Cd in *P. leucopus* collected from abandoned strip mines with significant seasonal variation in accumulation. Bank voles (*Clethrionomys glareolus*) at an abandoned Pb mine had significantly elevated levels of Pb and Cd when compared with reference individuals (Milton et al. 2003). Although tissue residue levels were not measured in my study, previous research at TCSFS and these studies on small mammals suggests that bioaccumulation may occur in *P. leucopus* at TCSFS.

Metaphase chromosome analysis revealed no significant increase in the frequency of structural chromosomal aberrations in *P. leucopus* at BC within TCSFS despite documented levels of elevated Pb, Cd, and Zn at the site (Moeller 2004; USEPA 2005). Percent aberrant cells per individual (1.82-2.04%) and mean lesions per cell (0.018-0.027) observed at BC, OWMA, and SNWR were consistent with other karyological studies. At a pristine site, Topashka-Ancheva et al. (2003) reported spontaneous chromosomal aberrations occurring at frequencies of 1.42 to 3.37% per individual in

yellow-necked wood mice (*Apodemus flavicollis*) and *C. glareolus.* Two studies of *P. leucopus* from Oklahoma found no significant increase in chromosomal aberrations in *P. leucopus* exposed to Aroclor 1254, a polychlorinated biphenyl (Shaw-Allen and McBee 1993), or heavy metals (Husby et al. 1999). Percent aberrant cells per individual presented herein fall within values reported by those studies (Shaw-Allen and McBee 1993, 0.5-1.73%; Husby et al. 1999, 2.5-7%). The mean number of lesions per cell detected at TCSFS, SNWR, and OWMA was slightly higher than values presented by Shaw-Allen and McBee (1993; range 0.005-0.018). Those studies also found chromatid breaks and acentric fragments to be the most commonly occurring aberrations in both contaminated and reference populations, accounting for approximately 90% of aberrations observed (Shaw-Allen and McBee 1993; Husby et al. 1999). Similar aberration frequencies were found in reference populations by McBee et al. (1987). None of the previously discussed studies presented mitotic index for *P. leucopus*. In a study of roof rats (*Rattus rattus*) trapped at a waste disposal plant, there was no significant difference in mean mitotic index between individuals from contaminated and reference sites and mitotic index ranged from 0.6-2.7% (Eckl and Rigler 1997). These values are consistent with observed mean mitotic indices at the three sites in this study (1.17-1.38%).

Bol'shakov et al. (2003) found species specific genotoxic responses to radionuclide contamination. Common voles (*Microtus arvalis*) exposed to radionuclide contamination had a significantly higher frequency of structural chromosome aberrations in bone marrow cells when compared with animals from reference sites; however, northern mole voles (*Ellobius talpinus*) from the same sites did not differ. *E. talpinus*

populations have smaller population fluctuations and live in more isolated colonies than *M. arvalis* and the authors suggested that these factors reduced clastogenic effects associated with population cycles and viral stress. This study concluded that genomic stability in *M. arvalis* and *E. talpinus* is dependent on population-demographic structure and population cycles (Bol'shakov et al. 2003). The between species differences in genomic stability observed by Bol'shakov et al. (2003) suggest that great care is required when comparing results of genotoxicity studies across species and populations.

AFLP analysis utilizes dominant markers and estimates of heterozygosity are made under the assumptions of Hardy-Weinberg equilibrium. This constraint makes meaningful comparison of heterozygosity estimates between AFLP and microsatellite analysis, which uses codominant markers, impossible (Meudt and Clarke 2007). Heterozygosity determined in this study is similar to values reported for contaminated and reference populations of the Sacramento sucker (*Catostomus occidentalis*) by Whitehead et al. (2003). That study used AFLP analysis and reported heterozygosity values ranging from 0.13-0.16 (Whitehead et al. 2003). Percent polymorphic loci in my study (40.45-62.92%) were higher than those reported for *C. occidentalis* (22-44%; Whitehead et al. 2003).

AFLP data presented herein showed clustering that corresponds with the geographic location of the populations observed. Group 1 is three populations located in the western portion of Oklahoma and Group 2 is made up of populations in the east. Significant results of the Mantel test indicate that observed population structuring is due to isolation by distance. A significant difference in population genetic structure was detected between BC and all reference populations in Group 2 (OWMA, OOL, and

SNWR). DSP differed only from one reference population in group 2, SNWR. SNWR is located approximately 158 km from DSP while OOL and OWMA are 95 and 103 km, respectively. Moeller (2004) reported higher bioavailable levels of Cd and Zn in sediment collected from BC (Cd: 4 mg/kg, Zn: 120 mg/kg) when compared with sediment from DSP (Cd: 1 mg/kg, Zn: 10 mg/kg); however, bioavailable Pb was higher in sediment from DSP (5.2 vs. 2.8 mg/kg). Different levels of metal exposure may partially explain the differences in population genetic structure between sites within TCSFS and reference populations.

Whitehead et al. (2003) found biogeographic factors at a variety of scales explained genetic variation detected in populations of *C. occidentalis* exposed to pesticides. Despite varying levels of contamination exposure, populations within the same river system were more genetically similar than those from different watersheds and downstream populations were more similar to each other than upstream populations from the same tributary. This study emphasized the necessity of including biogeographical factors in measuring genetic structure of populations exposed to contaminants (Whitehead et al. 2003).

In an allozymic study conducted in the Kansas portion of the Tri-State Mining District, Roark and Brown (1996) assessed several populations of mosquitofish (*Gambusia affinis*), bluntnose minnow (*Pimephales notalus*), and blackstripe topminnow (*Fundulus notatus*) exposed to Pb and Zn. Allozyme analysis detected significantly different allelic frequencies between contaminated and reference sites in all three species. *P. notalus* and *F. notatus* populations had a higher proportion of heterozygous genotypes in the contaminated creek, but no difference was detected in the *G. affinis* population.

Two of the enzymes studied showed differential allozyme sensitivity to high doses of Zn (*P. notalus*: > 237.6 mg/L and *F. notatus*: > 1,032 mg/L), but none showed differential sensitivity to Pb *in vitro.* The levels of Pb and Zn were significantly lower *in situ* than in the *in vitro* study; however, water samples from the contaminated creek contained significantly more Pb (0.035 mg/L) and Zn (0.735 mg/L) than reference samples (Pb: 0.011, Zn: 0.05 mg/L) (Roark and Brown 1996).

Bioavailable levels of Pb and Zn from sediment collected from BC and DSP within TCSFS were higher than those measured in water from Kansas streams, but no sediment levels were reported in that study (Roark and Brown 1996; Moeller 2004). The routes of exposure of *G. affinis, P. notalus, F. notatus,* and *P. leucopus* are very different and may explain the contrary findings in population-level genetic effects in these studies. Uptake of contaminants by fish and small mammals can occur through ingestion or absorption, but fish are more likely to absorb contaminants transdermally due to secondary circulation and are exposed to a wider variety of contaminants via the gills than mammals are in the lungs (Kleinow et al. 2008).

The population clustering seen in AFLP analysis is similar to the distribution of 2 well-defined chromosomal races of *P. leucopus* (Baker et al. 1983; Stangl 1986, Nelson et al. 1987). The races are distinguished by 3 euchromatic, pericentric inversions on chromosomes 5, 11, and 20 (Stangl and Baker 1984, Greenbaum et al. 1994). Both of the chromosomal races are widely distributed and are capable of hybridizing over a large area; however, the parental cytotypes have never been found to occur in sympatry (Stangl 1986). In Oklahoma, the northeastern and southwestern cytotypes meet in a narrow hybrid zone. Analysis of karyotypes, mitochondrial DNA restriction sites, and allozymic

patterns indicate asymmetrical introgression of the northeastern cytotype into southwestern cytotype populations (Stangl 1986; Nelson et al. 1987).

Several factors should be considered when interpreting the absence of significant increase of chromosomal aberrations in *P. leucopus* from TCSFS. All three metals of concern at TCSFS, Pb, Zn, and Cd, have been documented to be genotoxic in laboratory studies (Çelik et al. 2005; Hikiba et al. 2005; Nava-Hernández et al. 2009). Only one of these studies exposed animals to metal concentrations similar to the bioavailable levels seen at BC. Çelik et al. (2005) dosed *R. norvegicus* with 0.5 mg/kg/d intraperotineally for 4 months and found chromosomal aberrations frequencies of 7.0 % compared with 2.5% in control animals. Nava-Hernández et al. (2009) used the COMET assay and found increased DNA strand breaks only in *R. norvegicus* receiving doses of CdCl > 25 mg/L (42.33% strand breaks) and Pb acetate > 250 mg/L (58.53% strand breaks). ZnO induced aberrations in 66.7% of metaphases in *M. auratus* ovary cells at doses of 180µM (Hikiba et al. 2005).

Laboratory studies cannot adequately account for abiotic factors experienced *in situ* and these factors, including pH, temperature, moisture, ion composition, osmotic pressure, mineral content of the soil, and exposure to sunlight can influence the availability and uptake of contaminants (Babich and Stotzky 1983). Many metals, including Pb, Cd, and Zn, are capable of antagonistic and synergistic interactions in uptake, deposition, and bioavailability, depending on mixture composition (Komjarova and Blust 2008; Jihen et al. 2009). Age, stage of development, and immune status of the animal also can greatly influence uptake and storage of metals (Goyer and Clarkson 2001). *P. leucopus*, like many organisms, utilizes metallothioneins, which are low

molecular weight compounds capable of binding metals. Metallothionens can be induced by environmental stressors, including cold temperatures and nutritional stress, which would in turn affect the way metals are bound, transported, and distributed in the body (Hidalgo et al. 1990).

Dose and duration of contaminant exposure, age of the organisms collected, and the influence of biotic and abiotic conditions at the site are often unknown in *in situ* studies of wildlife. However, *in situ* bioassays are essential to adequately assess the effects of contaminants in real world scenarios and at multiple levels of organization. This study found no significant increase in frequency of structural chromosomal aberrations and significant alteration of population genetic structure was seen in only one population from TCSFS. Further study of these populations, including tissue residue and metallothionein analyses, may help clarify the population-level difference detected at BC and elucidate metal effects on other endpoints or mechanisms of adaptation or detoxification employed by *P. leucopus* at TCSFS.

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REFERENCES

Babich H, Stotzky G (1983) Developing standards for environmental toxicants: the need to consider abiotic environmental factors and microbe-mediated ecologic processes. Environ Health Persp 49:247-260

Bagley ML, Anderson SL, May B (2001) Choice of methodology for assessing genetic impacts of environmental stressors: polymorphism and reproducibility of RAPD and AFLP fingerprints. Ecotoxicol 10:239-244

Baker RJ, Robbins LW, Stangl Jr. FB, Birney EC (1983) Chromosomal evidence for a major subdivision in *Peromyscus leucopus*. J Mammal 64:356-359

Baker RJ, Qumsiyeh MB (1988) Methods in chiropteran mitotic chromosomal studies. In: Kunz T (ed) Ecological and behavioral methods for the study of bats. Smithsonian Institution Press, Washington D.C., pp 425-435

Belfiore NM, Anderson SL (2001) Effects of contaminants on genetic patters in aquatic organisms: an overview. Mut Res 489:97-122

Beyer WN, Dalgam J, Dudding S, French JB, Mateo R, Miesner J, Sileo L, Spann J

(2004) Zinc and lead poisoning in wild birds in the Tri-State mining district (Oklahoma, Kansas, and Missouri). Arch Environ Contam Toxicol 48:108-117

Beyersmann D, Hartwig A (2008) Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. Arch Toxicol 82:493-512

Bickham JW, Sandhu S, Hebert PDN, Chikhi L, Athwal R (2000) Effects of chemical contaminants on genetic diversity in natural populations: implications for biomonitoring and ecotoxicology. Mutat Res 463:33-51

Bol'shakov VN, Gileva EA, Yalkovskaya LE (2003) Species-specific features of inter- and intrapopulation variation in the level of chromosome instability in rodents. Russian J Ecol 34:314-319

Bryant PE (2007) Origin of chromosomal aberrations: mechanisms. In: Obe G, Vijayalaxmi (eds) Chromosomal alterations: methods, results, and importance in human health. Springer, New York, pp 177-199

Çelik A, Çömelekoglu Ü, Yalin S (2005) A study on the investigation of cadmium chloride genotoxicity in rat bone marrow using micronucleus test and chromosome aberration analysis. Toxicol Ind Health 21:243-248

Conder JM, Lanno RP (1999) Heavy metal concentrations in mandibles of white-tailed deer living in the Picher Mining District. Bull Environ Contam Toxicol 63:80-86

Eckl PM, Riegler D (1997) Levels of chromosomal damage in hepatocytes of wild rats living within the area of a waste disposal plant. Sci Tot Environ 196:141-149

Goyer RA, Clarkson TW (2001) Toxic effects of metals. In: Klassen CD (ed) Casarett and Doull's toxicology: the basic science of poisons. McGraw-Hill, New York, pp 811-867

Greenbaum IF, et al. (1994) Cytogenetic nomenclature of deer mice, *Peromyscus* (Rodentia): revision and review of the standardized karyotype. Cytogen Cell Genet 66:181-195

Hartwig A (1994) Role of DNA repair inhibition in lead- and cadmium- induced genotoxicity: a review. Environ Health Persp 102:45-50

Hartwig A, Asmuss M, Ehleben I, Herzer U, Kostelac D, Pelzer A, Schwerdtle T, Bürkle A (2002) Interference by toxic metal ions with DNA repair processes and cell cycle control: molecular mechanisms. Environ Health Persp 110:797-799

Hausbeck JS (1995) Analysis of trace metal contamination of coal stripmines and bioaccumulation of trace metals by *Peromyscus leucopus*. Thesis, Oklahoma State University

Hidalgo J, Borrás, Garvey JS, Armario A (1990) Liver, brain, and heart metallothionein induction by stress. J Neurochem 55:651-654

Hikiba H, Watanabe E, Barrett JC, Tsutsui T (2005). Ability of fourteen chemical agents used in dental practice to induce chromosome aberrations in Syrian hamster embryo cells. J Pharmacol Sci 97:146-152

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

Jihen EG, Imed M, Fatima H, Abdelhamid K (2009) Protective effects of selenium (Se) and zinc (Zn) on cadmium (Cd) toxicity in the liver of the rat: Effects of the oxidative stress. Ecotox and Environ Safety 72:1558-1564

Kleinow KM, Nichols JW, Hayton WL, McKim JM, Barron MG (2008) Toxicokinetics

in fishes. In: DiGiulio RT, Hinton DE (eds) The toxicology of fishes. CRC Press, Boca Raton, pp 55-152

Komjarova I, Blust R (2008) Multi-metal interactions between Cd, Cu, Ni, Pb and Zn in water flea *Daphnia magna*, a stable isotope experiment. Aquat Toxicol 90:138-144

Labra M, Di Fabio T, Grassi F, Regondi SMG, Braclae M, Vannini C, Agradi E (2003) AFLP analysis as biomarker of exposure to organic and inorganic genotoxic substances in plants. Chemosphere 52:1183-1188

Linzey AV, Grant DM (1994) Characteristics of a white-footed mouse (*Peromyscus leucopus*) population inhabiting a polychlorinated biphenyls contaminated site. Arch Environ Contam Toxicol 27:521-526

Martins N, Bollinger C, Harper RM, Ribeiro R (2009) Effects of acid mine drainage on the genetic diversity and structure of a natural population of *Daphnia longispina*. Aquat Toxicol 92:104-112

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

McBee K, Lochmiller RL (1996) Wildlife toxicology in biomonitoring and bioremediation:Implications for human health. In: Bloom AD, de Serres F (eds) Ecotoxicity and human health: a biological approach to environmental remediation. CRC Press, Boca Raton, pp 163-181

McMillan AM, Bagley MJ, Jackson SA, Nacci DE (2006) Genetic diversity and structure

of an estuarine fish (*Fundulus heteroclitus*) indigenous to sites associated with a highly contaminated urban harbor. Ecotoxicol 15:539-548

Meudt HM, Clarke AC (2007) Almost forgotten of latest practice? AFLP applications, analyses, and advances. Trends Plant Sci 12:106-117

Milton A, Cooke JA, Johnson MS (2003) Accumulation of lead, zinc, and cadmium in a wild population of *Clethrionomys glareolus* from an abandoned lead mine. Arch Environ Contam Toxicol 44:405-411

Moeller LA (2004) Effects of metal contamination on developing red-eared slider turtles (*Trachemys scripta*) and implications for the species as a biomonitor. Thesis, Oklahoma State University

Muller LA, Lambaerts HM, Vangronsveld J, Colpaert JV (2004) AFLP-based assessment of the effects of environmental heavy metal pollution on the genetic structure of pioneer populations of *Suillus luteus*. New Phytol 164:297-303

Nava-Hernández MP, Hauad-Marroquín LA, Bassol-Mayagoitia S, García-Arenas G, Mercado-Hernández R, Echávarri-Guzmán MA, Cerda-Flores RM (2009) Lead-, cadmium-, and arsenic-induced DNA damage in rat germinal cells. DNA Cell Biol 28:241-248

Nelson K, Baker RJ, Honeycutt RL (1987) Mitochondrial DNA and protein differentiation between hybridizing cytotypes of the white-footed mouse, *Peromyscus leucopus*. Evol 41:864-872

Peakall DB, McBee K (2001) Biomarkers for contaminant exposure and effects in mammals. In: Shore RF, Rattner BA (eds) Ecotoxicology of wild mammals. John Wiley & Sons, New York, pp 551-576

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol 6:288-295

Preston RJ, Dean BJ, Galloway S, Holden H, McFee AF, Shelby M (1987) Mammalian *in vivo* cytogenetic assays. Analysis of chromosome aberrations in bone marrow cells. Mut Res 189:157-165

Roark S, Brown K (1996) Effects of metal contamination from mine tailings on allozyme distributions of populations of Great Plains fishes. Environ Toxicol Chem 15:921-927

Schmitt CJ, Brumbaugh WG, Linder GL, Hick JE (2006) A screening-level assessment of lead, cadmium, and zinc in fish and crayfish from northeastern Oklahoma, USA. Environ Geochem Health 28:445-471

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

Sheffield SR, Sawicka-Kapusta K, Cohen JB, Rattner BA (2001) Rodentia and Lagomorpha. In: Shore RF, Rattner BA (eds) Ecotoxicology of wild mammals. John Wiley & Sons, New York, pp 215-314

Shugart LR (1999) Structural damage to DNA in response to toxicant exposure. In: Forbes VE (ed) Genetics and ecotoxicology. Taylor and Francis Publishers, Philadelphia, pp 151-167

Stangl Jr. FB, Baker RJ (1984) A chromosomal subdivision in *Peromyscus leucopus*: implications for the subspecies concept as applied to mammals. J Mamm 64:356-359

Stangl Jr. FB (1986) Aspects of a contact zone between two chromosomal races of *Peromyscus leucopus* (Rodentia: Cricetidae). J Mamm 67:465-473

Topashka-Ancheva M, Metcheva R, Teodorova S (2003) A comparative analysis of the heavy metal loading of small mammals in different regions of Bulgaria II: chromosomal aberrations and blood pathology. Ecotoxicol Environ Saf 54:188-193

USEPA, United States Environmental Protection Agency (2005) Five-year review report: third five-year review report for the Tar Creek Superfund Site, Ottawa County, Oklahoma. USEPA, Region 6, Dallas

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Friters A, Pot J, Paleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nuc Acid Res 23:4407-4414

Weidman S, Williams CF, Anderson CO (1932) Miami-Picher lead-zinc district. University of Oklahoma Press, Norman

Whitehead A, Anderson SL, Kuivila KM, Roach JL, May B (2003) Genetic variation among interconnected populations of *Catostomus occidentalis*: implications for distinguishing impacts of contaminants from biogeographical structuring. Mol Ecol 12:2817-2833

Table 3.1 Sequences of polymerase chain reaction primers and adapters used in AFLP analysis. An asterisk denotes fluorescently labeled primers.

Name	Sequence
Primers	
*EcoR*I - CAC*	5' - ACTGCGTACCAATTCCAC - 3'
*EcoR*I - CAT*	5' - ACTGCGTACCAATTCCAT - 3'
*Ase*I - TCT	5' - GATGAGTCCTGAGTAATTCT - 3'
*Ase*I - TAG	5' - GATGAGTCCTGAGTAATTAG - 3'
*Ase*I - TGT	5' - GATGAGTCCTGAGTAATTGT - 3'
*Ase*I – TAA	5' - GATGAGTCCTGAGTAATTAA - 3'
Adapters	
*Ase*I	5' - TACTCAGGACTCAT - 3'
	3' - GAGTCCTGAGTAGCAG - 5'
*EcoR*I	5' - AATTGGTACGCATCTAC - 3'
	3' - CCATGCGTCAGATGCTC - 5'

Table 3.2 Chromosomal aberrations and mitotic indices measured in *Peromyscus leucopus* from Beaver Creek (BC) and reference sites Sequoyah National Wildlife Refuge (SNWR) and Osage Wildlife Management Area (OWMA). Values in parentheses are ranges.

Locality	*n*	Number of Cells	Mean number aberrant cells/individual	Mean number of lesions/cell	Mean Mitotic Index (%)
BC	45	2250	1.02 (0-2)	0.022 (0-3)	1.17 (0.09-1.9)
SNWR	21	1050	1.24 (0-4)	0.027 (0-4)	1.38 (0.07-2.2)
OWMA	23	1150	0.91 (0-3)	0.018 (0-3)	1.25 (0.03-1.9)

Table 3.3 Total numbers of locally common bands, mean unbiased heterozygosity, and percent polymorphic loci observed in AFLP analysis (BC = Beaver Creek, COTT = Cotton Co., DSP = Douthat Settling Pond, LCB = Lake Carl Blackwell, OOL = Oolagah Wildlife Management Area, OWMA = Osage Wildlife Management Area, SNWR = Sequoyah National Wildlife Refuge, WOOD = Woodward Co.).

	BC	COTT	DSP	LCB	OOL	OWMA	SNWR	WOOD
Number of Locally Common Bands	68	64	75	71	68	65	65	64
Percent Polymorphic Loci	60.67%	53.93%	57.30%	62.92%	48.31%	47.19%	40.45%	56.18%
Mean Unbiased Heterozygosity	0.220	0.209	0.205	0.223	0.178	0.183	0.185	0.203
Locally Common Bands in ≤ 25% of Populations	1	0	1	0	0	0	0	0
Locally Common Bands in ≤ 50% of Populations	8	16	9	18	7	8	1	15

Table 3.4 Pairwise Φ_{PT} comparisons calculated from AFLP results (BC = Beaver Creek, COTT = Cotton Co., DSP = Douthat Settling Pond, LCB = Lake Carl Blackwell, OOL = Oolagah Wildlife Management Area, OWMA = Osage Wildlife Management Area, SNWR = Sequoyah National Wildlife Refuge, WOOD = Woodward Co.) * denotes $P \leq 0.05$, ** denotes $P \leq 0.01$

	BC	COTT	DSP	LCB	OOL	OWMA	SNWR	WOOD
BC	---							
COTT	0.897**	---						
DSP	0.012	0.903**	---					
LCB	0.834**	0.00	0.820**	---				
OOL	0.051*	1.042**	0.008	0.931**	---			
OWMA	0.095**	0.901**	0.011	0.800**	0.034	---		
SNWR	0.154**	0.828**	0.117*	0.809**	0.220**	0.196*	---	
WOOD	0.872**	0.01	0.817**	0.058	0.941**	0.746*	0.788**	---

Figure 3.1 Sampling localities used in metaphase chromosome analysis (4, 7, and 8 only) and AFLP analysis (1 = Woodward Co. (WOOD), 2 = Cotton Co. (COTT), 3 = Lake Carl Blackwell (LCB), 4 = Osage Wildlife Management Area (OWMA), 5 = Oolagah Wildlife Management Area (OOL), 6 = Douthat Settling Pond (DSP), 7 = Beaver Creek (BC), 8 = Sequoyah National Wildlife Refuge (SNWR)).

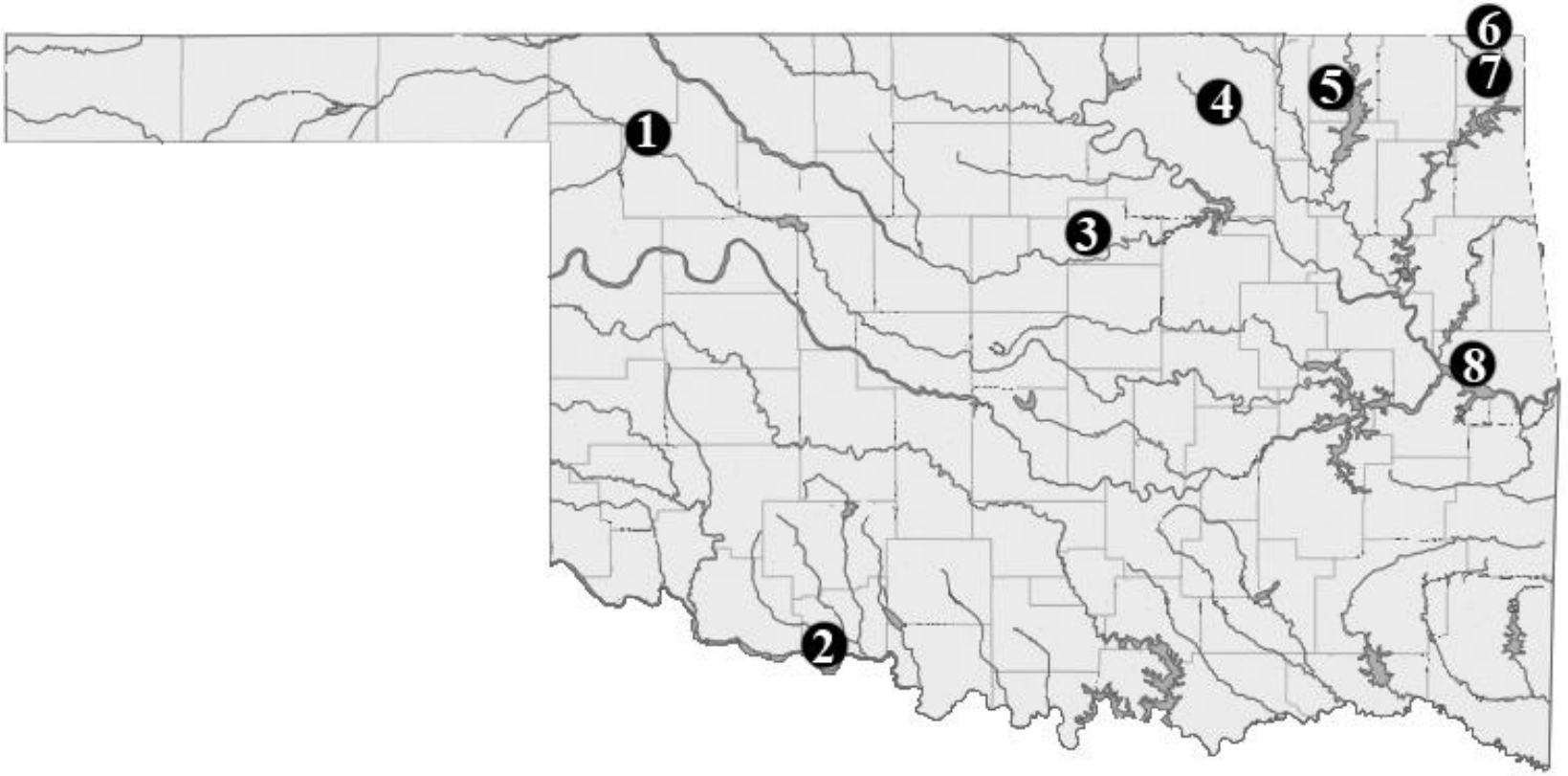

1
2
3
4
5
6
7
8

Figure 3.2 Chromosomal aberrations observed in *Peromyscus leucopus* (aberration denoted by arrow). A) chromatid break (OK 6313), B) chromosome break (OK 6265), C) ring chromosome (OK 6271), D) acentric fragment (OK 6277). OK numbers are catalog numbers in the Oklahoma State University Collection of Vertebrates Frozen Tissue Collection

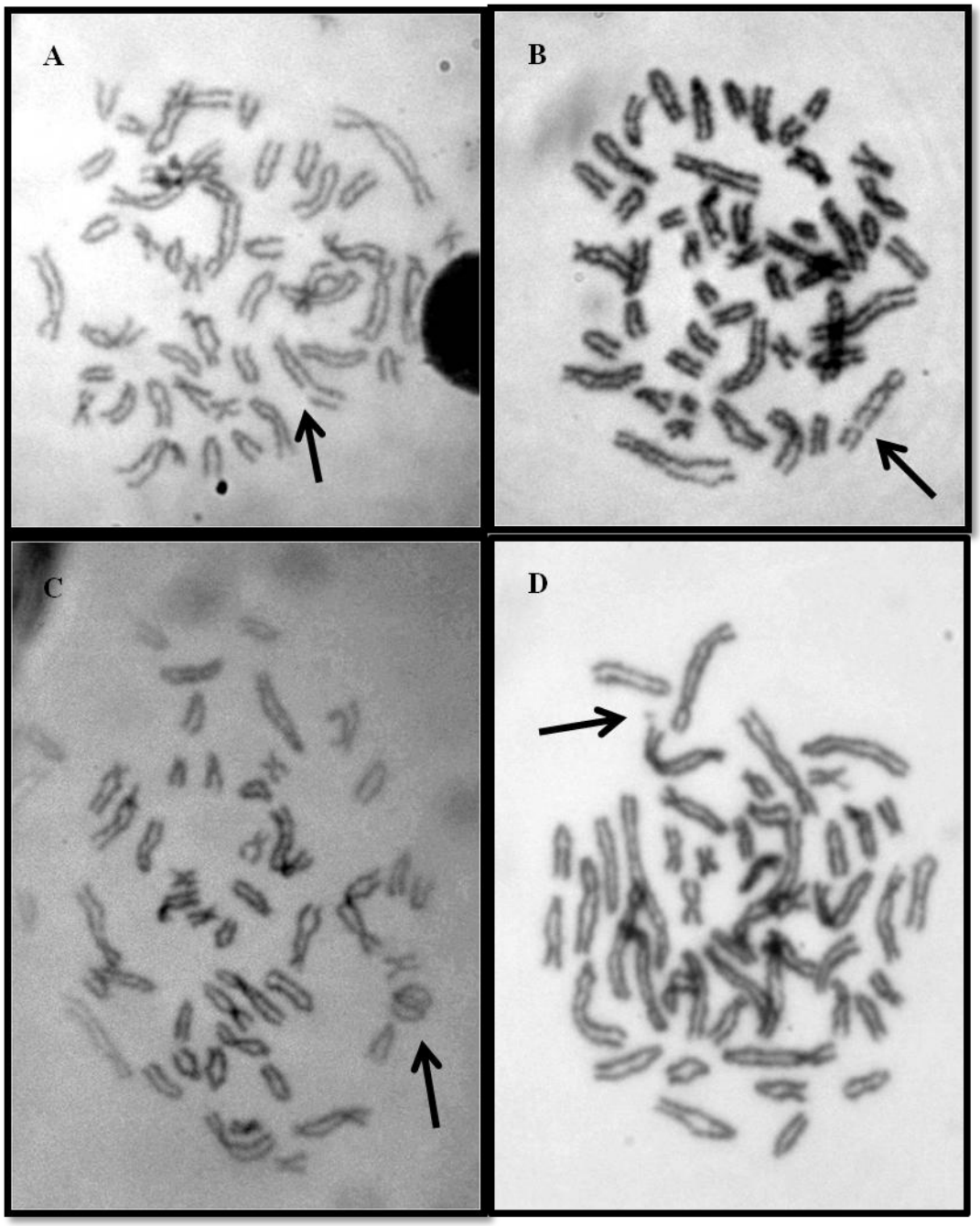

A
B
C
D

Figure 3.3 Frequency of chromosomal aberration classes observed in *Peromyscus leucopus* from Beaver Creek (BC), Sequoyah National Wildlife Refuge (SNWR), and Osage Wildlife Management Area (OWMA) (cB = chromatid break, CB = chromosome break, R = ring chromosome, ACF = acentric fragment).

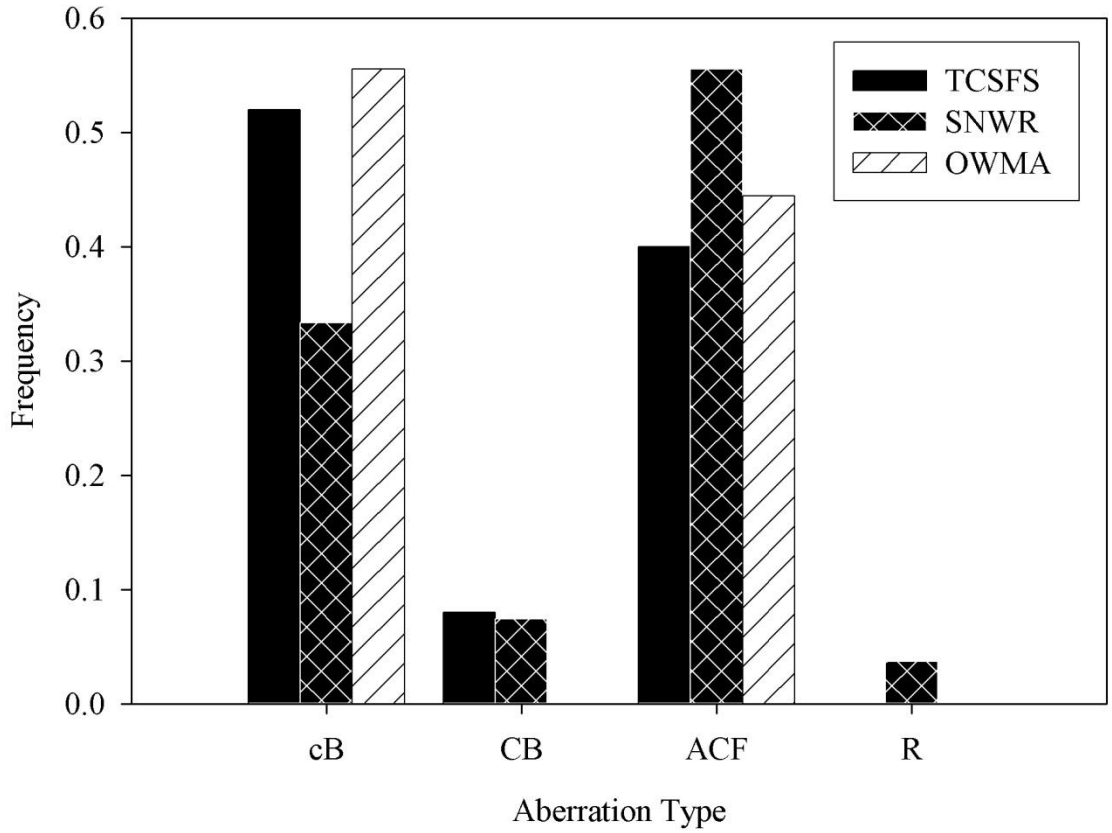
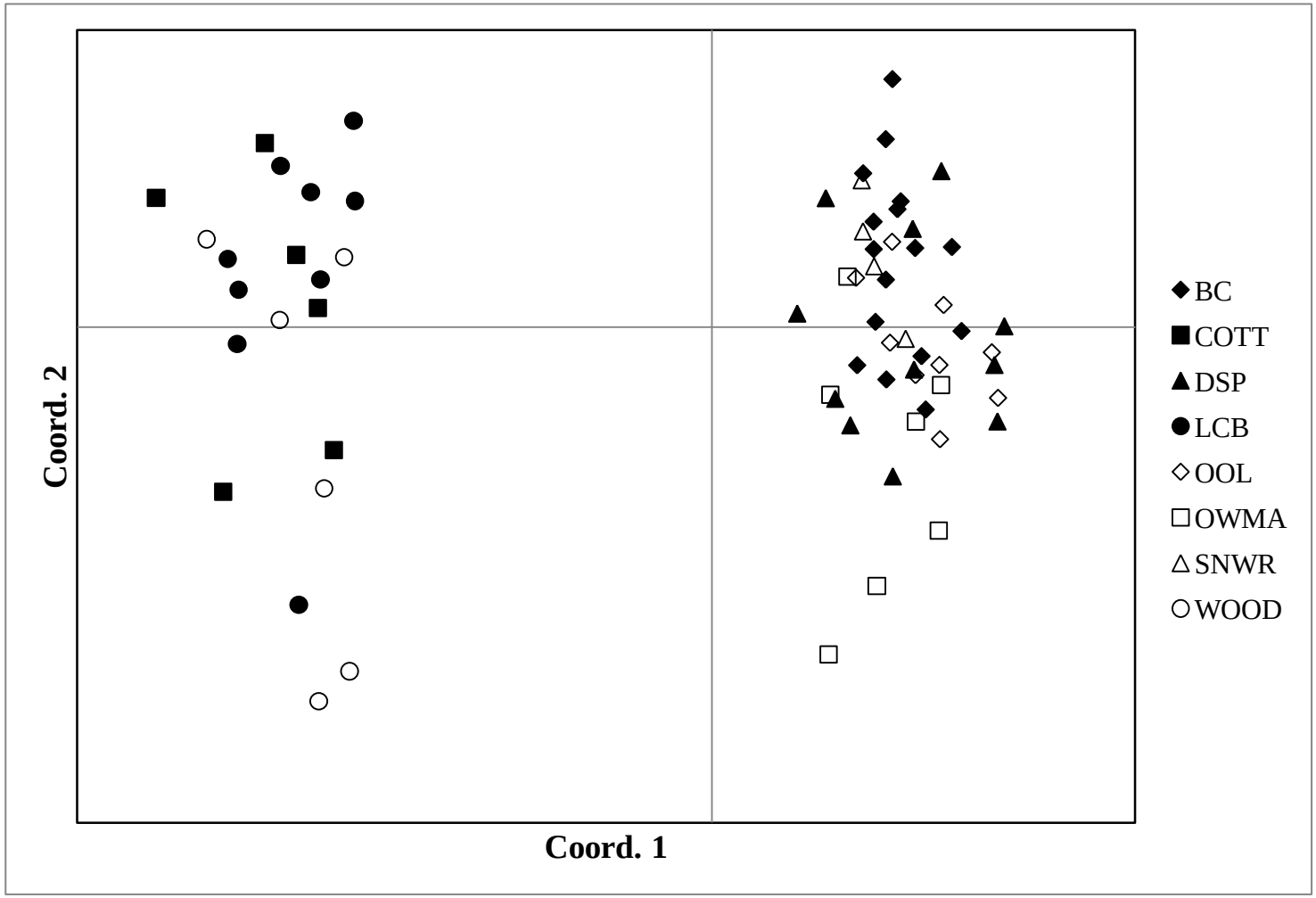

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Frequency
TCSFS
SNWR
OWMA
cB
CB
ACF
R
Aberration Type

Figure 3.4 Plot of the first 2 coordinates of principal coordinates analysis based on AFLP data representing *Peromyscus leucopus* from 8 sites in Oklahoma (BC = Beaver Creek, COTT = Cotton Co., DSP = Douthat Settling Pond, LCB = Lake Carl Blackwell, OOL = Oolagah Wildlife Management Area, OWMA = Osage Wildlife Management Area, SNWR = Sequoyah National Wildlife Refuge, WOOD = Woodward Co.).


Coord. 2
Coord. 1
BC
COTT
DSP
LCB
OOL
OWMA
SNWR
WOOD

POSTSCRIPT

Evaluation of wildlife populations exposed to contaminants *in situ* present challenges due to the effects of abiotic factors, environmental conditions, habitat heterogeneity, and organism interactions. However, *in situ* studies are necessary to understand the effects of xenobiotics on organisms in nature. I combined measurement of anogenital distance (AGD), metaphase chromosome analysis, and amplified fragment length polymorphism (AFLP) to assess potential deleterious effects of lead, cadmium, and zinc on white-footed mice (*Peromyscus leucopus*) from Tar Creek Superfund Site (TCSFS).

AGD, when corrected for body weight, did not differ significantly among TCSFS and two reference sites, Sequoyah National Wildlife Refuge (SNWR) and Osage Wildlife Management Area (OWMA). As expected, AGD was sexually dimorphic, being significantly longer in males than females at all sites sampled. Additionally, a significant positive correlation was seen between mass and AGD. There was a significant site*reproductive status interaction in males and females. *Post hoc* analyses revealed that these significant interaction terms were driven by small AGDs in non-reproductive males and females from OWMA.

Metaphase chromosome analysis was used to score standard karyotypes for 89 individuals from Beaver Creek within TCSFS, SNWR, and OWMA. Karyotypes were

scored for 6 types of structural aberrations, chromatid and chromosome breaks, ring chromosomes, dicentric chromosomes, translocation figures, and acentric fragments. This assay revealed no significant difference in the number of aberrant cells per individual or the number of lesions per cell among the 3 sites. Mitotic index did not differ significantly among the three sites. Chromatid breaks and acentric fragments were the most commonly observed aberrations at all sites

Peromyscus leucopus from 8 sites in Oklahoma were used in AFLP analysis to measure difference in population genetic structure. Two sites within TCSFS, Beaver Creek (BC) and Douthat Settling Pond (DSP), and 6 reference sites distributed throughout the state, OWMA, SNWR, Oolagah Wildlife Management Area (OOL), Lake Carl Blackwell (LCB), Cotton Co. (COTT), and Woodward Co. (WOOD). Analysis of molecular variance revealed that 68% of observed genetic variation occurred within populations. Isolation by distance was confirmed by a significant Mantel test. Distinct geographic clustering was detected by principal coordinates analysis. The three most western populations (LCB, COTT, and WOOD) clustered together into Group 1 and eastern populations (BC, DSP, SNWR, OOL, and OWMA) clustered together into Group 2. Pairwise Φ_{PT} comparisons revealed that all Group 1 populations were significantly different than all Group 2 populations. BC differed significantly from all populations except for DSP; however, DSP differed significantly only from LCB, COTT, WOOD, and SNWR. These results indicate some genetic differentiation of both contaminated populations from reference populations; however, the degree of differentiation varied between the two sites.

VITA

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Scope and Method of Study: The purpose of this study was to assess the effects of heavy metals on white-footed mice from Tar Creek Superfund Site. Animals used in the study were collected from Tar Creek Superfund Site, and two reference sites, Osage Wildlife Management Area and Sequoyah National Wildlife Refuge. Tissues from additional sites were obtained from the Oklahoma State University Collection of Vertebrates Frozen Tissue Collection. Assessment included measurement of anogenital distance, metaphase chromosome analysis, and amplified fragment length polymorphism analysis.

Findings and Conclusions: There was no significant decrease in anogenital distance in individuals from TCSFS. There was significant sexual dimorphism and positive correlation between mass and AGD at all three sites. Metaphase chromosome analysis revealed no significant difference in the incidence of structural chromosomal aberrations between TCSFS and two matched reference sites. Chromatid breaks and acentric fragments were the most commonly observed type of aberration at all sites. There was no significant difference in mitotic index among sites. Amplified fragment length polymorphism revealed a significant difference in population genetic structure between metal-exposed populations and geographically similar reference populations in Oklahoma; however, only one of the metal-exposed populations differed from all reference populations. Principal coordinates analysis did reveal strong geographic clustering of the populations with the three most western populations clustering and the five most eastern populations clustering together. The population genetic structure measured in AFLP analysis is similar to distribution of two karyotypic races of white-footed mice. The two races differ by three pericentric inversions and group into northeastern and southwestern groups with a hybrid zone in central Oklahoma. The eastern cluster from this study corresponds to the distribution of the northeastern cytotype and the western cluster corresponds to the southwestern cytotype.

ADVISER'S APPROVAL: Dr. Karen McBee