ASSESSMENT OF INDIVIDUAL AND
POPULATION-LEVEL ENDPOINTS IN RED-EARED
SLIDER TURTLES (*TRACHEMYS SCRIPTA*) FROM A
METAL-CONTAMINATED SUPERFUND SITE

By

KIMBERLY ANNE HAYS

Bachelor of Science
Jacksonville State University
Jacksonville, Alabama
2002

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 2005
ASSSESSMENT OF INDIVIDUAL AND POPULATION-LEVEL ENDPOINTS IN RED-EARED SLIDER TURTLES (*TRACHEMYS SCRIPTA*) FROM A METAL-CONTAMINATED SUPERFUND SITE

Thesis Approved:

Karen McBee
Thesis Adviser

Stanley Fox

Joseph Bidwell

A. Gordon Emslie
Dean of the Graduate College
Three chapters composing this thesis have been formatted for independent publication. The second chapter, “Effects of anthropogenic disturbance on population demographics of red-eared slider turtles (*Trachemys scripta*) in a former mining district”, will be submitted to *Biological Conservation*. The third chapter, “Flow cytometric analysis of effects of heavy metal contamination on red-eared slider turtles (*Trachemys scripta*) from the Tar Creek Superfund Site”, will be submitted to *Ecotoxicology*. The fourth chapter, “Time-to-event analysis to assess effects of heavy metals on neurobehavioral response in turtles”, will be submitted to *Bulletin of Environmental Contamination and Toxicology*. 
ACKNOWLEDGEMENTS

I must express my appreciation to my major advisor, Dr. Karen McBee, for her constant encouragement, enthusiasm, constructive criticism, and abundant patience as I waded through not only this project, but my life for the past several years. I also thank my other committee members, Drs. Stanley Fox and Joseph Bidwell for guidance on all aspects of this project and Drs. Mark Payton and Zhiang Zhang for invaluable statistical advice. I could never have completed the field portion of this study without the assistance of fellow graduate students, undergraduates, and friends who were willing to help me in the field, particularly Emily AcklandAry, Maria Harrington, Kyle Wesneski, Makiko Yabuhara, Jennifer Meyers, Joey Rushing, Courtney Lynd, Kendra Phelps, Miwa Hara, Kate Wilson, and Michael Kibbe. I must also thank Theresa Crum and Drs. George Cline and Frank Romano who not only taught me to do good science, but also encouraged me to teach others about it. I thank my family and friends who have remained supportive and encouraging even though they did not understand my “special weekend trips”.

The financial support of the Society of Environmental Toxicology and Chemistry, EA Engineering, Sigma Xi, Robberson and Lochmiller families, and Oklahoma State University Graduate College and Department of Zoology made this project possible.
TABLE OF CONTENTS

Chapter                                              Page

I. MULTI-ENDPOINT TOXICOLOGICAL EVALUATION OF RED-EARED SLIDER TURTLES (*TRACHEMYS SCRIPTA*) FROM TAR CREEK SUPERFUND SITE ........................................................................................................... 1
   Introduction......................................................................................................... 2
   Literature Review ................................................................................................ 5
       The important role of reptiles as biomonitors ........................................... 5
       Reptiles and environmental contamination ................................................ 7
       Natural history of *Trachemys scripta* ...................................................... 9
       A multi-endpoint approach at TCSFS ......................................................... 11
   References......................................................................................................... 17

II. EFFECTS OF ANTHROPOGENIC DISTURBANCE ON POPULATION DEMOGRAPHICS OF RED-EARED SLIDER TURTLES (*TRACHEMYS SCRIPTA*) IN A FORMER MINING DISTRICT ........................................................................ 26
   Abstract............................................................................................................. 27
   Introduction....................................................................................................... 28
   Methods ............................................................................................................ 31
       Study Sites .................................................................................................. 31
       Field Methods .............................................................................................. 32
       General Population Characteristics ......................................................... 33
       Demographic Analysis .............................................................................. 34
   Results............................................................................................................... 36
       General Characteristics ............................................................................. 36
       Demographic Analysis ............................................................................... 37
   Discussion......................................................................................................... 39
   Acknowledgements ........................................................................................... 46
   References......................................................................................................... 46
III. FLOW CYTOMETRIC ANALYSIS OF EFFECTS OF HEAVY METALS ON RED-EARED SLIDER TURTLES (TRACHEMYS SCRIPTA) FROM THE TAR CREEK SUPERFUND SITE, OKLHOMA............................................60

Abstract ............................................................................................................. 61
Introduction ....................................................................................................... 62
Methods ............................................................................................................ 65
   Study Sites .................................................................................................. 65
   Field Methods .......................................................................................... 66
   Flow Cytometric Analysis ......................................................................... 67
   Metal Analysis .......................................................................................... 68
   Quality Control ......................................................................................... 69
   Statistical Analysis ................................................................................... 70
Results ............................................................................................................... 71
   Flow Cytometry ....................................................................................... 71
   Metals Analyses ....................................................................................... 73
Discussion ......................................................................................................... 74
Acknowledgements ......................................................................................... 81
References ....................................................................................................... 82

IV. TIME-TO-EVENT ANALYSIS TO ASSESS EFFECTS OF HEAVY METALS ON NEUROBEHAVIORAL RESPONSE IN TURTLES.......................... 101

Introduction ..................................................................................................... 102
Methods .......................................................................................................... 104
Results and Discussion .................................................................................... 106
Acknowledgements .......................................................................................... 113
References ...................................................................................................... 114

V. SUMMARY ..........................................................................................................117
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.1. Mean plastron length and mass (± 1 S.E.) and percentage of adult male <em>Trachemys scripta</em> exhibiting melanism from mined site, Tar Creek Superfund Site (TCSFS) and reference sites, Lake Carl Blackwell (LCB) and Sequoyah National Wildlife Refuge (SNWR)</td>
<td>52</td>
</tr>
<tr>
<td>I.2. Mean (± 1 S.E.) mass, plastron length, and carapace length of adult male and female <em>Trachemys scripta</em> collected from mined Tar Creek Superfund Site (TCSFS) and reference sites, Lake Carl Blackwell (LCB) and Sequoyah National Wildlife Refuge (SNWR)</td>
<td>53</td>
</tr>
<tr>
<td>IV.1. Results of Cox Regression Analysis - Initiating Righting</td>
<td>109</td>
</tr>
<tr>
<td>IV.2. Results of Cox Regression Analysis – Completing Righting</td>
<td>110</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>II.1. Distribution of adult and juvenile, female (F) and male (M) <em>Trachemys scripta</em> collected from mined Tar Creek Superfund Site (TC) and reference sites, Lake Carl Blackwell (LC) and Sequoyah National Wildlife Refuge (SN)</td>
<td>55</td>
</tr>
<tr>
<td>II.2. Proportional distribution of size classes based on plastron length (mm) of <em>Trachemys scripta</em> collected from mined Tar Creek Superfund Site (TCSFS) and reference sites, Lake Carl Blackwell (LCB) and Sequoyah National Wildlife Refuge (SNWR)</td>
<td>57</td>
</tr>
<tr>
<td>II.3. Recapture estimates ($\rho$) as generated by Program MARK for monthly trapping intervals in 2003 and 2004 at mined Tar Creek Superfund Site and reference sites, Lake Carl Blackwell (LCB) and Sequoyah National Wildlife Refuge (SNWR)</td>
<td>59</td>
</tr>
<tr>
<td>III.1. Mean coefficient of variation of animals collected August – September 2003 from Tar Creek Superfund Site (TCSFS) and reference sites, Lake Carl Blackwell (LCB) and Sequoyah National Wildlife Refuge (SNWR)</td>
<td>90</td>
</tr>
</tbody>
</table>
III.2. Mean coefficient of variation (+ 1 S. D.) of animals collected April – October 2004 from A) Tar Creek Superfund Site (TCSFS) and reference sites, B) Sequoyah National Wildlife Refuge (SNWR), and C) Lake Carl Blackwell (LCB). \( P \) values were generated in ANOVA to test for significant differences among months at each site. ................................................................................................................................. 92

III.3. DNA flow histograms of normal (A and B) and aneuploid (C and D) individuals from the Tar Creek Superfund Site (TCSFS) population. Arrows point to the aneuploid peaks or shoulders adjacent to the main \( G_1 \) peak (highest peak). Specimens illustrated include: A) OK 7170 B) OK 7259 C) OK 7188 D) 7184.................................................................................................................................94

III.4. Blood levels of Pb and Cd for a subset of animals collected from TCSFS and SNWR in spring (April), summer (June), and fall (September) during the 2004 collection season.........................................................................................................................96

III.5 Levels of Pb detected in bone for a subset of animals collected from TCSFS and SNWR mid and late during the 2004 collection season.................................................................98

III.6 Relationships between tissue metal levels and mean CV for a subset of animals collected from contaminated Tar Creek Superfund Site (TCSFS) and reference site, Sequoyah National Wildlife Refuge (SNWR) in spring (April), summer (June/July), and fall (September) 2004.................................................................100
IV.1. Kaplan-Meier curve representing initiating righting time for *T. scripta* populations at TCSFS and reference sites, SNWR and LCB. Log-rank statistics revealed a significant difference between reference sites (*P* = 0.0054). Reference sites were considered separately for all subsequent analyses.................................107

IV.2. Kaplan-Meier curve representing complete righting time for *T. scripta* populations at TCSFS and reference sites, SNWR and LCB. Log-rank statistics revealed no significant difference between reference sites (*P* = 0.25). Reference sites were combined for all subsequent analyses.................................................................108
CHAPTER 1

MULTI-ENDPOINT TOXICOLOGICAL EVALUATION OF RED-EARED SLIDER TURTLES (TRACHEMYS SCRIPTA) FROM TAR CREEK SUPERFUND SITE
The Tri-State mining district, made up of portions of NE Oklahoma, SW Missouri, and SE Kansas, was an area of heavy lead and zinc mining from 1891 to 1970 (Weidman, 1932; Parkhurst et al., 1988). In Oklahoma, there were three major mining areas, the Peoria District, located east of the Spring River, and the Miami and Quapaw Districts located west of the Spring River. After 1914, the Miami and Quapaw Districts were combined and referred to as the Picher Field mining area. Zinc ores (sphalerite, smithsonite, and calamine) and lead ore (galena) were mined by means of the room-and-pillar method in which rooms are dug out beneath the water table and pillars of ore are left intact to support the room. During years of active mining, water was pumped out of mines into flotation ponds to separate mine tailings from desired minerals. In the 1920’s, the region generated 300,000 to 400,000 tons of zinc per year and was responsible for 75% of zinc production in the United States (Weidman, 1932). After mines were abandoned in the 1970’s, pumping ceased and mines began to fill with water. Discharge of heavily contaminated acid mine drainage began in 1979 (USEPA, 2000). Picher Field mining area was proposed for listing on the Environmental Protection Agency’s National Priority List in September 1980 and was officially listed in 1983 as Tar Creek Superfund Site (TCSFS). Formal listing made the site eligible for federal and state funding for remediation and restoration. The municipalities of Picher, Cardin, Quapaw, North Miami, and Commerce, with a combined human population of approximately 20,000, are located within TCSFS. Also, 70% of the site is located on tribal land owned by the Quapaw Tribe of Oklahoma, thereby requiring involvement of additional federal and tribal agencies in remediation efforts and increased cultural sensitivity to land and wildlife species (USEPA, 2005). Acidic water, contaminated with lead, zinc, cadmium, arsenic,
and other metals, continues to flow from mines into Tar Creek and several smaller creeks on the site, which feed the Neosho River and later empty into Grand Lake O’ the Cherokees. Mine water also leaches into the shallow Boone aquifer, which overlies the drinking water source for local communities, Ribidoux aquifer (Parkhurst et al., 1988; USFWS, 2000). Seventy-five million tons of mine tailings left over from the ore extraction process and 800 acres of both wet and dry flotation ponds still remain on the site (USEPA, 2005).

Studies of wildlife from sites with known contamination, such as TCSFS, often are used in protection of human health, but also provide critical data necessary for remediation and restoration of wildlife habitats. Reptiles, such as turtles, are the most understudied group of vertebrates in toxicology (Hopkins, 2000), yet they can provide valuable insight into effects of environmental contamination. Characteristics such as high site fidelity, delayed sexual maturity, and broad geographic distribution outweigh difficulties encountered when using reptiles in toxicological studies (Hopkins, 2000). Only a few studies have used turtles as biomonitors (Bickham et al., 1988; Meyers-Schöne et al., 1993; Lamb et al., 1995), but these animals bridge aquatic and terrestrial habitats, have a wide range of dietary strategies over their lifetimes, and lead long lives (Gibbons, 1990a). Beaver Creek runs through a 40-acre portion of TCSFS owned by the Quapaw Tribe of Oklahoma and known as the Catholic 40. A low water crossing built across Beaver Creek to provide access to a mine resulted in formation of a pond that we observed flooding over onto surrounding chat piles during periods of heavy rainfall. *Trachemys scripta* (red-eared slider) is common in Beaver Creek at the Catholic 40 (TCSFS), and at Sequoyah National Wildlife Refuge (SNWR) and Lake Carl Blackwell
unmined reference sites. The goal of my study was to use multiple assays and endpoints to assess *T. scripta* present at Beaver Creek within TCSFS.

Habitat alteration and exposure to metals can have a wide range of effects including alteration of demographic parameters, behavioral functions, and genetic integrity (Kazantzis and Lilly 1979; Sharma and Talduker, 1987). Impacts on basic demographic parameters can include alteration of birth rate, clutch size, and mortality rate and induction of malformations. Behavioral endpoints reflect sub-lethal toxicity (Little, 1990). Chromosomal lesions are associated with reduced fertility, induced birth defects, and carcinogenesis, and may have long-term, multi-generational effects on exposed biota (Shugart, 1990). Monitoring frequencies of chromosomal lesions makes it possible to identify effects of contaminants in seemingly healthy populations. Analysis of tissue metal levels in *T. scripta* from TCSFS is an imperative component in establishing a link between metal exposure and population, genetic, and behavioral effects.

Investigation of effects of heavy metals on *T. scripta* at TCSFS will provide valuable information on potential impacts on multiple endpoints, help establish level of risk, and provide critical information for implementation of cleanup programs before human and wildlife health is further affected. Specifically, I investigated effects of exposure to environmental metals on: 1) population demography, 2) genetic structure, 3) neurobehavioral response, and 4) tissue-metal content, in *T. scripta* inhabiting Beaver Creek at TCSFS and two unmined, reference sites, LCB and SNWR. I monitored population size and structure for all three populations using mark-recapture methods to test the hypothesis that the TCSFS population would show altered population structure compared to populations at reference sites. By monitoring variation in DNA content
using flow cytometry, I hypothesized that animals from TCSFS would show increased frequencies of chromosomal lesions, indicated by larger mean coefficients of variation, when compared with reference animals. Righting time was used as a measure of neurobehavioral response, and I hypothesized that animals exposed to heavy metals at TCSFS would show longer times to complete righting as a result of impaired neurological function. Finally, TCSFS animals also were expected to have higher blood and bone concentrations of lead and cadmium as determined by atomic absorption spectrometry. The use of population, genetic, and behavioral endpoints in combination with classical analysis of tissue metal levels provides a more complete understanding of detrimental effects of exposure to heavy metals on wildlife populations and aids in establishing turtles, *T. scripta* in particular, as valuable environmental sentinels.

LITERATURE REVIEW

*The important role of reptiles as biomonitors*

Much emphasis has been placed on finding suitable species to serve as biomonitors for environmental contamination. Reptiles are the least studied of all vertebrates in toxicology, yet they have many characteristics that make them a desirable choice for field based toxicological studies (Heppell, 1998; Hopkins, 2000). Life span and generation time of many reptiles exceed that of most mammals, birds, and amphibians (Tinkle, 1961). Animals with long lives and delayed sexual maturity can be exposed to environmental conditions for long periods of time in immature, juvenile, and
reproductively active, adult stages (Ernst et al., 1994). Although these characteristics may present challenges in toxicological studies, reptiles can often provide data more relevant to the human lifespan than short-lived animals.

Wide geographic distribution of reptiles allows for large-scale studies from a variety of geographic locations (Hopkins, 2000). The fairly sedentary lifestyle of reptiles also promotes them as ideal biomonitors. Movements of females occur only during nesting and breeding seasons, and males make larger movements during periods of courtship (Ernst et al., 1994). Females often exhibit strong philopatry, returning to the same nesting sites season after season. Strong philopatry coupled with small home ranges makes reptiles fairly easy to track and locate over the course of their lifetime. This trait also removes some uncertainty about location of exposure, an important consideration in toxicological field work. Reptiles often can be tied directly to a contaminated area in which the source and levels of contamination are known, whereas organisms with large home ranges and low site fidelity can encounter numerous sources of extraneous contamination, making evaluation of type, location, and duration of exposure problematic.

Reptiles demonstrate several types of reproductive strategies. Turtles are oviparous and employ a wide variety of nesting strategies with variable amounts of contact with substrate and water. Contact with substrate and water may provide a route of contaminant uptake in hatchlings (Webb 1961; Zug et al., 2001). Turtles and many other reptiles undergo temperature dependant sex determination (TSD) and can provide valuable insight into global temperature changes resulting from natural phenomena, human induction, or environmental contamination. Even small variations in nest
temperature during the incubation period can drastically alter sex ratios (Ferguson and Joanen, 1982). Most reptiles occupy a high trophic level at some point in their life cycle, thereby increasing the probability of exposure to contaminants passed through lower trophic levels (Lower and Kendall, 1990).

Reptiles and environmental contamination

Only a small percentage of toxicological studies involve reptiles; however, reptiles are exposed to a wide variety of contaminants including organic compounds, radionuclides, and heavy metals. The long biological half-lives of many of these contaminants increase their probability of bioaccumulation (Hall, 1980).

Reptiles may be exposed to a variety of organic compounds. Many organic-based pesticides are highly lipophilic in nature and are often introduced to reptiles via trophic transfer (Hall, 1980). Poikilotherms, including reptiles, may be less able to metabolize or detoxify contaminants than homeotherms due to periods of depressed metabolic activity (Hall, 1980). Some organic compounds act as endocrine disruptors either blocking or mimicking naturally produced androgens or estrogens or altering synthesis or degradation of these hormones (Crain and Guilette, 1998; Guilette, 2000). Trachemys scripta eggs dosed with PCB metabolites (2’, 4’, 6’ – Trichloro - 4 - biphenylol and 2’, 3’, 4’, 5’ - Tetrachloro - 4 - biphenylol) showed complete sex reversal when incubated at 26°C, a temperature that typically produces 100% males. When mixed, these two compounds synergized and significantly increased the amount of ovarian development at doses as low as 1 ppm (Crews et al., 1995). Willingham (2000) showed that eggs treated with
pesticides (chlordan, *trans*-Nonachlor or *p, p’* – DDE) yielded hatchlings with altered sex ratios and growth rates.

Radioactive waste disposal and large-scale radioactive disaster can serve as sources of radionuclide contamination. Several studies address the effects of cesium and strontium on red-eared sliders from the Savannah River Ecology Lab in Aiken, South Carolina (Bickham et al., 1988; Lamb et al., 1991; Lamb et al., 1995). *Trachemys scripta* exposed to $^{134,137}$Cs and $^{89,90}$Sr in radioactive seepage basins showed chromosomal damage in red blood cells examined by flow cytometry. Plastron length and DNA content variation were positively correlated, suggesting that longer exposure leads to more dramatic damage (Lamb et al., 1991; Lamb et al., 1995). Meyers-Schöne et al. (1993) suggested that DNA insult via radionuclide exposure is directly related to diet. Common snapping turtles (*Chelydra serpentina*) are piscivorous and showed more DNA damage and a higher body burden of radionuclides than did *T. scripta*, a more herbivorous turtle (Meyers-Schöne et al., 1993).

Metals occur naturally and many are essential for normal physiological functions. However, 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. Thirty metals, both essential and non-essential, are known to have toxic effects (Goyer and Clarkson, 2001). Cadmium, lead, selenium, chromium, and manganese have been detected in turtle egg shells; lead, mercury, and selenium were found in high concentrations in egg contents of *T. scripta* (Burger and Gibbons, 1998). Presence of heavy metals in both shell and egg contents suggests that females are capable of
sequestering metals in eggs and eggshells, thereby possibly reducing the metal load in their own bodies (Meyers-Schöne and Walton, 1994; Moeller, 2004).

Metals have a variety of effects in reptiles, including behavioral effects, hematological alterations, depression of enzyme activity, and DNA strand breakage. Common snapping turtles (C. serpentina) exposed to lead at three sites within Missouri’s Old Lead Belt showed a significant negative correlation between tissue Pb concentrations and activity of δ–ALAD (aminoleuvilinic acid dehydratase), a blood enzyme necessary for heme synthesis. Turtles from the most contaminated site showed a 93 to 94 % reduction in δ–ALAD activity when compared with turtles from less contaminated sites (Overmann and Krajicek, 1995). Meyers-Schöne et al. (1993) showed that T. scripta and C. serpentina bioaccumulate Hg in both kidney and muscle tissues and had higher frequencies of DNA breakage than control animals.

*Natural history of Trachemys scripta*

I used *T. scripta* as a biomonitor of heavy metal contamination at TCSFS. *Trachemys scripta* is found throughout the Mississippi Valley of the United States and ranges south from Illinois to the Gulf of Mexico. The natural range extends south through Mexico and Central America and into Columbia and Venezuela. Slider turtles are found worldwide due to introduction via the pet trade (Gibbons, 1990a). Sliders often inhabit slow-moving bodies of water with soft bottoms such as lakes, ponds, roadside ditches, and slow-moving streams. Basking on rocks, logs, and other projections allows sliders to raise their internal temperature, which increases metabolism and digestion rates.
(Parmenter and Avery, 1990). Sliders cannot tolerate water temperature below 10°C and will hibernate in stumps, abandoned muskrat burrows, and soft mud at the bottom of ponds or lakes to avoid extreme cold (Gibbons, 1990a).

Sexual maturity of female *T. scripta* has been a topic of debate. Gibbons et al., (1981) claims that female sliders mature between 6 to 10 years; conversely, data from Lake Texoma, Oklahoma, suggest that females mature at approximately 174 mm plastron length regardless of age (Webb, 1961). Males mature at 90 to 110 mm typically between 3 to 5 years of age (Gibbons et al., 1981). Sliders can live for 20 to 30 years in wild populations, making them one of the few biomonitors with exposure duration similar to that of human populations (Gibbons, 1990a). Home ranges are significantly larger in males than females. Larger females exploit larger home ranges than smaller females; but body size in males does not significantly influence home range size (Schubauer et al., 1990). Mating occurs in the spring (Gibbons, 1990a). Nests are dug by females in sandy or loamy soil usually in disturbed areas such as road banks, dikes, or dams with little plant cover (Webb, 1961). Eggs are laid in early spring and incubate for approximately 3 months before hatching. Hatchlings typically overwinter in the nests and do not emerge until the following spring. Eggs have a highly calcareous shell with a large amount of yolk, which is necessary to maintain hatchlings until their emergence from the nest. The calcareous shells are somewhat flexible and porous to allow gas and water exchange. Females can produce 2 clutches per reproductive season and require 2 weeks between laying events (Gibbons, 1990a). Webb (1961) estimated reproductive capacity of *T. scripta* to be as high as 26 eggs per season. *Trachemys scripta* lack sex chromosomes and undergo TSD. Male-biased populations are produced at 26°C, whereas female
populations are produced at 31°C (Hall, 1980). Sex ratios can vary among populations of *T. scripta* and among years of collection. Generally, wild populations of *T. scripta* yield male-biased adult sex ratios due to different rates of male and female maturity (Gibbons, 1990b).

*Trachemys scripta* is considered an opportunistic omnivore and eats vertebrates, invertebrates, and vegetation (Parmenter and Avery, 1990). Seasonal and age-related shifts are evident in eating patterns. Juveniles are often omnivorous and consume large amounts of protein-rich food to aid in development. Adults are considered herbivores, but omnivory tends to dominate in summer months and herbivory in cooler seasons (Parmenter and Avery, 1990). Slider turtles are responsive to olfactory cues but do not rely heavily on olfaction for prey detection and capture. They are active foragers attracted to movements in the water column. Microhabitat exclusion is seen between the age classes; adults forage in deeper waters possibly due to maneuverability constraints in shallow areas. Juveniles forage in shallower areas that are not as easily accessible to larger, adult turtles (Parmenter and Avery, 1990).

*A multi-endpoint approach at TCSFS*

In order to assess the impact of heavy metals on *T. scripta* at TCSFS, I combined measures of genetic integrity, population demography, and neurobehavioral response with classical tissue metal analyses.

Life history traits, such as age-specific birth and death rates, fecundity, and sex ratios, can have a major impact on community structure and ecosystem health. Mark-
recapture studies are non-invasive and can help provide valuable information about population demography. Turtles can be easily marked on marginal scutes without causing harm to the animal. For example, Frazer et al. (1991) used mark-recapture methods over a 25-year period to estimate growth, survivorship, and longevity on painted turtles (*C. picta*) in Michigan. Increases in density and growth rates were linked with weather patterns during the sampling period. Mark-recapture methods also were used to correlate population density of the Sabine map turtle (*Graptemys ouachitensis sabinensis*) with various environmental variables, including depth, flow rate, and light penetration (Shivley and Jackson, 1985). Investigation of population demography in exposed, but seemingly healthy populations, is a good first step in assessing the impacts of various types of anthropogenic disturbance, including mining (Marchand and Litvaitis, 2004; Conner et al., 2005). Slight alterations in size of individuals, sex ratio, or the proportion of juveniles could have lasting effects on long-term success of the population.

Genetic damage is a useful biomarker with which to assess effects of environmental contamination in natural populations because many anthropogenic and natural contaminants are genotoxicants and alter structure and function of DNA (Sharma, 1985; Shugart 1990). Genetic insults often occur only as a small number of structural alterations in the entire genome, but persistence of genetic insults can lead to further response at other levels of organization (Shugart, 1999). Genetic damage occurring in inert or non-coding sections of DNA may not be phenotypically expressed and is more difficult to identify than phenotypically expressed alterations. Genotoxicants can cause genetic damage via several pathways: damage irreparable by normal enzymatic repair systems, inhibition of enzymatic repair systems, or alterations in DNA replication.
Genotoxic chemicals or their metabolites can covalently bind with nucleotides of DNA, typically guanine, resulting in the formation of DNA adducts (Jackson, 1971; Sharma, 1985; Shugart, 1999). Adduct formation and spindle disruptions are genetic insults that do not directly change the structure of chromosomes, whereas chromosomal lesions are structural alterations of chromosomes. Chromosomal lesions can lead to decreased fertility, induced birth defects, and carcinogenesis, and include strand breaks, exchange figures, and deletions (Sharma, 1985; Shugart, 1990; Shugart, 1999).

Several metals, including Cd, Zn, and Pb, can act as genotoxicants, but the exact mechanisms by which they elicit chromosomal lesions are unknown (Tachi et al., 1985; Hartwig, 1994). Cd can compete with calcium and Zn for binding sites on DNA repair enzymes and prevent the repair of normally occurring DNA damage (Hartwig, 1994; Satoh et al., 2002). Some studies show that Cd and Pb can cause chromosomal lesions by altering the redox state within cells and releasing free radicals, which can damage the structure of DNA (Hartwig et al., 2002; Lin et al., 2005). Zn, although an essential cofactor for many enzymes, also has been shown to induce chromosome gaps and breaks in vivo (Hikiba et al., 2005).

Flow cytometry (FCM) is an ideal assay for the evaluation of chromosomal lesions because it allows for highly precise observation of large numbers of cells (Watson, 1987; Dallas and Evans, 1990). Flow cytometry measures the amount of DNA present in each nucleus of a population of cells. Variation among the nuclei of an individual is represented as the coefficient of variation (CV) around the mean for all cells in the G1 stage of the cell cycle. Increased CVs result from chromatin breaks that alter amounts of DNA being distributed to daughter cells (Shapiro, 1983). Flow cytometric
analysis of multiple tissue types including blood, heart, spleen, and kidney has been used extensively to assess *T. scripta* inhabiting radioactive seepage basins at Savannah River Ecology Lab. Those data showed multiple peaks indicative of aneuploidy caused by increased DNA content variation. The assay was highly repeatable between studies and between different tissues (Bickham et al., 1988; Lamb et al., 1991; Lamb et al., 1995). Flow cytometry has also been used in populations of birds and mammals exposed to contaminants (Bickham et al., 1992; Husby and McBee, 1999; Whittier and McBee, 1999).

Behavioral endpoints reflect sub-lethal toxicity ranging in magnitude from learning and retention difficulties to decreased predator avoidance to alteration in calling or courtship behaviors to decreased righting times (Little, 1990). Investigation of behavioral effects in turtles has taken several routes. Hatchlings dosed with contaminants in the lab are most commonly used in behavioral studies because exposure concentration and duration are known. Burger et al. (1998) measured survival and used righting trials to assess impacts of lead exposure on motor skills on hatchling slider turtles. Hatchlings dosed with lead showed decreased survival, and individuals injected with 1.0 mg/g took significantly longer to completely right themselves when placed on their carapace (Burger et al., 1998). PCB contamination was inversely related to righting response in hatchling snapping turtles exposed to PCBs in the Sheboygan River, Michigan, with approximately 35% of turtles from highly contaminated sites showing no response (Patnode et al., 1998; Portelli and Bishop, 2000). Behavioral toxicology is subject to criticism because of the difficulty in tying behavioral effects to ecological effects; however, classical laboratory assays alone are insufficient because they may not provide
a complete representation of sub-lethal contaminant effects in a field setting (Little, 1990).

Contaminant levels in tissues and the environment must be determined in any toxicological study to provide a link between environmental contamination and effects within the organism of interest. Unlike laboratory studies, dose and duration of environmental contamination is often unknown. When investigating effects of contaminants in the environment it is essential that both dose and bioavailability are taken into consideration. In the environment, only a portion of contaminants are available for uptake because of physicochemical properties of contaminants and matrices in which they are found. Metals, for example, can exist in a variety of oxidation states, which can have very different effects (Kendall et al., 2001). Most metals, with the exception of lead, are transported by water. Much of the total metal load is carried on different particles in the water column, including dissolved organic carbon, and eventually becomes deposited in sediment. Sequestration or tight sorption of contaminants in soil or sediments can lead to decreased bioavailability over time (Meyers-Schöne and Walton, 1994). However, changes in chemical and physical factors of the water and sediment, including pH and microbial activity, can alter binding properties and make previously deposited metals available for uptake (Beijer and Jernelöv, 1979). Many contaminants undergo changes during the deposition process that affect bioavailability. Mercury, for example, may be methylated during its incorporation into the sediment producing methylmercury, which is more bioavailable than the inorganic form (Meyers-Schöne and Walton, 1994). Toxicity and bioavailability of divalent cationic metals, including Cd,
Zn, and Pb, can be reduced by binding action of acid volatile sulfides in anoxic sediments (Ankley, 1996).

Contaminant levels, from a variety of tissues, are commonly used in the investigation of exposed wildlife because they often provide an accurate representation of bioavailable contaminants. Swartz et al. (2003) collected blood, liver, and fat samples to assess micronuclei counts, mercury levels, and pesticides in turtles exposed to industrial waste in Azerbaijan. Moeller (2004) used atomic absorption spectrometry to determine zinc and cadmium levels in substrate from the Catholic 40 and from lab-exposed *T. scripta* eggshells, tissue, and turtle shell samples. This assay has also been used to determine levels of trace metals in blood of Kemp’s Ridley sea turtles (*Lepidochelys kempii*) (Kenyon et al., 2001). Clark et al., (2000) used atomic absorption spectrometry to determine metal levels of several wetland reptiles, including *T. scripta*. Analysis of tissue metal levels in *T. scripta* from TCSFS allows us to link exposure to heavy metals and genetic, population, and behavioral effects.

Toxicants can be a major threat to long-lived organisms, but these animals often do not receive attention due to the time and expense of long-term toxicological studies. However, it is imperative that we study these organisms at contaminated sites, like TCSFS, to protect wildlife populations and generate data applicable to the protection of human health. Due to their life history characteristics and low metabolic rate, reptiles, such as turtles, may be less able to metabolize or detoxify contaminants than birds and mammals and can serve as biomonitors for environmental contamination (Hall, 1980). TCSFS has been listed on EPA’s National Priority list since 1983, but major risk to humans and wildlife still exist. I seek to establish *T. scripta* as a valuable biomonitor at
Beaver Creek within TCFS. I also seek to combine multiple endpoints to assess and provide a more complete picture of possible negative anthropogenic effects on *T. scripta* present at TCSFS.

REFERENCES


Moeller, L.A. 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, Stillwater, OK, USA.


United States Environmental Protection Agency (USEPA). 2005. TCSFS Fact Sheet, Tar Creek (Ottawa County) Washington D.C., USA.

United States Fish and Wildlife Service (USFWS). 2000. Final partial restoration plan and environmental assessment addressing injuries to migratory birds and threatened and endangered species at the Tar Creek Superfund Site Ottawa County, Oklahoma. Tulsa, OK, USA.


CHAPTER 2

EFFECTS OF ANTHROPOGENIC DISTURBANCE
ON POPULATION DEMOGRAPHICS OF RED-EARED SLIDER TURTLES

(Trachemys scripta) IN A FORMER MINING DISTRICT
Abstract

Understanding the effects of anthropogenic disturbances on wildlife species and habitats can aid in formation of appropriate land use practices and management of natural disturbances. Mining is a major industry worldwide and often results in severe physical and chemical alteration of landscapes. Tar Creek Superfund Site, located in NE Oklahoma, was mined extensively from the early 1900’s to 1970 and remains heavily damaged. We investigated population parameters, including body size, size distributions, sexual dimorphism, sex ratios, recruitment, and recapture and survival rates to assess a population of red-eared slider turtles (*Trachemys scripta*) from Beaver Creek within Tar Creek Superfund Site. Our findings indicate that male *T. scripta* at Tar Creek Superfund Site are significantly smaller than males at reference sites. The Tar Creek Superfund Site population also yielded significantly higher values in a sexual dimorphism index. Sex ratios were closer to 1:1 and more juvenile males were present in the Tar Creek Superfund Site population than reference populations. Survival and recapture rates did not vary significantly among the three sites. The combination of basic population parameters suggests that males at Tar Creek Superfund Site are not successfully being recruited into the adult population. Although active disturbance at the site ceased over 35 years ago, *T. scripta* at Tar Creek Superfund Site may be continuing to show deleterious effects of mining disturbance.

*Keywords:* Anthropogenic disturbance; Population demographics; *Trachemys scripta*; Tar Creek Superfund Site
1. Introduction

Habitat loss and degradation can elicit deleterious effects at the individual, population, and community level (Mitchell and Klemens, 2000). Anthropogenic disturbance events may be one source of habitat loss and degradation in many landscapes; however, due to the complexity and varying spatial and temporal scales of many of these events there is discrepancy in objectively defining and categorizing anthropogenic disturbance. White and Pickett (1985:7) define a disturbance as “any relatively discrete event in time that disrupts ecosystem, community or population structure and changes resource, substrate availability, or the physical environment”. Based on this definition, anthropogenic activities such as logging, mining, and settlement spread can be classified along with natural events as disturbances (Paine et al., 1998). Investigation of the effects of anthropogenic disturbances on wildlife species can help establish standards by which land use decisions are made in the future.

Mining is an important economic activity worldwide that includes extraction and supplemental preparation of solid ores and minerals, crude petroleum, and natural gas (UN, 2005). Mining operations vary greatly based on the substance of interest and can include wells, quarries, and surface and underground mines (UN, 2005). Surface and underground mines are the most commonly used in the United States, both require extensive removal of overburden and can result in physical alteration of the landscape (USEPA, 1995). Mining can be considered a compounded anthropogenic disturbance; not only does mining physically disturb landscapes, but contamination from overburden or mine drainage also affects wildlife and human populations.
The United States participates heavily in the mining industry with over 14,000 active mining operations, the majority of which are surface mines, reported in 2003 (NIOSH, 2003). Mining for coal and metals has a long history in Oklahoma (ODOM, 2003). 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 and zinc were extracted using a variety of mining strategies, including strip, room-and-pillar, cave, and surface mining. Due to the lack of reclamation requirements at the time mining ceased, much of the Tri-State Mining District remains heavily damaged (USEPA, 2005). Tar Creek Superfund Site (TCSFS) is a 40-mi$^2$ portion of the Tri-State Mining District located in Ottawa County, in extreme NE Oklahoma. Currently, 75 million tons of mine tailings and 800 acres of both wet and dry flotation ponds are present on the site. In addition to physical alterations to the landscape, TCSFS is contaminated with lead, zinc, cadmium, and other heavy metals. Concerns about effects of contaminants on health of human residents led to the placement of TCSFS on the Environmental Protection Agency’s National Priority List in 1983 (USEPA, 2005).

Red-eared slider turtles, *Trachemys scripta*, have a wide geographic distribution and due to the pet trade can be found virtually worldwide (Ernst et al., 1994). Despite the now wide distribution of *T. scripta*, some conservation concerns are being raised. *Trachemys scripta* is in a lower risk category on the IUCN Red List; however, it is categorized as close to qualifying for vulnerable status (IUCN, 2004). A long-lived, broadly distributed species like *T. scripta* lends itself to general natural history studies that can provide information valuable for wildlife conservation efforts. Short-lived
mammalian biomonitors can be used to investigate effects of disturbance on multiple generations. Use of a long-lived species, however, provides data more relevant to the human lifespan. Investigation of basic life history characteristics and demography at sites with anthropogenic disturbance, like TCSFS, serve as an additional means by which to assess the impact of disturbances on wildlife species. Connner et al., (2005) used community composition, sex ratios, and sexual dimorphism to assess aquatic turtle assemblages in altered urban landscapes. Demographic responses have also been used to investigate the response of painted turtles (*Chrysemys picta*) to various levels of habitat loss (Marchand and Litvaitis, 2004). Mark-recapture studies have also proven useful in assessing demography of terrestrial and aquatic turtle species (Dodd 1990; Lindeman, 1990; Bowen et al., 2004).

This study is a small portion of a larger project using multiple endpoints to investigate the impact of mining disturbance on *T. scripta* at Beaver Creek within TCSFS. Basic population demography and knowledge of natural history provide a solid background necessary for investigating specific endpoints. In this study, we sought to measure basic characteristics including sex ratio, percent of melanistic animals, size distribution, sexual dimorphism, and population demography as a means to assess the impact of mining disturbance on *T. scripta* from TCSFS and less critically disturbed reference sites, Lake Carl Blackwell (LCB) and Sequoyah National Wildlife Refuge (SNWR). We hypothesized that animals from TCSFS would be physically smaller and have a lower incidence of melanism than reference populations because they are not surviving to old ages. We also expected the TCSFS population to have altered sex ratio, percent juveniles, and lower survival and recapture compared to reference populations.
2. Methods

2.1. Study Sites

Animals for this study were collected from TCSFS and from unmined sites, SNWR and LCB. At TCSFS, animals were collected from a 40-acre portion of the site known as the Catholic 40, located in Ottawa County, Oklahoma. The Catholic 40 is owned and managed by the Quapaw Tribe of Oklahoma and the Bureau of Indian Affairs, respectively. The site is highly disturbed and is predominately mine tailings with little vegetative cover. A low water crossing built across Beaver Creek to provide access to a mine site has resulted in the formation of a pond which periodically floods over onto contaminated chat piles. Emigration and immigration of *T. scripta* at the Catholic 40 is possible, but due to the extensive disturbance in the area it is likely that the home ranges of *T. scripta* lie completely within the disturbed area. Public access is not permitted at the site and no restoration has taken place since mining ceased. LCB is a recreational lake located in Payne County, Oklahoma, that is owned and managed by Oklahoma State University. We collected animals from Pine Grove Slough; an area that is closed to boat traffic but is adjacent to a main road through the grounds. Pine Grove Slough is connected to the main lake via drainage systems and a large number of turtles were seen basking and active throughout the collecting period. SNWR spans approximately 21,000 acres in Muskogee, Haskell, and Sequoyah Counties, Oklahoma. We collected animals from Little Vian Creek located in Sequoyah County just SE of refuge headquarters. Little Vian Creek is flood-controlled and empties into Sally Jones Lake, which is part of the
Arkansas River System. Flood control efforts keep the water level fairly constant with the exception of periods of very heavy rainfall. Basking turtles were abundant in all water bodies at SNWR. References sites were selected to reduce the likelihood of downstream or airborne contamination or residual disturbance effects from TCSFS were possible.

2.2. Field Methods

Efforts were made to collect animals from all sites monthly from July through September 2003 and April through October 2004. Four to six hoop nets, anchored with wooden stakes and baited with canned sardines, were set in the late afternoon at each site. Traps were checked early the following morning and captured animals removed. All animals were handled following IACUC approved field methods (IACUC ACUP No. AS0315). Traps were checked periodically throughout the day for a maximum of 48 hours or until 20 animals were collected. Upon collection, animals were placed in a 62-L plastic tub with 6-9 cm of local stream water.

Sex was determined by observation of cloacal position and elongation of foreclaws (Ernst et al., 1994). Mass was measured to the nearest gram using a Pesola spring scale. Straight-line carapace and plastron length and width were recorded to the nearest mm using a ruler. Animals were recorded as melanistic or non-melanistic. Melanism is a progressive increase of carapace and plastron pigmentation that occurs naturally in many species of aquatic turtles. In *T. scripta*, melanism typically occurs in males and extreme cases can result in complete loss of the striped pattern on the head and neck (McCoy, 1968). Each animal was given a unique mark on marginal scutes with a
Dremel rotary saw. Marking allowed us to determine if animals were recaptured on subsequent trapping occasions. A carapace sample collected during marking was stored in a 3-ml polystyrene tube and deposited in the Oklahoma State University Collection of Vertebrates Frozen Tissue Collection. Marked animals were reweighed and remeasured at each recapture. All animals were released at the point of capture.

2.3. General Population Characteristics

When investigating multiple populations in the field, it is difficult to accurately and consistently age *T. scripta*. We used values published by Gibbons et al. (1980) to delineate mature and immature animals. We considered as adults, males $\geq 100$ mm and females $\geq 167$ mm straight-line plastron length (SPL). Adult sex ratios were generated for each site and compared using Chi-squared tests. For animals collected on multiple occasions, a capture was randomly chosen for inclusion in analyses. Percentage of melanistic animals was generated for each site and relative proportions were compared among sites using Chi-squared tests. The relative proportion of juveniles in each population was compared among sites using Chi-squared tests. Relative frequency distributions of plastron length were generated based on 10-mm size classes of SPL. Due to low cell counts, animals with plastron lengths $< 110$ mm were pooled and plastron lengths $\geq 200$ mm were pooled to generate frequency distributions. Frequency distributions of sizes were compared between sites using Chi-squared tests.

To compare the degree of sexual size dimorphism (SSD) between critically disturbed TCSFS and reference populations, we generated a sexual dimorphism index
(SDI) based on Lovich and Gibbons (1992). The SDI was calculated for adult *T. scripta* using the formula:

\[
\text{SDI} = \frac{\text{Female SPL}}{\text{Male SPL}} - 1
\]

Negative values indicate that males are larger than females and positive values indicate that females are larger than males. Variation in the degree of SSD was tested using a 3x2 factorial design in analysis of variance. Plastron length was log transformed to reduce variance and used to test for size differences between site of collection and sex. A significant interaction term (site x sex) indicates variation in degree of SSD by site (Ritke and Kennedy, 1993; Lovich et al., 1998; Stevens and Kennedy, 2005).

2.4. Demographic Analysis

Survival (Φ) and recapture (ρ) estimates were generated using the Cormack-Jolley-Seber (CJS) model for open populations in Program MARK (Version 4.1; White and Burnham, 1999). The CJS model requires that the data set meet four assumptions: (1) every animal present at the time of the *i*th sample has an equal probability of capture, (2) every marked animal that is present in the population immediately after *i* has the same probability of survival until the next sampling period, *i* + 1, (3) marks cannot be lost or overlooked, (4) all samples are instantaneous and all animals are released immediately after capture (Pollock et al., 1990). We think that assumptions 3 and 4 were met in our sampling design and mark-recapture methods. We used goodness-of-fit tests in RELEASE (version 3.0, embedded in Program MARK) to test assumptions 1 and 2 which are related to capture and survival, respectively. We generated a saturated global
model that included effects for group (site) and time (month) variation in \( \Phi \) and \( \rho \), and interactions. Using this global model we performed a goodness-of-fit test to determine if the data were overdispersed. Overdispersion indicates that not enough explanatory factors were included in the model to explain the variation. The mean expected deviance calculated from 1000 bootstrapping simulations was 162.03, whereas the global model deviance was 192.29; this yielded an overdispersion factor (\( \hat{c} \)) of 1.19. A \( \hat{c} \) value greater than 1 is indicative of overdispersion (Pryde, 2003). To account for overdispersion, model likelihoods were divided by \( \hat{c} \) and used to adjust future models accordingly. In order to determine the best model for our data, we tested 16 pre-defined models in Program MARK. We determined the most appropriate model from those tested by using an information theoretic criterion, Akaike’s Information Criterion (AIC). Information theoretic criteria seek to optimize the likelihood function while minimizing the number of parameters to yield the model most parsimonious for the data set. Use of an information theoretic criterion, like AIC, in model selection helps prevent the arbitrary assignment of significance levels that often have no biological significance (Akaike, 1973; Lebreton et al., 1992; Cooch and White, 2005). In Program MARK, AIC is adjusted for specified \( \hat{c} \) values and small sample sizes which are common in mark-recapture studies and reported as the QAIC\( _c \) value. The most parsimonious model is that which yields the lowest QAIC\( _c \) value. Two models are considered equally valid if QAIC\( _c \) values differ by less than 2 (Pryde, 2003; Cooch and White, 2005).
3. Results

3.1. General Characteristics

A total of 331 animals was collected during the 2003 and 2004 sampling periods. After random removal of replicate animals and animals with incomplete collection data, 305 animals (TCSFS, \( n = 123 \); SNWR, \( n = 126 \); LCB, \( n = 56 \)) were used for the following analyses. Adult sex ratios (F:M) were 1:1.26 for animals from critically disturbed TCSFS \( (n = 95) \) and 1:3.48 and 1:2.08 from SNWR \( (n = 112) \) and LCB \( (n = 37) \), respectively. Sex ratios differed significantly among the three sites \( (\chi^2 = 11.25, \ P = 0.0036) \). The majority of melanistic animals at each site were male with only one melanistic female present at each of the three sites. Size of melanistic males did not vary greatly among the three sites, but reference sites yielded significantly higher \( (\chi^2 = 8.28, \ P = 0.02) \) percentages of adult males showing melanism than TCSFS (Table 1).

Proportion of juveniles at TCSFS was 0.23 \( (n = 28) \). Reference sites SNWR \( (n = 14) \) and LCB \( (n = 19) \) yielded proportions of 0.11 and 0.34, respectively. The relative proportion of juveniles differed significantly among the three sites \( (\chi^2 = 13.60, \ P = 0.0011) \). When data were partitioned by sex, all three sites showed a higher proportion of juvenile females than males (Fig. 1). Three juveniles for which sex could not be determined \( (SNWR, n = 1; LCB, n = 2) \) were excluded from this analysis. Thirty-six percent of females at TCSFS were juveniles \( (n = 24) \), while only 7% of males were juveniles \( (n = 4) \). Similarly at SNWR, 32% of females were juveniles and only 1% of males were juveniles. Fifty-seven percent of females trapped at LCB were juveniles and
3.8% of males were juveniles. When partitioned by sex neither the proportion of female \( (\chi^2 = 4.68, P = 0.10) \) or male \( (\chi^2 = 3.54, P = 0.17) \) juveniles differed significantly among sites. The distribution of size classes (based on plastron length) differed significantly among sites \( (\chi^2_{20} = 57.71, P < 0.0001) \) (Fig. 2).

All sites yielded positive SDI Values, indicating that females are larger than males. TCSFS was the highest at 0.33, while reference sites SNWR and LCB had SDI values of 0.17 and 0.19, respectively. Analysis of variance performed on log transformed plastron lengths revealed a significant interaction between sex and site \( (P = 0.0047) \), suggesting that the degree of sexual size dimorphism was significantly different among the three sites. Further analysis of least squared means showed that adult males from TCSFS were significantly smaller than adult males from both SNWR and LCB \( (P = 0.04, 0.0002) \). Adult females from TCSFS were significantly larger than adult females from SNWR \( (P = 0.03) \). Mean values and standard errors of carapace and plastron length and mass for adults are reported in Table 2.

3.2. Demographic Analysis

A total of 331 animals were collected 454 times in 2003 and 2004. Goodness-of-fit tests to assess the assumptions of capture homogeneity (assumption 1) were not significant \( (\chi^2_{16} = 12.70, P = 0.69) \), indicating that animals from all sites had an equal capture probability. Similarly, goodness-of-fit tests to assess survival homogeneity (assumption 2) were not significant \( (\chi^2_{18} = 7.63, P = 0.98) \), indicating that animals from all sites had an equal probability of being encountered again regardless of being marked.
before or at sample period $i$. Overall, the data fit the global model as indicated by the lack of significance in combined tests of assumptions 1 and 2 ($\chi^2_{34} = 20.33, P = 0.97$).

We selected a model in which survival probability varied by site and recapture probability varied by both site and month of collection. QAIC$_c$ values ranked this model (QAIC$_c = 612.45$) second behind a model in which survival was held constant and recapture probability varied by site and month (QAIC$_c = 608.07$). The QAIC$_c$ values of the two models differed by more than two; however, likelihood ratio tests were conducted to determine if a truly significant difference was present. LRT tests showed no significant difference between the models ($\chi^2_2 = 0.02, P = 0.88$) so we chose to use the more expanded of the two models. Survival probability was lowest at SNWR (0.74 ± 0.23) and equal at TCSFS and LCB (0.86 ± 0.06 and 0.86 ± 0.04, respectively). We also looked at the percentage of overwinter survivors at each site. Overwinter survivors were those animals collected in 2003 that were also collected in 2004, indicating that these animals survived the winter hibernation period. LCB yielded the highest percentage of overwinter survival (44%). Although LCB and TCSFS showed similar survival probabilities, TCSFS showed a lower percentage (28%). SNWR showed the lowest overwinter survival with 4.8%.

Recapture probability varied within each of the three sites. LCB showed overwhelmingly the highest recaptures rates with rates approaching 50% between April and May 2004 and May and June 2004. TCSFS showed recapture rates consistently lower than LCB at all time intervals except September 2003 to April 2004 and August to September 2004. Few recaptures ($n = 6$) in the SNWR population led to missing estimates of recapture probability (Fig. 3). A single individual from LCB was collected 8
times over the 10 month study; six collections were in six consecutive months. Recaptures from TCSFS were generally few recaptures of many individuals. TCSFS had 37 animals captured multiple times with an average of 2.32 captures per individual. LCB, however, yielded 3.27 captures per individual with a total of 28 animals captured multiple times.

4. Discussion

Although conservationists have no control over the effect natural disturbances have on native habitats and species, we can use the lessons learned from those disturbances to prevent deleterious effects of anthropogenic disturbance. Habitat loss, the complete elimination of viable habitat, has quickly become one of the primary causes of extinctions. Habitat degradation, which does not eliminate habitats completely, but greatly alters the physical and often chemical properties of systems, must also be considered for its potential effects on wildlife (Mitchell and Klemens, 2000). TCSFS provides a unique area in which to study the effects of anthropogenic disturbance on wildlife.

A major concern in any population is the recruitment of juveniles into the reproductively active adult population. Ultimately, lack of adult recruitment can lead to genetic bottlenecks and severe declines in the effective size of the populations. *Trachemys scripta* illustrate a classic Type III survivorship curve in which juvenile survival is very low, often with high mortality before emergence from the nest due to predation or disease (Gibbons, 1990a). Anthropogenic disturbances, as seen at TCSFS,
may significantly alter growth and recruitment of individuals, thereby altering the
dynamic of entire populations.

By measuring population characteristics such as sex ratio, proportion of juveniles,
and size distributions we can draw some conclusions about the success of adult
recruitment in natural populations (Marchand and Litvaitis, 2004; Conner et al., 2005).
Natural populations of *T. scripta* are expected to show male biased sex ratios because of
differential maturity between males and females. Males mature at a given size, usually
around 100 mm plastral length, whereas females reach maturity based on age, generally
around 8 years old. In fast-growing populations, males reach maturity at younger ages
and females at larger sizes (Ernst et al., 1994). In populations in which adult recruitment
is occurring, more adult males than adult females will be present. Based on this
information, we hypothesized that the TCSFS population would show an altered adult sex
ratio. As expected, male-biased sex ratios were present at SNWR and LCB, while TCSFS
had a sex ratio closer to 1:1. Heavily male-biased sex ratios at both reference sites
suggest that more male recruitment is occurring in those populations compared to
TCSFS. However, Gibbons (1990b) found wide fluctuations in sex ratio between years
in South Carolina populations of *T. scripta*, indicating that resources available for growth
in a given year can drastically effect recruitment and sex ratio. Variation in sex ratios
between TCSFS and reference sites may be due to slight differences in resource
availability or hatchling success among the three sites. Based on Gibbons (1990b)
findings, we know that long term conservation decisions about a population cannot be
made based on short-term sampling and that repeated sampling efforts are necessary to
constantly evaluate populations.
At larger sizes, male *T. scripta* are also documented as developing melanism. McCoy (1968) documented melanism occurring in males at approximately 145 mm plastral length at LCB, but size at which onset of melanism occurs varies greatly. Loss of bright coloration may play a role in female choice and also may contribute to selection for maturity at small sizes in males (Lovich et al., 1990). As hypothesized, the TCSFS population shows a lower incidence of melanism, which may also be indicative of low adult male recruitment. Mean plastron length for melanistic males showed little difference among sites, but the high difference in occurrence suggests that few old males were present at TCSFS.

The presence of a higher percentage of female juveniles than male juveniles at all sites was expected based on differential maturity of the sexes. Although it had the adult sex ratio closest to 1:1, TCSFS had a higher, but not significant, percentage of juvenile males which may suggest a younger population of males at TCSFS. We hypothesized that TCSFS would show an altered size distribution. SNWR and LCB yielded unimodal size distributions with peaks at 160 and 170 mm plastron length, respectively (Fig. 2b and 2c). TCSFS animals, however, produced a relatively even size distribution with slightly larger proportions of animals present in the smallest and largest size classes (Fig. 2a). Based on the size differential between the sexes, the data suggest that females surviving to recruitment are reaching large sizes at a faster rate or living longer, thereby reaching bigger sizes. Gibbons and Greene (1990) have found that plastron length in females positively correlates with clutch size in *T. scripta*. The ability of female *T. scripta* to attain large sizes at TCSFS increases the possibility of large clutches and may explain the slightly higher percentage of juveniles present in the population.
In a species that shows Type III survivorship, recruitment of juveniles into the adult population is a key factor in the ultimate sex ratio of the adult population, however, Wilson (1975) proposed sex ratio of hatchlings also can greatly affect adult sex ratio. *Trachemys scripta*, like many other reptilian species, demonstrates temperature dependant sex determination. Males are generally produced at low nest temperatures (approximately 26°C), whereas females are produced at nest temperatures around 31°C. Vogt and Bull (1984) found that *Graptemys outachitensis* (Ouachita map turtle) and *Graptemys pseudogeographica* (False map turtle) produced all-male nests in areas with heavy vegetative cover and all-female nests were found in open areas receiving full sun. Similarly, nests of *T. scripta* from South Carolina receiving sun were 5-10°C warmer at the surface and 180 mm below the surface than shaded nests (Congdon and Gibbons, 1990). Although our study did not collect hatchling turtles, the area surrounding Beaver Creek at TCSFS has been disturbed due to former mining activities and has much less vegetative cover than both SNWR and LCB. The difference in cover and substrate may contribute to slight differences in nest temperature between the sites and thereby contribute to more equal sex ratio at TCSFS.

Sexual dimorphism, with females being larger, is expected in *T. scripta* and many other species of turtles. Mode of selection for sexual dimorphism in *T. scripta* has been the subject of great debate, but it is thought that it is the result of sexual selection and natural selection acting in opposition to the size and age at which maturity is attained (Gibbons and Lovich, 1990; Janzen et al., 2000). Natural selection favors males and females attaining larger sizes before maturity because they would be less susceptible to predation during mating and nesting activities (Janzen et al., 2000). Sexual selection
confers advantage to males that mature at smaller sizes, thereby younger age, because of
the increased number of potential mating opportunities over a lifetime. Females that
mature at larger body sizes, however, have an advantage because clutch size and body
size are strongly correlated (Gibbons and Greene, 1990; Gibbons and Lovich, 1990).

The degree of sexual dimorphism is greater in the TCSFS population. We
hypothesized that male and female turtles from TCSFS would be physically smaller than
turtles from reference sites. Males from TCSFS support our hypothesis and were
significantly smaller than both reference populations; however, females were larger than
both reference populations, yet only significantly larger than SNWR. Conner et al.
(2005) published SDI values of 0.16 and 0.20 for *T. scripta* that are consistent with
values from LCB and SNWR. Gibbons and Lovich (1990) found highly variable SDI
values in populations in South Carolina. The SDI generated from TCSFS (0.33) is still
higher that the average (0.25) found by Gibbons and Lovich (1990). The presence of
significantly smaller adult males at TCSFS suggests that males surviving to sexual
maturity are not reaching sizes comparable to males at both reference sites.

Mark-recapture studies to assess survival and capture rates have been used in
several species of turtles (Cagle, 1950; Langtimm et al., 1996; Kazmaier et al., 2001;
Fonnesbeck and Dodd 2003). Turtles can be easily marked on marginal scutes and can be
followed for several years with little concern of losing marks (Cagle, 1939). Mark-
recapture was an ideal way to assess *T. scripta* from TCSFS without removing animals
from what may be a compromised population. We hypothesized that survival and
recruitment would be lower in the TCSFS population. Unexpectedly, all three sites
showed high survival rates, exceeding 70% at all sites. The slightly lower survival and
Recapture probabilities at SNWR may not be an accurate representation due to the high probability of movement within the refuge. Over 60% of SNWR is water and provides ideal habitat for *T. scripta*. The large number of turtles observed combined with the small number of recaptures suggests a large population with extensive movement. Ideally, long term studies are used to report annual survivorship because several studies have found that within season survivorship is high for some species (Langtimm et al., 1996; Fonnesbeck and Dodd, 2003). Overwinter survival is a basic characteristic that provides a good annual assessment of survival and would be useful in long-term studies. In our study, however, the unequal recapture probability may have contributed greatly to the disparity in overwinter survival estimates among the sites.

Recapture rates in our study were higher than for many published results of other aquatic and terrestrial species. Fonnesbeck and Dodd (2003) found recapture rates ranging from 0.17 to 0.09 for *Sternotherus odoratus* in Alabama. *Terrapene ornata* in fragmented habitats in Illinois had recapture rates ranging from 0.08 to 0.16 (Bowen, et al., 2004). Recapture rates were higher at LCB than TCSFS in seven of nine trapping intervals. More turtles were seen basking and active at LCB than TCSFS, yet many of the turtles recaptured were captured over multiple months. We must also consider that Beaver Creek at TCSFS is not accessible to the public, while LCB and SNWR are commonly used for fishing. Different levels of activity among the sites may contribute to both survival and recapture probabilities.

Ideally, multiple forms of capture would be used in population studies to minimize trap bias. Long term studies of *T. scripta* and other species of aquatic turtles at Savannah River Ecology Lab, Aiken, South Carolina, USA, found that aquatic traps are
more selective for males, whereas terrestrial traps are more selective for females due to overland movements for nesting. Also, males are generally smaller and may be more able to get into aquatic traps than females (Gibbons, 1990b). We reduced temporal effects of trapping by sampling over the course of the active season. Based on the models we investigated in Program MARK, we also found little indication of sex being a significant factor in recapture probability. However, we must acknowledge that our data may show some male bias due to the use of aquatic traps only.

Short-term studies as described herein do not illustrate long-term population trends, however they are useful in establishing conservation efforts in anthropogenically disturbed areas. Combined endpoints suggest that recruitment of male *T. scripta* into the adult population at TCSFS may be lower (but not significantly so) than less disturbed reference sites, but short-term survival does not appear to be significantly affected. Females are reaching large sizes at TCSFS, indicating that resource availability is not a major problem at the site. Based on the definition of White and Pickett (1985), TCSFS can be classified as a disturbed habitat and our study indicates that *T. scripta* have been adversely affected at TCSFS. Active disturbance at TCSFS ceased at least 35 years prior to our study. Based on the life span of *T. scripta*, the turtles we examined most likely entered the breeding population after mining ceased. We might speculate that the levels of adult recruitment that we observed may be higher than when disturbance was occurring.

Long-term study of basic characteristics and demography is necessary at TCSFS and other anthropogenically disturbed sites to determine the initial response and ability of populations to recover after active disturbance has ceased. Studies of this nature also
provide the background information necessary for investigation of specific endpoints. The results of this study, when combined with continuing work at TCSFS on behavioral and genetic effects, will aid in providing a more complete picture of effects of mining disturbance.

5. Acknowledgements

We would like to thank Quapaw Tribe of Oklahoma, Oklahoma State University, and U.S. Fish and Wildlife Service for site access; numerous graduate and undergraduate students for field assistance, particularly Jennifer Meyers, Courtney Lynd, Maria Harrington, and Kendra Phelps; Drs. Mark Payton and Eric Hellgren for statistical assistance; Drs. Joe Bidwell and Stanley Fox for advice and editorial assistance; and Society of Environmental Toxicology and Chemistry, EA Engineering, Oklahoma State University Graduate College, and Sigma Xi for funding.

References


Bowen, K.D., Colbert, P.L., Janzen F. J. 2004. Survival and recruitment in a human-impacted population of Ornate Box Turtles, Terrapene ornata, with
recommendations for conservation and management. Journal of Herpetology 38, 562--568.


Dodd, C.K. 1990. Effects of habitat fragmentation on a stream-dwelling species, the flattened musk turtle *Sternotherus depressus*. Biological Conservation 54, 33--45.


Table 1. Mean plastron length and mass (± 1 S.D.) and percentage of adult male *Trachemys scripta* exhibiting melanism from Tar Creek Superfund Site (TCSFS) and reference sites, Lake Carl Blackwell (LCB) and Sequoyah National Wildlife Refuge (SNWR).

<table>
<thead>
<tr>
<th></th>
<th>TCSFS</th>
<th>SNWR</th>
<th>LCB</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>54</td>
<td>15</td>
</tr>
<tr>
<td>Percentage of Melanistic adult males</td>
<td>38%</td>
<td>62%</td>
<td>60%</td>
</tr>
<tr>
<td>Mass (g)</td>
<td>961 ± 209</td>
<td>876 ± 149</td>
<td>1032 ± 235</td>
</tr>
<tr>
<td>Plastron Length (mm)</td>
<td>173 ± 16</td>
<td>164 ± 11</td>
<td>178 ± 13</td>
</tr>
</tbody>
</table>
Table 2. Mean (± 1 S.D.) mass, plastron length, and carapace length of adult male and female *Trachemys scripta* collected from Tar Creek Superfund Site (TCSFS) and reference sites, Lake Carl Blackwell (LCB) and Sequoyah National Wildlife Refuge (SNWR).

<table>
<thead>
<tr>
<th></th>
<th>TCSFS</th>
<th></th>
<th>SNWR</th>
<th></th>
<th>LCB</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>53</td>
<td>42</td>
<td>87</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Mass (g)</td>
<td>658 ± 344</td>
<td>1382 ± 251</td>
<td>755 ± 275</td>
<td>1120 ± 163</td>
<td>894 ± 384</td>
</tr>
<tr>
<td></td>
<td>Plastron Length (mm)</td>
<td>148 ± 30</td>
<td>198 ± 12</td>
<td>154 ± 21</td>
<td>183 ± 6</td>
<td>167 ± 30</td>
</tr>
<tr>
<td></td>
<td>Carapace Length (mm)</td>
<td>164 ± 33</td>
<td>213 ± 14</td>
<td>173 ± 24</td>
<td>201 ± 12</td>
<td>187 ± 35</td>
</tr>
</tbody>
</table>
Fig. 1 Distribution of adult and juvenile, female (F) and male (M) *Trachemys scripta* collected from mined Tar Creek Superfund Site (TC) and reference sites, Lake Carl Blackwell (LC) and Sequoyah National Wildlife Refuge (SN). Males $\geq 100$ mm and females $\geq 167$ mm plastral length (Gibbons et al., 1980) were considered adults for this study.
Fig. 2 Proportional distribution of size classes based on plastron length (mm) of 
*Trachemys scripta* collected from A. Tar Creek Superfund Site (TCSFS), B. Sequoyah 
National Wildlife Refuge (SNWR), and C. Lake Carl Blackwell (LCB)
Fig. 3 Recapture estimates (ρ) as generated by Program MARK for monthly trapping intervals in 2003 and 2004 at A. Lake Carl Blackwell (LCB), B. Sequoyah National Wildlife Refuge (SNWR) and C. Tar Creek Superfund Site (TCSFS).
CHAPTER 3

FLOW CYTOMETRIC ANALYSIS OF EFFECTS OF HEAVY METALS ON RED-EARED SLIDER TURTLES (TRACHEMYS SCRIPTA) FROM TAR CREEK SUPERFUND SITE
ABSTRACT

Tar Creek Superfund Site was an area of lead and zinc mining from the 1890’s to 1970 and is currently contaminated with lead, zinc, and cadmium. This study used flow cytometry to measure variation in nuclear DNA content of red blood cells collected from *Trachemys scripta* at Tar Creek Superfund Site and reference sites, Lake Carl Blackwell and Sequoyah National Wildlife Refuge. We also used atomic absorption spectrometry to measure Pb and Cd in blood and Pb in bone samples. Coefficients of variation around the G1 peak ranged from 5.33 – 5.48 and showed no significant difference between contaminated and reference populations. On a month-by-month basis CVs from Tar Creek Superfund Site showed a u-shaped distribution with highest CVs present early and late in the season. Aneuploidy was present in 32 animals from Tar Creek Superfund Site, while reference populations contained 11 and 10 aneuploid individuals, respectively. Blood Pb levels were not significantly different between Tar Creek Superfund Site and Sequoyah National Wildlife Refuge populations and ranged from undetectable – 0.09 mg/L. Pb levels in carapace samples did not differ significantly between sites; however, Pb levels were higher in carapace than blood for both populations. Blood Cd was significantly higher at Tar Creek Superfund Site and showed a step-wise increase in concentration throughout the collection year. High numbers of aneuploid animals in the Tar Creek Superfund Site population indicate that heavy metals may be having a deleterious effect on *T. scripta*.

*Keywords:* flow cytometry, Tar Creek Superfund Site, aneuploid, *Trachemys scripta*
INTRODUCTION

Metals occur naturally in the environment and many are essential for physiological functions in humans and wildlife. However, anthropogenic activities, such as mining, smelting, and burning of fossil fuels, play a large role in worldwide redistribution of metals into air, soil, water, and food (Goyer and Clarkson, 2001). The Tri-State Mining District comprised portions of Oklahoma, Kansas, and Missouri and was mined heavily for zinc and lead from the 1890’s to 1970. After mining in the Tri-State District ceased, mines located below the water table began to flood. Acid mine drainage into Tar Creek and smaller creeks in Oklahoma was detected in 1979 (Parkhurst et al., 1988; USEPA, 2000 USEPA, 2005).

In 1983, the Oklahoma portion of the Tri-State Mining District was placed on the Environmental Protection Agency’s National Priority List as Tar Creek Superfund Site (TCSFS). Seventy-five million tons of chat, a gravel-like substance left over from the ore extraction process, and over 800 acres of flotation ponds, which were used to separate tailings from desired minerals, remain on the 40 mi² site. The main contaminants at TCSFS are lead (Pb) and zinc (Zn), which were originally mined at the site, and cadmium (Cd), a typical byproduct of zinc mining (USEPA, 2005). Sediment collected from Douthat Settling Pond and the Catholic 40 within TCSFS showed significantly higher levels of all three metals when compared with reference sediments. Cadmium ranged from 20 to 56 mg/kg, Zn from 3000 to 9300 mg/kg, and Pb from 440 to 540 mg/kg (Moeller, 2004). An earlier study found higher variation in Pb levels, ranging from 37 to >1000 mg/kg (Parkhurst et al., 1988).
Genetic damage is a useful biomarker with which to assess effects of environmental contaminants in wildlife populations. Many contaminants can be classified as genotoxicants and alter structure and function of DNA (Sharma, 1985; Shugart, 1990; Preston and Hoffmann, 2001). Chromosomal lesions, structural alterations of chromosomes such as strand breaks, exchange figures, and deletions, can be caused by damage irreparable by normal enzymatic repair systems, inhibition of enzymatic repair systems, or alteration in DNA replication. Chromosomal lesions can lead to decreased fertility, birth defects, and carcinogenesis (Shugart, 1990; Shugart, 1999; Preston and Hoffmann, 2001). Cd, Pb, and Zn can act as genotoxicants, but the exact mechanisms by which they elicit chromosomal lesions is unknown (Tachi et al., 1985; Hartwig, 1994). Cd can compete with calcium and Zn for binding sites on DNA repair enzymes and prevent repair of normally occurring DNA damage (Hartwig, 1994; Satoh et al., 2002). Some studies show that Cd and Pb can cause chromosomal lesions by altering the redox state within cells and releasing free radicals, which can damage the structure of DNA (Hartwig et al., 2002; Lin et al., 2005). Cd may also interfere with the mitotic apparatus and cause aneuploidy in exposed cells (Güerci et al., 2000; Seoane and Dulout, 2001; Seoane et al., 2002) Zn, although an essential cofactor for many enzymes, also has been shown to induce chromosome gaps and breaks in vivo (Hikiba et al., 2005).

Flow cytometry is a rapid, cost effective method used to detect chromosomal lesions (Watson, 1987; Dallas, 1990). Proportions of a cell population in each of the three stages of interphase, G₀/G₁ with diploid DNA following mitosis, S during which DNA is being synthesized, and G₂ with twice the amount of DNA immediately prior to division, can be measured using flow cytometry. Variation in nuclear DNA content among cells
within an individual is measured as the coefficient of variation (CV) around the mean for all cells in the G0/G1 stage of the cell cycle. Larger CVs result from unequal amounts of DNA being distributed to daughter cells from dividing cells with chromosomal lesions. Animals with higher frequencies of chromosomal lesions have larger CVs compared to animals with lower frequencies of chromosomal lesions (Otto et al., 1981; Shapiro, 1983). Flow histograms also can reveal aneuploid subpopulations of cells as subpeaks around the G1 peak. Aneuploid cells contain an abnormal number of chromosomes due to nondisjunction events occurring during mitotic division (Irons and Stillman, 1985; Cimini and Degrassi, 2005). Studies that use flow cytometry as an indicator of effects of genotoxicants on wildlife are limited (Lamb et al., 1991; Custer et al., 1994; Lamb et al., 1995; Husby and McBee, 1999; Wittier and McBee, 1999; Wickliffe et al., 2000; Matson et al., 2005); however, these studies demonstrated that flow cytometry can serve as a tool to evaluate risk to wildlife populations.

Few studies have been done using turtles as biomonitors (Meyers-Schöne et al., 1993; Meyers-Schöne and Walton, 1994; Lamb et al., 1995), but these animals are ideal for monitoring the riparian zone because they regularly move between land and water. Turtles also exhibit higher site fidelity than many birds and mammals, thereby reducing uncertainty about sites and routes of exposure. Short-lived mammalian biomonitors can be used to investigate effects of disturbance on multiple generations, but more long-lived species like turtles can provide data on multiple years of exposure to environmental contaminants (Gibbons, 1990; Hopkins, 2000). Turtles, like other lower vertebrates, have nucleated red blood cells, which lends favorably to their use in flow cytometric and other genetic toxicology studies (Fossi et al., 1994; Clark et al., 2000).
The objective of our study was to use flow cytometry to identify possible genetic effects of exposure to heavy metal contamination on *Trachemys scripta* at Beaver Creek within TCSFS. Specifically, we monitored *T. scripta* inhabiting Beaver Creek within TCSFS in relation to reference animals from two unmined sites in Oklahoma. We investigated relationships among sex, site, and month of capture, blood metal levels, and levels of genetic damage measured as CVs of G₁ peaks. We hypothesized that animals from TCSFS would show significantly larger CVs for G₁ peaks than animals collected from both reference populations because of high levels of Pb, Cd, and Zn present at TCSFS. We also hypothesized that females from all sites would show lower CVs for G₁ peaks than males from matched sites because egg laying provides females an additional means to rid their bodies of contaminantants (Meyers-Schöne and Walton, 1994). Largest CVs also were expected early (April/May) in the trapping period because metabolic rate is greatly depressed over the winter months, preventing normal DNA repair and red blood cell synthesis.

**METHODS**

*Study Sites*

Our TCSFS population of *T. scripta* was located within Beaver Creek at the Catholic 40 in Ottawa County, Oklahoma, on tribal land owned and managed by the Quapaw Tribe of Oklahoma and Bureau of Indian Affairs. A low-water crossing built across the creek resulted in the formation of a pond that floods over onto chat piles during periods of heavy rain. Animals also were collected from two unmined, reference sites,
Lake Carl Blackwell (LCB) and Sequoyah National Wildlife Refuge (SNWR). LCB is a recreational lake maintained by Oklahoma State University and located west of Stillwater in Payne County, Oklahoma. Collection at LCB was done in Pine Grove Slough, which is closed to boat traffic, but connected to the main lake via drainage systems and adjacent to a road. SNWR is approximately 21,000 acres located in Muskogee, Haskell, and Sequoyah counties, Oklahoma, and is used for hunting and cooperative agriculture on a seasonal basis. Animals collected at SNWR were trapped in a small portion of Little Vian Creek located directly SE of refuge headquarters in Sequoyah County. Reference sites were selected to reduce the possibility of downstream or airborne contamination from TCSFS.

Field Methods

All animals were handled following IACUC approved field methods (IACUC ACUP No. AS0315). Efforts were made to collect T. scripta from TCSFS, LCB, and SNWR monthly from July to September 2003 and April to October 2004. Four to six hoop nets baited with sardines were used at each trapping effort. Traps, anchored with wooden stakes, were set in the late afternoon or evening and checked early the next morning. Captured animals were removed and traps were rebaited, replaced, and checked periodically throughout the day for new animals. Traps remained in place for ca. 48 h during each trapping effort or until 20 animals were collected. Animals were placed in 62 L plastic tubs after removal from traps. For all animals collected, sex was determined by presence of longer foreclaws in males and position of cloaca relative to the rim of the
carapace (Ernst et al., 1994). Straight-line carapace and plastron length and width were taken to the nearest mm using a ruler and mass measurements were taken to the nearest gram using a Pesola spring scale. Blood samples were collected from a maximum of 20 animals per site per month. Blood samples were taken only from animals weighing more than 200 g. Blood (ca. 2 ml) was drawn from the subcarapacial sinus with a 23-gauge needle and 1-cc syringe. Samples were transported back to the lab in heparinized vacutainers stored on ice. Blood samples were transferred into 3 ml polystyrene tubes and stored at -86°C. Blood draw sites were swabbed with Betadine to prevent infection and animals were maintained in plastic tubs with 6 to 9 cm of local stream water for 3 h post-sampling to ensure recovery before release at the point of capture. Prior to release, all individuals were given unique notches on marginal scutes with a Dremmel rotary saw (D. Ligon, pers. comm.). Animals recaptured during subsequent trapping efforts were reweighed and remeasured to make note of any changes. Blood samples were taken from recaptured animals collected in a different month than their original capture. Blood samples were not taken from any animal twice in a given sampling month.

Flow cytometric analysis

Sample preparation for flow cytometric analysis followed the protocol of Bickham (J.W. Bickham, pers. comm.) and Matson et al., (2005). For each blood sample (n = 385), one drop was combined with 50 µl of citrate buffer and added to 450 µl of trypsin solution in a 1.5-ml tube. The tube was then inverted 3 times and allowed to rest at room temperature for 10 min. Trypsin inhibitor solution (375 µl) was added, followed
by a 10-min resting period at room temperature. The solution was filtered through 30-µl mesh nylon into a 5-ml Falcon tube to remove any large particles. The solution was stained with 375 µl of propidium iodide stain solution. Tubes were wrapped in foil to prevent light degradation of the stain and allowed to incubate on ice for 15 min.

Chicken erythrocyte nuclei (CEN; BioSure, Inc.) served as a standard and were prepared by combining 1 drop of commercial standard and 1 ml of propidium iodide stain solution in a 5-ml tube. The tube was wrapped in foil and allowed to incubate at room temperature for 10 min.

Flow cytometric analysis was performed on a FACScan flow cytometer (laser with excitation wave length = 488 nm). DNA data files were acquired with CellQuest® software for three replicates of 10,000 cells each for each animal. DNA profiles were analyzed using ModFit LT® analytical software and profiles generated for each replicate included a DNA histogram, total number of nuclei measured, mean number of cells for each stage of the cell cycle, and coefficient of variation for mean number of cells in G1.

After analysis of the 2003 samples was complete, we determined that poor laser alignment led to inconsistent results that did not fall within our quality control standards. Following laser correction and recalibration of the flow cytometer, the 2003 samples were prepared and reanalyzed using the methods above.

**Metal Analysis**

Furnace atomic absorption spectrometry was used to analyze one aliquot of whole blood (ca. 250 µl) for Pb and Cd in a subset of males and females collected from
both TCSFS and SNWR, respectively, in spring (April; \( n = 6, n = 5 \)), summer (late June/July; \( n = 5, n = 6 \)), and fall (September; \( n = 6, n = 6 \)) in the 2004 trapping period. Samples were run in duplicate and the mean concentration of metals was presented in µg/L. Analyses were performed by the Oklahoma Animal Disease Diagnostic Laboratory.

Carapace samples from the same subset of animals (TCSFS, \( n = 16 \); SNWR, \( n = 15 \)) were weighed and digested using a modified method of Giesy and Weiner (1977). Digestate was diluted to 10% with the addition of reagent grade water and stored in acid washed polystyrene bottles. Samples were analyzed for Pb in duplicate by Oklahoma Animal Disease Diagnostic Laboratory.

**Quality Control**

CEN standard solutions prepared in the flow cytometry lab in the OSU Center for Veterinary Health Sciences were run daily to prepare the instrument for use. A minimum of 5 replicate CEN standards prepared in our lab were run prior to sample analysis. Flow cytometric analysis proceeded only if CEN standards prepared by OSU Center for Veterinary Health Sciences and in our lab showed CV ≤ 2. Three replicate CEN standards were run after every 5 samples to maintain laser alignment and prevent instrumental drift. If standards showed CV > 2 at any point the run was aborted. A maximum of 30 samples was analyzed per run to prevent blood clotting and dye quenching. A random sample of 5 individuals from a previous run was included with each set of 25 new individuals as additional quality control. Samples for which three
replicates were not completed were excluded from statistical analysis. Also, excluded from statistical analyses were samples for which triplicate CVs varied by more than one.

Analysis of blood and bone by Oklahoma Animal Disease Diagnostic Laboratory followed EPA protocols for metal analysis. The spectrometer was calibrated by generating a standard curve with commercial metal standards and spiked samples were run after every sample.

Statistical Analyses

Mean CVs were calculated for each individual based on the 3 replicates of 10,000 cells measured for each blood sample. Only the first capture of an animal in which multiple samples of blood were collected was considered in this analysis. Plastron length, an indicator of age, was plotted against mean CV to determine if there was a relationship between age and CV. Mean CVs were analyzed using three-way analysis of variance (ANOVA, \( \alpha = 0.05 \)) to test for significant differences between site, month, and sex and the interactions among those variables. Because samples from 2003 and 2004 were handled differently (multiple thaws vs. one thaw), we analyzed years separately.

CVs from animals collected during more than one month within a sampling period were analyzed to test for changes in CV through time. A slope was generated for each animal by plotting CV versus month. Animals had from two to five points on this line. Slopes \((n = 28)\) were then analyzed using one-way ANOVA to test for differences among the three sites.
The number of aneuploid individuals present at each site in 2004 was counted. Individuals with an aneuploid peak or shoulder present in at least two of three replicate histograms were considered aneuploid for our study. Frequency of aneuploid individuals was compared among the sites using a Chi-squared test.

Pb and Cd levels were not normally distributed and did not show homogeneity of variance in blood or carapace samples. Kruskal-Wallis k-sample tests were used to test for significance differences in metal levels based on season and site of capture and sex of animals.

RESULTS

A total of 331 animals were collected 454 times throughout the course of the study, and 393 blood samples were collected. Blood samples for which three replicate CV values were not generated and samples that did not meet quality control criteria (n = 91) were excluded from statistical analyses.

Flow cytometry

Sample size was uneven among sites (TCSFS, n = 46; LCB, n = 31; SNWR, n = 18), but even among months of collection and sex. No strong correlation was seen between plastron length and mean CV in 2003 samples (TCSFS, R² = 0.09; LCB, R² = 0.00007; SNWR, R² = 0.0009). Analysis of variance of 2003 samples revealed no significant interactions between site, month of capture, and sex, but did show a significant site effect (P = 0.03). Reference sites did not differ significantly from one
another, but both yielded significantly larger mean CVs than TCSFS animals (Fig. 1).

Neither month of collection nor sex had a significant effect on CV.

A total of 244 blood samples were analyzed for the 2004 season. After the removal of duplicate samples from single individuals and samples that did not meet quality control standards, the 2004 season yielded 207 samples (TCSFS, \(n = 70\); LCB, \(n = 39\); SNWR, \(n = 98\)) for statistical analysis. No strong correlation was seen between plastron length and mean CV (TCSFS, \(R^2 = 0.0002\); LCB, \(R^2 = 0.11\); SNWR, \(R^2 = 0.07\)). Mean CVs were consistent across all three sites (TCSFS = 5.49; LCB = 5.35; SNWR = 5.33). There was no significant three-way interaction between site, month of capture, and sex; however, a significant month*site interaction (\(P < 0.0001\)) was detected.

Additionally, sex did not significantly affect CV. One-way analysis of variance to test for significant differences among months at each site was significant for TCSFS (\(P = 0.005\)) and SNWR (\(P < 0.0001\)). LCB did not differ significantly (\(P = 0.23\)) across months of collection (Fig. 2).

Twenty-eight animals were collected during more than one sampling month in 2004. Analysis of variance of slopes generated from these animals revealed no significant effect of site (\(P = 0.37\)) on slope of CVs. Approximately 73% of animals yielded negative slopes, indicating that CV was decreasing over the 2004 sampling period. Six animals yielded positive slopes: four females from TCSFS, one male from LCB, and one male from SNWR.

All complete histograms generated by flow cytometry were investigated for the presence of aneuploidy. Aneuploidy was observed in animals from all sites. There was a significant difference in the frequency of aneuploidy among the three sites (\(\chi^2 = 24.51\),
LCB and SNWR had 14.7% and 9.4% of animals showing aneuploidy, respectively. More aneuploid females \( n = 7 \) were collected than aneuploid males \( n = 4 \) at LCB; however, SNWR had more aneuploid males \( n = 6 \) than females \( n = 4 \).

TCSFS had 37.2% of animals showing aneuploidy and 63% of these were female. Eighty-eight percent of animals from TCSFS exhibiting aneuploidy were collected between April and June 2004. Similar temporal trends were seen at LCB, but aneuploid animals collected at SNWR were collected throughout the 2004 trapping season. All three sites had at least one animal that exhibited aneuploidy in multiple blood samples. Exemplary histograms of animals exhibiting aneuploidy are presented in Fig. 3.

**Metals Analyses**

Cadmium levels were measured in blood samples collected from TCSFS \( n = 17 \) and SNWR \( n = 17 \) during spring, summer, and fall 2004. Samples were selected to keep size consistent within season at each site and an even distribution of males and females. CV was unknown at the time samples were selected for metals analysis. All animals from SNWR with the exception of one male collected during mid-season had blood cadmium levels below detection limits of 0.01 mg/L. In TCSFS animals, blood cadmium levels ranged from below detection levels to 0.45 mg/L. Kruskal-Wallis tests revealed a significant site effect, animals from TCSFS pooled across season and sex had a significantly higher \( \chi^2_1 = 5.91, P = 0.015 \) mean blood Cd level than animals from SNWR (Fig. 4). Blood Cd levels did not vary significantly when partitioned by season \( \chi^2_2 = 1.56, P = 0.46 \) or sex \( \chi^2_1 = 3.10, P = 0.08 \). Blood samples from both sites
showed Pb levels ranging from below detection limits to 0.09 ppm. There was no significant site ($\chi^2_1 = 1.52, P = 0.22$), sex ($\chi^2_1 = 0.10, P = 0.75$) or season ($\chi^2_2 = 1.21, P = 0.55$) effect on blood Pb concentrations (Fig. 4). Carapace samples ($n = 31$) showed highly variable concentrations of Pb at both TCSFS and SNWR (Fig. 5); however there was no significant site ($\chi^2_1 = 0.55, P = 0.46$), sex ($\chi^2_1 = 3.23, P = 0.07$), or season ($\chi^2_2 = 0.0013, P = 0.97$).

Neither blood Cd nor blood Pb levels showed strong correlation with CV. Although not strongly correlated, blood Pb and Cd samples from TCSFS had negative slopes (-0.71 and -3.7, respectively) and samples from SNWR had positive slopes (0.93 and 3.1, respectively) (Fig. 6 A and B). Carapace Pb levels showed a stronger correlation with CV and mean CV decreased at both sites as carapace Pb increased. SNWR showed a stronger correlation and rate of decrease (slope = -0.05) than TCSFS (slope = -0.01) (Fig. 6 C). This is expected because Pb present in bone is not readily available to cause effects.

**DISCUSSION**

The genetic effects of heavy metals on wildlife are difficult to study. Source, dose, and duration of field exposure are difficult to pinpoint; laboratory studies, however, may not be environmentally realistic. A few studies at TCSFS have monitored body burdens and enzymatic activity of various wildlife species, but none to our knowledge include genetic analyses. Northern cardinals (*Cardinalis cardinalis*), American robins (*Turdus migratorius*), and water fowl collected from throughout TCSFS showed
increased tissue concentrations of Pb and depressed activity of delta-aminolevulinic acid dehydrogenase (ALAD), an enzyme necessary for heme synthesis (Beyer et al., 2004). Elevated levels of Zn and degenerative abnormalities were detected in pancreas and liver of free-ranging Canada geese (Branta canadensis) and mallards (Anas platyrhynchos) collected at TCSFS (Sileo et al., 2004). White tailed deer (Odocoileus virginianus) collected in the immediate mining area had increased Pb levels in mandibles (Conder and Lanno, 1999).

Several studies have successfully employed flow cytometry to investigate wildlife populations exposed to genotoxicants. Spleens of white-footed mice (Peromyscus leucopus) from a site contaminated with a mixture of oil, grease, polychlorinated biphenols, hexachlorobenzene, and heavy metals showed significantly higher CVs in DNA content than reference animals (McBee and Bickham, 1988). A significant increase in DNA CV was also seen in spleen cells of hatchling night herons (Nycticorax nycticorax) exposed to petroleum waste in both Texas and Louisiana (Custer et al., 1994). Trachemys scripta has been used in flow cytometric studies of the effects of low-level radiation exposure from cooling reservoirs at Savannah River Ecology Lab. These studies used both blood and spleen tissue and found that sliders inhabiting the contaminated reservoirs showed significantly higher CV in DNA content (Bickham et al., 1988; Lamb et al., 1991; Lamb et al., 1994).

The use of blood samples from our three populations of T. scripta allowed us to amass a sample size larger than many previously published studies and we were also able to track changes in CV of twenty-eight animals collected multiple times in 2004. The significantly smaller mean CVs of TCSFS animals in 2003 were unexpected. Based on
known effects of heavy metals and other genotoxicants (Shugart, 1999) we expected to see a higher mean CV at TCSFS. However, 2003 blood samples endured multiple thaws due to necessary additional preparation of samples for analysis. The CVs generated for the 2003 samples therefore may not be indicative of the populations and may be an artifact of the multiple rounds of thawing and freezing. We found that mean CVs from all sites were smaller in the 2003 samples than 2004 samples. In order to prevent deleterious effects of multiple freezes on blood samples we recommend that samples be stored as aliquots and be thawed only prior to analysis.

Flow cytometric results from 2004 samples also were unexpected based on previously published studies. Significance in the month*site term in ANOVA is somewhat misleading; ANOVA tested and compared all combinations of month and site and significance in comparisons from different sites in different months hold little or no biological significance. However, significance in one-way ANOVA of mean CVs from the TCSFS and LCB explain the slight u-shaped curves seen through the 2004 season. Mean CVs were large in April and decreased slightly as the collection season progressed, reaching a minimum value in August. CVs from September and October, however, increased slightly.

High CVs early in the season may be related to the winter months that *T. scripta* spend hibernating, often under sediment at the bottom of the pond (Ernst et al., 1994). Just prior to hibernation, there is an increase in the number of red blood cells and a marked decrease in metabolic rate. Following springtime emergence, the number of red blood cells decreases (Sypek and Borysenko, 1988). Altland and Brace (1962) reported the mean life span of turtle red blood cells, as measured in *Terrapene ornata*, as 600-800
days. They also suggested that red blood cell turnover is positively correlated with metabolic rate, explaining why poikilotherms, like turtles, have erythrocytes with long life spans. Less specialized than mammalian erythrocytes, reptilian red blood cells can presumably undergo nucleic acid and protein synthesis throughout the long life span of the cells (Brace and Altland, 1955; Sypek and Borysenko, 1988). The depressed metabolic rate during hibernation can reduce nucleic acid synthesis and prevent normal DNA repair. Under normal metabolic conditions, these processes can repair naturally occurring and induced DNA damage. However, decreased action of these processes due to low metabolic rate may be responsible for increased mean CVs upon emergence from hibernation in spring.

Coefficients of variation decreased at all sites throughout the study period and reached their lowest mean values at TCSFS and LCB in July and August, respectively. Cline and Waldman (1962) found that mean life span of red blood cells in alligators (Alligator mississippiensis) decreased at warmer ambient temperatures due to increased metabolism. Life spans of erythrocytes were prolonged by a factor of at least three at cooler ambient temperatures. Increased metabolism in T. scripta during mid-summer months possibly could shorten lifespan of red blood cells and increase the efficiency of DNA repair enzymes and nucleic acid synthesis. Later in the season (September and October) cooler temperatures would depress metabolic rate and possibly explain slightly higher mean CVs.

The presence of mosaic, aneuploid G0/G1 peaks are also indicative of genetic damage. The presence of shoulders or additional peaks in the G0/G1 stage indicates a subpopulation of cells with different DNA content (Cimini and Degrassi, 2005).
Typically the presence of aneuploidy alters the CV G1 of the population (Irons and Stillman, 1985). We found no distinct site difference in mean CV values across the 2004 collecting period; however, the TCSFS population showed a significantly higher percentage of aneuploid individuals than either reference population. Individuals with aneuploidy from TCSFS were generally collected early in the season (April and June). Few animals were collected in the May 2004 trapping period which accounts for the low number of aneuploid animals collected in that month. There was no distinct pattern of size or sex of aneuploid animals among the three sites. Seven aneuploid animals from LCB were captured later in the trapping season and did not exhibit aneuploidy possibly due to DNA repair or red blood cell turnover. Due to low recapture numbers at SNWR, no aneuploid animals had additional captures. Ten animals from TCSFS were trapped multiple times in 2004. Six of these animals did not show aneuploidy in subsequent samples. However, four animals showed normal flow cytometry histograms early in the season and aneuploid peaks in later months. The presence of aneuploid animals early in the season is consistent with the decreased metabolism and low erythrocyte turnover during and just following hibernation.

Bickham et al., (1988) reported increased CV and the presence of aneuploid *T. scripta* collected from radioactive seepage basins at Savannah River Ecology Lab. Interestingly, a later study on the same population revealed no difference in mean CV and no detection of aneuploidy (Lamb et al., 1995). Neither study indicated in which month samples were collected. Two mallard ducks (*Anas platyrhynchos*) from the same site also showed DNA aneuploidy. After removal from exposure and later flow cytometric
analysis, DNA aneuploidy was seen only in one animal (George et al., 1991). Our study also shows that the presence of aneuploidy can vary through time.

Some studies have found that Cd can induce aneuploidy in a variety of cell types. Cadmium chloride and cadmium sulfate significantly increased the frequency of hypodiploid cells, those containing fewer chromosomes than normal, in human diploid fibroblasts (Güerci et al., 2000; Seoane et al., 2002). A dose response increase in lagging fragments and chromosomes, indicative of erroneous migration of chromosomes during mitosis, also was seen in Chinese hamster ovary cells dosed with cadmium chloride (Seoane and Dulout, 1994). Selby et al. (1992) found hypodiploid and hyperdiploid aneuploid populations of cells in testes of rats dosed with cadmium chloride. Although our study presents limited data on the levels of Cd present in the blood of *T. scripta*, the association of aneuploidy and cadmium contamination in other studies suggests that future study of this association at TCSFS is prudent.

Many flow cytometric studies utilize lethal sampling and are unable to comment on trends in CV and DNA aneuploidy over time. As seen in work on *T. scripta* and *A. platyrhinchos* from Savannah River, repeated, non-lethal sampling provides valuable and often more ecologically relevant information than short-term studies (Bickham et al., 1988; George et al., 1991; Lamb et al., 1991; Lamb et al., 1995). Lamb et al. (1995) reported that CVs may undergo temporary fluctuations due to changes in population structure or metabolic responses to contaminants of the organisms of interest. Studies on Cesium contamination have found that uptake and excretion in largemouth bass (*Micropterus salmoides*) and yellow-bellied slider turtles (*Pseudemys scripta*) show asymmetrical patterns and are more dependent upon individual patterns of activity and
physical condition than size (Peters and Brisbin, 1988; Peles et al., 2000). Although toxicokinetics of heavy metals and radionuclides differ, the occurrence of monthly variation in CV suggests that physiological processes, such as metabolic rate, possibly alter effects of contaminants on exposed populations.

Levels of circulating Pb in *T. scripta* from TCSFS were higher than values previously reported at TCSFS. Beyer et al. (2004) reported blood Pb levels ranging from 1.3 – 4.0 mg/kg dw in songbirds from TCSFS. Similarly, Sileo et al., (2004) found circulating Pb levels ranging from 1.7 – 5.9 mg/kg dw in waterfowl. Low circulating Pb levels in our study may be due to partitioning of metals into bone and other tissues. Levels of Pb present in carapace samples were slightly higher for animals from TCSFS than SNWR and concentrations increased from spring to fall at both sites. Levels of Pb below detection limits in the spring sampling period at both TCSFS and SNWR were cause for concern. All samples from that period were digested on the same day and laboratory errors may be responsible for non-detectable levels. Few studies have reported bone metal levels for reptiles; however, bone Pb levels detected in fall and summer sampling periods in this study were much higher than levels detected in mandibles of white-tailed deer at TCSFS. Conder and Lanno (1999) reported Pb levels < 1.5 mg/kg dw in mandibles collected from TCSFS.

Circulating Cd levels were significantly higher in the TCSFS population in spring, summer, and fall trapping periods than at SNWR. At TCSFS, blood Cd levels were lowest in the spring trapping period and increased through summer and reached highest concentrations in the fall trapping period (Fig. 4). A previous study showed that waterfowl from TCSFS had blood Cd levels < 0.1 mg/kg dw (Sileo et al., 2004). For both
Cd and Pb, highest blood levels were expected in spring due to long term exposure to contaminated substrate during winter months; however, these levels indicate that animals are accumulating metals throughout the active season.

Based on previous studies on avian and mammalian species from TCSFS and blood and bone metal levels collected in this study, further study of the herpetofauna at TCSFS is necessary. Reptiles are often more sensitive to contaminants than birds and mammals and provide information relevant to the human lifespan (Hall, 1980). The variability seen in tissue metal concentrations, DNA CV, and aneuploidy in this and other studies further emphasize the need for long term studies that use non-lethal sampling efforts combined with periodic sacrifice of animals to determine contaminant loads in other tissues. The large metabolic depression that turtles undergo each winter must also undergo further study to determine the role life strategies have on the ability of *T. scripta* to sustain successful populations at TCSFS.

ACKNOWLEDGEMENTS

We thank Quapaw Tribe of Oklahoma, U.S. Fish and Wildlife Service, and Oklahoma State University for site access; numerous field assistants, including Emily AcklandAry, Kendra Phelps, Courtney Lynd, Maria Harrington, and Jennifer Meyers; Dr. John Bickham and Cole Matson from Texas A&M University for assistance in method development; Terry Colberg in the flow cytometry lab at Oklahoma State University Center for Veterinary Health Sciences for assistance in sample analysis; Dr. Mark Payton for statistical assistance; Drs. Stanley Fox and Joe Bidwell for editorial assistance; and


Moeller, L.A. (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, Stillwater, OK, USA.


USEPA, United States Environmental Protection Agency (2000). Five year review: Tar Creek Superfund Site Ottawa County, Oklahoma. Washington D.C., USA.

USEPA, United States Environmental Protection Agency (2005). TCSFS Fact Sheet, Tar Creek (Ottawa County), Washington D.C., USA.


Fig. 1 - Mean coefficient of variation (+ 1 S.D.) of animals collected August – September 2003 from Tar Creek Superfund Site (TCSFS) and reference sites, Lake Carl Blackwell (LCB) and Sequoyah National Wildlife Refuge (SNWR). Sites with the same letter are not significantly different ($\alpha = 0.05$).
Fig. 2 - Mean coefficient of variation (+ 1 S. D.) of animals collected April – October 2004 from A) Tar Creek Superfund Site (TCSFS) and reference sites, B) Sequoyah National Wildlife Refuge (SNWR), and C) Lake Carl Blackwell (LCB). P values were generated in ANOVA to test for significant differences among months at each site.
A. 

$P = 0.005$

B. 

$P < 0.0001$

C. 

$P = 0.23$
Fig. 3 - DNA flow histograms of normal (A and B) and aneuploid (C and D) individuals from the Tar Creek Superfund Site (TCSFS) population. Arrows point to the aneuploid peaks or shoulders adjacent to the main G₁ peak (highest peak). Specimens illustrated include: A) OK 7170 B) OK 7259 C) OK 7188 D) 7184
Fig. 4 - Blood levels of Pb and Cd (mg/L + 1 S.D.) for a subset of animals collected from TCSFS and SNWR in spring (April), summer (June), and fall (September) during the 2004 collection season. Collection periods within a site that share the same lower case different letter are not significantly different. Sites with the same capital letter are not significantly different ($\alpha = 0.05$).
Fig. 5 - Levels of Pb (mg/kg dw + 1 S.D.) detected in bone for a subset of animals collected from TCSFS and SNWR mid and late during the 2004 collection season. Sites within a season that share the same lower case different letter are not significantly different. Seasons with the same capital letter are not significantly different (\( \alpha = 0.05 \)).
Fig. 6 – Relationships between tissue metal levels and mean CV for a subset of animals collected from contaminated Tar Creek Superfund Site (TCSFS) and reference site, Sequoyah National Wildlife Refuge (SNWR) in spring (April), summer (June/July), and fall (September) 2004. A) Blood Cd levels in relation to mean CV; B) Blood Pb levels in relation to mean CV; C) Carapace Pb levels in relation to mean CV (carapace Pb levels were not available for Spring 2004). Legend serves all three graphs.
CHAPTER 4

TIME-TO-EVENT ANALYSIS TO ASSESS THE EFFECTS OF HEAVY METALS ON NEUROBEHAVIORAL RESPONSE IN TURTLES
Lead plays no biological role, yet it is a ubiquitous component of all inorganic and biological systems. The use of lead-based products, such as leaded gasoline and paint, has decreased since the 1970’s, yet industrial and mining activities still release lead into the environment (Goyer and Clarkson 2001). As of 1999, 1,026 of 1,467 sites listed on the Environmental Protection Agency’s National Priority List have lead levels above baseline (ATSDR 1999). Tar Creek Superfund Site (TCSFS), located in extreme NE Oklahoma, was an area of heavy lead and zinc mining from the 1890’s to 1970. The 40-mi² area was mined using the room-and-pillar method in which ore was extracted from rooms dug out below the water table. Acid mine drainage was detected in 1979 and still flows into Tar Creek and other creeks on the site. Seventy-five million tons of mine tailings and 800 acres of both wet and dry flotation ponds are still present on the site. Five municipalities (Picher, Cardin, Quapaw, Commerce, and North Miami) with a combined population of approximately 20,000 are encompassed by the site (EPA 2005).

Effects of lead on the central nervous system are a major concern in areas of heavy lead contamination, like TCSFS. Children are highly susceptible to lead poisoning because their nervous system is not completely developed and thereby more susceptible to harm via contaminants. Children also have high levels of hand-to-mouth contact, which is often responsible for intake of heavy metals (Goyer and Clarkson 2001). Blood lead levels less than the CDC recommended 10µg/dL can still cause decreased cognitive ability in children (Canfield et al. 2003). In Oklahoma, the statewide average blood lead concentration is exceeded by only 2% of children; however, a large portion of children
from TCSFS (38.3% in Picher, 62.5% in Cardin and 13.4% in Quapaw) have blood lead levels that exceed the recommended level (EPA 2000).

Due to potential deleterious effects of heavy metals, much emphasis has been placed on public health studies; however, extensive studies of human populations can be difficult. Use of sentinel wildlife species at contaminated sites can provide information valuable to habitat, wildlife species, and public health concerns (Lower and Kendall 1990). A variety of different endpoints can be employed to study contaminated populations of wildlife without permanently removing animals from potentially at risk populations. Behavioral toxicology is a discipline that helps combine ecological, physiological, and biochemical effects of environmental contaminants (Peakall 1992). Sublethal behavioral effects can include lethargy and tremors in birds and mammals and altered avoidance behaviors in fish (Little 1990). Using behavioral endpoints to effectively assess contaminated populations requires selection of behaviors that are ecologically significant to the organism.

Several studies have assessed effects of various contaminants on righting response in hatchling turtles. Righting is a necessary behavior because turtles that take longer to right themselves are more susceptible to predation and desiccation. Hatchling snapping turtles (C. serpentina) exposed to PCBs in the Sheboygan River, Michigan, showed decreased righting response that was inversely related to PCB exposure. Thirty-five percent of hatchlings from the most contaminated sites showed no response and made no effort to right (Portelli and Bishop 2000). Burger et al. (1998) showed that hatchling slider turtles
exposed to 1.0 mg/g Pb in the laboratory took significantly longer to initiate righting than those receiving lower doses.

Incorporation of behavioral assays with more classical assays of toxicity aids in establishing ecologically significant endpoints that can be measured in field settings (Peakall 1992). We seek to establish righting response, the ability of a turtle to completely flip over when placed on its back, as an appropriate field method with which to assess neurobehavioral impacts of heavy metal contamination on *Trachemys scripta* collected from TCSFS. We hypothesize that *T. scripta* from TCSFS will take longer to initiate and complete righting than animals from reference sites.

**METHODS**

*Trachemys scripta* were collected monthly from July to September 2003 and April to October 2004 from TCSFS and two unmined reference sites: Lake Carl Blackwell (LCB, Payne Co., OK) and Sequoyah National Wildlife Refuge (SNWR, Sequoyah Co., OK) as part of a larger genetic study (Hays, 2005). All animals were trapped using hoop nets baited with sardines. Traps were set with wooden stakes in the late afternoon and checked the following morning. All trapped animals were removed and placed in plastic tubs. Traps were rebaited, if necessary, and reset for a maximum of 48 hours per trapping period or until 20 animals were collected. Righting trials were conducted on a random sample of animals (ca. half of the total animals collected during a trapping session) during the July, August and September trapping periods of 2003 and 2004 (TCSFS, n =
Ambient air temperatures were consistently over 24° C during this period to reduce any effect of temperature on righting response. Each animal was placed on its carapace on a synthetic grass mat. Latency to begin righting (in seconds), defined as the first movement of a limb beyond the plane of the carapace, and complete righting time, defined as the time required to completely turn onto the plastron from the initiation of the test, were recorded. Animals were observed for a maximum of 1200 seconds. Animals that showed no response or initiated but did not complete righting received designations of >1200. After the righting trial was completed, animals were weighed to the nearest gram with a Pesola spring scale and straight-line carapace and plastron length and width were measured to the nearest mm. Sex was determined by the observation of secondary sexual characteristics, such as elongated foreclaws and cloacal position in relationship to carapacial rim (Ernst et al., 1994). All animals were given a unique notch on marginal scutes to identify animals recaptured in subsequent trapping efforts. Carapace and blood samples were collected and deposited in the Oklahoma State University Collection of Vertebrates Frozen Tissue Collection. Animals were released at the point of capture. All animals were handled following Institutional Animal Care and Use Committee (IACUC) approved field methods (IACUC ACUP No. AS0315).

We used time-to-event analysis with the Cox proportional hazards (CPH) regression model to analyze both initiation and completion of righting time (Piegorsch and Bailer 1997). Right censoring occurs when an animal does not complete the event (i.e. initiating or righting) in the observed time period and can be accounted for by this analysis. The CPH model allows us to account for variation in righting time, including censored
animals, and generates a hazard ratio that is an estimate of relative likelihood that an event will occur in any small time interval, as affected by specified covariates. CPH regression can be employed to look at main or interaction effects of these covariates while simultaneously controlling for other variables. Both discrete and continuous covariates can be employed in this analysis (Piefforsch and Bailer 1997). We specified sex and site as discrete covariates and mass as a continuous variable in both tests. We used two-tailed tests with a significance level of 0.05.

Kaplan-Meier curves and associated log-rank statistics can be generated for discrete covariates of main interest prior to CPH regression to determine if multiple groups can be combined. Each curve represents a different site and traces percent turtles that did not complete the righting event as a function of time. A log-rank statistic value of $P > 0.1$ indicates that sites are not significantly different. Kaplan-Meier curves do not control for other variables and have a higher Type III error rate then the CPH model. However, Kaplan-Meier curves provide a valuable graphical representation of discrete covariates that can be used in model development.

RESULTS AND DISCUSSION

We used initial observation of Kaplan-Meier curves to determine if our two reference populations could be combined for later analyses. Kaplan-Meier curves and log-rank tests for initiation of righting showed that the two reference sites (LCB and SNWR)
Fig. 1 - Kaplan-Meier curve representing initiating righting time for *T. scripta* populations at TCSFS and reference sites, SNWR and LCB. Log-rank statistics revealed a significant difference between reference sites (*P* = 0.0054). Reference sites were considered separately for all subsequent analyses.

...differed significantly (*P* = 0.0054) (Fig. 1). In further analyses of righting initiation, all three sites were coded separately. However, in analysis of complete righting data, log-rank statistics revealed that LCB and SNWR did not differ significantly (*P* = 0.2489). Thus they were combined to form one reference group that was compared with TCSFS for further analyses (Fig. 2).

Although the Kaplan-Meier estimates suggested that sites may show a significant difference in time to righting initiation, results of the CPH model showed that site was
Fig. 2 - Kaplan-Meier curve representing completion of righting time for *T. scripta* populations at TCSFS and reference sites, SNWR and LCB. Log-rank statistics revealed no significant difference between reference sites ($P = 0.25$). Reference sites were combined for all subsequent analyses.

not a significant factor. It is important to note that Kaplan-Meier curves do not take into account effects of other covariates and can show invalid significance. Sex, mass, and interaction of sex and mass contributed significantly to time to righting initiation based on the CPH model. Hazard ratios, which yield percentages useful in determining the rate at which measured endpoints are occurring, were highly significant (0.097, respectively) mass (Table 1). Sex may also be considered a marginally significant factor. Interactions between all variables were also tested with the only marginally significant interaction occurring between sex and mass. The interaction was generated a hazard ratio of 3.375
Although only marginally significant, males initiated righting at a 59.8% lower rate than female turtles. Also, as mass increased by one unit (kg) the rate of righting initiation significantly decreased by 90.3%. The marginally significant interaction of sex and mass indicated that heavy males were the least likely to initiate righting. The percentage of censored animals, those that did not initiate the righting event in the observation period, was highly variable among the 3 sites. LCB had the fewest number of censored animals (14%), while TCSFS and SNWR had 31% and 41% of animals censored, respectively. Marginally significant differences were seen in the distribution of censored animals among the 3 sites ($\chi^2 = 5.81, P = 0.0547$).

### Table 1 Results of Cox Regression Analysis - Initiating Righting

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(95% Confidence Interval)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>0.402 (0.141 - 1.143)</td>
<td>0.09</td>
</tr>
<tr>
<td>Mass</td>
<td>0.097 (0.013 - 0.741)</td>
<td>0.02</td>
</tr>
<tr>
<td>Sex*Mass</td>
<td>3.375 (0.921 - 12.377)</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Under normal conditions, females do not initiate or complete neurobehavioral events more quickly than males. However, Burger and Gibbons (1988) found that female turtles are capable of sequestering metals in egg shells and contents. This may allow females to
decrease metal loads in their bodies and reduce neurobehavioral effects of lead and other heavy metals.

One variable were also identified that jointly and independently influenced the complete righting time of *T. scripta*, mass. The hazard ratio for mass relative to other variants was 0.384 and highly significant, whereas site was not significant with a hazard ratio of 1.662 (Table 2). We also tested for effect of sex and pairwise interactions between all factors and found none to be significant ($P > 0.2$). Hazard ratios generated for time to complete righting indicates that as mass increased by one unit (kg), and all other variables were held constant, the rate of righting completion decreased by 61.6%.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass</td>
<td>0.384 (0.161 - 0.912)</td>
<td>0.03</td>
</tr>
<tr>
<td>Site</td>
<td>1.662 (0.881 - 3.135)</td>
<td>0.12</td>
</tr>
</tbody>
</table>

A larger percentage of animals were censored in complete righting data than initiation data, however there was no significant difference in the number of censored animals between animals from TCSFS and the combined reference sites ($\chi^2_1 = 2.06, P = 0.15$).
Moeller (2004) found lead, zinc, and cadmium present at levels above baseline in substrate collected from TCSFS. Zn concentrations were the highest (3000 ppm), followed by Cd and Pb (220 ppm and 440 ppm, respectively). In contrast, commercial reference substrate yielded undetectable levels of Cd and Pb and 6.2 ppm of Zn. Hatchlings incubated on TCSFS substrate accumulated more Cd in turtle shells (carapace and plastron) than soft tissues, but Zn and Pb accumulated in soft tissues. Zn was found in the highest concentration (503.6 ppb) in the soft tissue, followed by Pb and Cd (50 ppb and 7 ppb, respectively) (Moeller, 2004).

*Emys scripta* is directly exposed to substrate in all life stages, thereby making uptake of contaminants likely. Eggs are laid in underground nests dug by adult females, hatchlings overwinter in the nest, and juveniles and adults bury themselves in sediment during winter months (Gibbons 1990). In a concurrent study (Hays 2005), we tested whole blood collected from a subset of *Emys scripta* trapped at TCSFS and SNWR in 2004 to measure circulating levels of Pb and Cd. Blood Zn was not tested because serum was not available. Blood Pb levels were not significantly different between the two sites. Animals trapped at TCSFS had significantly higher levels of blood Cd than animals from SNWR. Due to the lack of a significant difference in blood Pb concentrations between the sites and existing studies that indicate Pb can be stored in bone and shell (Moeller 2004), we also tested carapace samples for Pb. We found significantly higher levels of Pb in carapace than blood at SNWR, but the difference was not significant at TCSFS (Hays 2005).
Low levels of circulating Pb and significantly higher levels of Pb in bone indicate that *T. scripta* from SNWR is storing Pb in bone. Significant differences were not seen in the TCSFS population. Non-circulating Pb is not readily available to elicit a deleterious effect on neurobehavioral response and may explain why no significant difference in righting initiation or completion was seen between TCSFS and reference animals. Burger et al. (1998) dosed animals with a single metal in a laboratory setting; however, this may not accurately model field settings in which dose of contaminant and duration of exposure are often unknown. It is imperative that we look at other major contaminants at TCSFS, Cd and Zn, to provide possible explanations for the absence of neurobehavioral effects. Zinc is an essential metal that serves as a cofactor for > 300 enzymes and plays an essential role in the central nervous system. Cadmium is often released as a byproduct of the zinc mining process. When present with Zn, Cd often replaces it in active sites and more Zn is present in soft tissues and blood (Goyer and Clarkson 2001). Some studies have found that infants and children receiving Zn supplements show increased fine and gross motor skills, high locomotor scores, and increased amounts of high motor activities and functional and vigorous activity (Sandstead et al. 1998; Bhatnagar and Taneja 2001). Zinc present with Cd at TCSFS could possibly elicit a similar response in *T. scripta*. Further investigation of the effects of Cd and Zn on neurobehavioral response is necessary before drawing broad conclusions about *T. scripta* at TCSFS.

Despite the difficulty of selecting relevant endpoints, behavioral toxicology serves as a complement to traditional toxicological assays. Populations that are already at-risk can be monitored behaviorally in the field without permanently removing individuals from
the population. Our study has shown that righting time may be a useful behavioral endpoint that can be incorporated in field studies of turtles and time-to-event analysis is a successful means by which to analyze these data which often include censored values. Very few behavioral studies have used reptiles and an even smaller number have completed these tests on field collected animals. In order to further establish behavioral endpoints as a field tool further research is necessary to determine appropriate endpoints and methodology.

Acknowledgements

We would like to thank numerous field assistants; Oklahoma State University, Quapaw Tribe of Oklahoma, and U.S. Fish and Wildlife Service for site access; Society of Environmental Toxicology, EA Engineering, Sigma Xi, and Oklahoma State University for funding; Dr. Zhigang Zhang and Dr. Stephen Cox for statistical assistance; Dr. Joe Bidwell, Dr. Stan Fox, and Kendra Phelps for editorial assistance.

REFERENCES


EPA (2000) Five year review, Tar Creek Superfund Site, Ottawa County, Oklahoma. US Environmental Protection Agency, Washington DC

EPA (2005) Fact Sheet: Tar Creek Superfund Site Ottawa County, Oklahoma. US Environmental Protection Agency, Washington DC


Environ Tox Chem 9:1-2


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, Stillwater


Classical, laboratory-based toxicology tests have proved valuable in establishing public health standards and regulations. However, when studying wildlife populations exposed to contaminants, laboratory-based toxicity assays may not provide enough information for evaluation or conservation strategies. Comprehensive toxicological studies combine classical assays with natural history, demography, and behavior to accurately assess the overall condition of wildlife populations exposed to contaminants. By combining flow cytometric analysis and tissue metal analysis with population demography and behavior, this study was able to assess possible anthropogenic effects on red-eared slider turtles (Trachemys scripta) from Beaver Creek within Tar Creek Superfund Site (TCSFS).

Multiple measures of population demography, including adult sex ratios, percent juveniles and melanistic animals, and a sexual dimorphism index,, indicate differing population structures between contaminated and reference populations. Males from TCSFS are significantly smaller and show a lower incidence of melanism than animals from both reference sites. Also, the adult sex ratio is more heavily male biased and fewer juvenile males are present in reference populations than at TCSFS. Although estimates of survival did not differ among the sites, these data suggest that the TCSFS population is negatively impacted. These data suggest that male T. scripta at TCSFS are showing less adult recruitment than is occurring at both reference sites. Lack of adult recruitment can lead to reduction of effective population size and genetic bottlenecks.

Flow cytometric analysis, used to measure the coefficient of variation (CV) around the mean of cells in the G1 stage of the cell cycle, revealed a significant site difference in 2003, which is thought to be an artifact of freezing and thawing blood
samples on multiple occasions. Samples collected in 2004 did not reveal a significant site
difference, but the TCSFS population showed a significant difference among months of
collection. CVs were high just following emergence from hibernation and just prior to
hibernation. These data, combined with knowledge of season metabolic patterns of *T.
scripta*, suggest that depressed metabolic rates associated with hibernation prevent or
reduce nucleic acid synthesis and naturally occurring DNA repair enzymes and slow
erythrocyte turnover. TCSFS population showed a higher incidence of aneuploidy,
indicating the presence of a sub-population of cells with different DNA content, than both
reference populations. Although CV did not differ significantly among sites, the
increased presence of aneuploidy may indicate that heavy metals are having a deleterious
effect on the genetic integrity of the TCSFS population.

Blood Pb levels in *T. scripta* from TCSFS were higher than values published for
other taxa, but did not differ significantly from a reference population. Carapace Pb did
not differ significantly between contaminated and reference sites, but was higher at
TCSFS in both sampling periods and showed a step-wise increase in concentration
throughout the season at both sites. Low levels of circulating Pb and higher levels of
carapace Pb indicate *T. scripta* are capable of storing Pb in bone. Blood Cd levels were
significantly larger at TCSFS and showed a step-wise increase throughout the season at
both sites.

Righting time allows for assessment of neurobehavioral response in the field with
little stress to the turtle. Using time-to-event analysis we found that site was not a factor
in how quickly *T. scripta* initiated righting, but TCSFS animals completed the righting
event more quickly than reference animals. These data are contrary to knowledge about
the effect of Pb on neurobehavioral response; however, Zn can cause a stimulatory effect on motor skills in children and may elicit a similar effect in *T. scripta*.

Evaluation of population demography, genetics, and behavior indicate that *T. scripta* from TCSFS have been negatively impacted by the presence of heavy metals at the site. Deleterious effects are still evident in *T. scripta*, many of which entered the effective population after mining ceased approximately 35 years ago. Further comprehensive studies of this and other wildlife populations at contaminated sites like TCSFS will aid in better regulations, conservation strategies, and public health policies.
Kimberly Anne Hays
Candidate for the Degree of
Master of Science

Thesis
ASSESSMENT OF INDIVIDUAL AND POPULATION-LEVEL
ENDPOINTS IN RED-EARED SLIDER TURTLES (TRACHEMYS SCRIPTA)
FROM A METAL-CONTAMINATED SUPERFUND SITE

Major Field: Zoology

Biographical:

Personal Data: Born in Lakenheath, England, on 4 May 1980, to Joseph R. and Myrna C. Hays

Education: Graduated from Wetumpka High School, Wetumpka, Alabama, in May 1998. Received Bachelor of Science in Biology from Jacksonville State University, Jacksonville, Alabama, in April 2002. Completed the requirements for the Master of Science Degree with a major in Zoology at Oklahoma State University in December of 2005.

Experience: Employed as a teaching assistant, Oklahoma State University, Department of Zoology, Fall 2002 – Spring 2005 and Fall 2005.

Professional Memberships: Society of Environmental Toxicology and Chemistry, Southwestern Association of Naturalists
Name: Kimberly Anne Hays                     Date of Degree: December 2005
Institution: Oklahoma State University       Location: Stillwater, Oklahoma
Title of Study     ASSESSMENT OF INDIVIDUAL AND POPULATION-LEVEL ENDPOINTS IN RED-EARED SLIDER TURTLES (TRACHERMYS SCRIPTA) FROM A METAL-CONTAMINATED SUPERFUND SITE

Pages in Study: 119               Candidate for the Degree of Master of Science
Major Field: Zoology

Scope and Method of Study: The purpose of this study was to assess the effects of heavy metals on red-eared slider turtles from Tar Creek Superfund Site. Animals used in the study were collected from Tar Creek Superfund Site, and two reference sites, Lake Carl Blackwell and Sequoyah National Wildlife Refuge. Assessment included population, genetic, metal, and neurobehavioral studies.

Findings and Conclusions: The population of red-eared slider turtles from Tar Creek Superfund Site shows decreased male recruitment into the adult population based on altered adult sex ratios, decreased incidence of melanism and increased percentage of juvenile males. Three-way ANOVA showed no significant difference in CV as measured by flow cytometry; however, increased presence of aneuploidy in turtles from the Superfund Site is indicative of genetic damage. Blood and shell lead levels were not significantly different between sites, but more lead was present in shells. Blood cadmium levels were significantly higher at Tar Creek Superfund Site. Righting time was used to assess neurobehavioral response and time-to-event analysis showed no significant difference in righting response between animals from Tar Creek Superfund Site and reference animals.

ADVISER’S APPROVAL: Karen McBee