

Assessment Plan Addendum

Southeast Missouri Lead Mining District

Natural Resource Damage

Assessment

Effects of lead (Pb) exposure on songbirds breeding within the Southeast Missouri Pb Mining District

Rebecka Brasso, PhD. Southeast Missouri State University, Department of Biology, One University Plaza, MS 6200, Cape Girardeau, MO 63701

Frank R Thompson III, PhD. USDA Forest Service, Northern Research Station, 202 Anheuser-Busch Natural Resources Building, Columbia, MO 65211, USA

Melissa Roach, Department of Fisheries and Wildlife Sciences, University of Missouri, 302 Anheuser-Busch Natural Resources Building, University of Missouri, Columbia, MO 65211, USA

Julia Lankton, D.V.M., D.A.C.V.P. U.S. Geological Survey National Wildlife Health Center, 6006 Schroeder Road
Madison, WI 53711, USA

Barnett Rattner, PhD. U.S. Geological Survey Patuxent Wildlife Research Center, Beltsville Laboratory, c/o BARC-East, Building 308, 10300 Baltimore Avenue, Beltsville, MD 20705 USA

Natalie Karouna-Renier, PhD. U.S. Geological Survey Patuxent Wildlife Research Center, Beltsville Laboratory, c/o BARC-East, Building 308, 10300 Baltimore Avenue, Beltsville, MD 20705 USA

Danielle Cleveland, PhD. U.S. Geological Survey Columbia Environmental Research Center, 4200 New Haven Road, Columbia, MO 65201, USA

Abstract

The US Fish and Wildlife Service (FWS), along with the U.S. Forest Service, and Missouri Department of Natural Resources are pursuing a Natural Resource Damage Assessment (NRDA) in the Southeast Missouri Lead Mining District (SEMO), where lead mining dates back over 100 years. Residues from smelting, tailings and chat have contaminated soils and water. This study will expand upon an earlier investigation by Beyer et al. 2013 that determined that ground feeding songbirds have been injured from lead (Pb) associated with mining in SEMO. This study will evaluate nesting songbirds to determine whether exposure to lead has adversely affected their reproduction. Nesting success in open nests and nest boxes will be evaluated for two or three seasons. In addition, nestlings and adult songbirds will be captured and bled and analyzed for lead and delta-aminolevulinic acid dehydratase (ALAD) activity. A subset of the collected birds will be sacrificed for analysis of Pb in the kidney and liver tissues; a portion of these tissues will also be examined for microscopic evidence of lead poisoning toxicity and indicators of oxidative stress. Soil and earthworms will also be collected and analyzed for Pb and other heavy

metals including zinc (Zn), cadmium (Cd), copper (Cu), cobalt (Co), and nickel (Ni) to better understand the dietary relationship of heavy metal exposure. The work is designed to meet the needs of the FWS and other NRDA co-trustees and will include a report as well as one or more scientific publications.

Background and Justification

The Southeast Missouri Lead Mining District (SEMO) has been mined for a hundred years and has the largest source of lead ore in the U.S. The District is located within the Mississippi Flyway bird migration route. In a previous study, Beyer et al. (2013) found ground-feeding songbirds (northern cardinals *Cardinalis cardinalis* and American robins *Turdus migratorius*) contained lead concentrations in tissues that approached or exceeded adverse effect levels reported in the literature (Franson and Pain, 2011). In addition, Beyer et al. (2013) found over 50% inhibition of the delta-aminolevulinic acid dehydratase (ALAD) enzyme activity in the majority of study birds collected at SEMO compared to non-contaminated reference areas. Several other researchers have identified adverse effects of heavy metals to organisms in SEMO (Niethammer et al. 1985, Schmitt et al. 2007, Besser et al. 2009, Allert et al. 2008, Struckhoff et al. 2013), but potential reproductive effects in terrestrial organisms have not been studied. The U.S. Fish and Wildlife Service (FWS), along with co-trustees, is pursuing a Natural Resource Damage Assessment (NRDA) in SEMO and has trusteeship over natural resources and their services which FWS manages, including migratory birds. This study will help quantify injury to avian resources as part of the ongoing NRDA.

Several sites are being evaluated for study. Two to four sites in the “Old Lead Belt” may include mine/mill sites in St. Francois and Madison Counties and/or locations in the Big River floodplain in Jefferson, Washington, and/or St. Francois Counties, located 50 to 90 miles south-southwest of St. Louis; additional sites may be located near the Buick Smelter in the Viburnum Trend, or “New Lead Belt,” about 50 miles west of the Old Lead Belt. A reference site will be located at Reis Biological Field Station in Crawford County, with a potential back-up site at Johnson’s Shut-Ins State Park in Reynolds County. The Old Lead Belt sites may include Washington State Park and an adjacent private property within the Big River floodplain in Washington County; The Doe Run Pile in St. Francois County; and/or the Anschutz Mine in Madison County.¹ The Viburnum Trend sites will be located on U.S. Forest Service property north and/or south of the Buick Smelter. Two of these sites, namely Washington State Park, and areas near the Buick Smelter, were previously sampled by Beyer et al. 2013. Both of these sites demonstrated songbird exposure to lead. Sites will be chosen to represent a variety of exposure scenarios currently or historically represented in SEMO, including mine/mill waste piles, smelter-contaminated soils, alluvial outwash from mining and milling sites, and combinations of multiple mining, milling and smelting-related sources. Since many of the mine/mill waste piles in St. Francois County have been capped by remedial actions, sites in the Viburnum Trend and/or Madison County of similar soil lead concentrations will be studied to represent pre-remedial conditions in St. Francois County. Biologists from the FWS office in Columbia, MO will provide their expertise on the local sites. Final site selection will depend on property access, logistical considerations, habitat suitability, likelihood of anthropogenic disturbance during the breeding season, and soil lead concentrations that represent a range of exposure scenarios including those that are deemed to be potentially injurious to songbirds.

¹ The Trustees will obtain permission to access private property prior to conducting the study. Alternative locations may need to be used in the event that permission is not obtained.

Study objectives

- 1) To determine Pb exposure in songbirds breeding within the SEMO using both non-destructive (blood) and destructive sampling techniques (liver and kidney samples).
- 2) To compare Pb concentrations in bird tissues with soil Pb concentrations at each site (see below for site descriptions).
- 3) To assess physiological harm associated with Pb exposure in songbirds using biochemical and hematological biomarkers, ALAD, indicators of oxidative stress and damage (IOS), and/or hematocrit or packed cell volume (PCV). Additional harm at the level of the individual will be assessed through the harvesting and microscopic examination of kidney and liver tissue from a sub-set of individuals for microscopic examination of lesions associated with Pb toxicity from a sub-set of individuals. These measurements will be used to assess and quantify injury by comparing the various endpoints and the rate of abnormalities in contaminated study sites to the reference sites.
- 4) To assess potential reproductive effects of Pb exposure, basic reproductive parameters (clutch size, number of young hatched, nestling mass, number of young fledged, and nest success) of both nest box and open-cup nesting songbirds in contaminated and reference areas reflecting baseline conditions across a variety of habitats. The relation of reproductive parameters and biochemical and hematological measurements to tissue Pb concentrations will be used to determine whether physiological effects from Pb in adult birds are associated with adverse effects on reproduction in contaminated habitats.

Study sites

Sampling will occur at several contaminated sites within the SEMO as well as one or more reference sites. Previous studies by Beyer et al. 2013 demonstrated that pathological lesions in ground-feeding songbirds occurred at soil Pb concentrations of approximately 1000 mg Pb/kg, while ALAD inhibition could occur at lower Pb concentrations ranging from 345-1000 mg Pb/kg (Stratus 2014). For the purposes of this study, contaminated sites will be those with soil lead concentrations exceeding 1,000 mg/kg. Reference sites will have Pb concentrations at or below the mean background soil concentration for the Southeast Missouri Ozarks, generally <80 mg Pb/kg. Roux et al. 2007 demonstrated that rural songbirds during the nesting season had low concentrations of Pb compared to urban birds captured near Washington D.C., while SEMO birds previously sampled by Beyer et al. 2013 had significantly higher blood-Pb levels than the urban birds sampled by Roux et al. 2007. Therefore, it is expected that the rural reference sites selected in this study will be an unlikely source of significant Pb exposure to songbirds.

Owing to the diversity of habitat types and quality thereof associated with the study sites, not all species will be sampled at each site and sample sizes may vary based on local abundance. Bird species sampled in study sites will be compared to the same species sampled in reference areas. The selected sites will allow for evaluation across a range of lead concentration, sources of lead (e.g. smelter, mill waste, flood plain deposition), and habitat conditions (e.g. upland forest, old field/savanna, flood plains). Site selection will be based on access, exposure potential, and habitat quality.

Potential Contaminated sites: Buick Smelter, Madison Mines (Anschutz Mine), Washington State Park, Leadwood, Missouri Mines, and/or other private property in the Big River floodplain in Jefferson or St. Francois counties.

Reference sites: Reis Environmental Research Station and/or Johnson Shut-Ins State Park

Target species

This study will focus on regionally abundant species that, owing to their ground-feeding foraging habits, have a high probability of incidentally ingesting soil (Beyer et al. 1994). Target species include (but are not limited to):

Migratory species

Field sparrows (*Spizella pusilla*) are migrant songbirds that breed in brushy grasslands, second growth scrub, glades, and savannas and open woodlands. Field sparrows nest on or near the ground in clumps of grass at the base of shrubs and forages on the ground or in low vegetation on invertebrates and seeds (Carey et al. 2008).

Indigo buntings (*Passerina cyanea*) are migrant songbirds that breed in brushy old fields, second growth scrub, glades, savannas, open woodlands and also in forests wherever there are breaks in the canopy such as along rivers, roads, and tree fall gaps. Indigo buntings nest in low vegetation generally < 1m above the ground and forage on the ground and in low shrubs and trees on invertebrates, seeds, and fruits (Payne 2006).

Ovenbirds (*Seiurus aurocapilla*) are migrant songbirds that breed in mid- to late-successional deciduous and mixed deciduous and pine forest in Missouri. Ovenbirds nest and forage in leaf litter on forest floor invertebrates (Pomeluzi et al. 2011).

Kentucky warblers (*Geothlypis formosa*) are migrant songbirds that breed in mesic deciduous forest with dense understories, often near streams. Kentucky warblers nest on or very close to the ground and forage on invertebrates in the leaf litter or on low vegetation (McDonald 2013).

Louisiana waterthrush (*Parkesia motacilla*) are migrant songbirds that breed in closed canopy deciduous or mixed evergreen-deciduous forest along low-order streams and in bottomland hardwoods. Louisiana waterthrushes forage on invertebrates in stream channels, low herbaceous plants, leaf litter, soil, rocks, and moss and nest in stream banks, roots of upturned trees and under logs (Mattsson et al. 2009)

Worm-eating warblers (*Helmitheros vermivorum*) are migrant songbirds that breed in mature deciduous, mixed evergreen-deciduous, and disturbed or successional forest with dense understories or shrubs, typically in hilly terrain. Worm-eating warblers nest on the ground in leaf litter and forage on invertebrates on the forest floor, low shrubs, and trees (Vitz et al. 2013).

Wood thrush (*Hylocichla mustelina*) are migrant songbirds that breed in mid- to late-successional deciduous and mixed deciduous evergreen forest in Missouri. Wood thrush nest in low to medium height trees in the understory and forage in leaf litter on forest floor invertebrates (Evans et al. 2011).

Red-winged blackbirds (*Agelaius phoeniceus*) are migrant songbirds that breed in wetlands, around retention ponds, and in drier habitats such as fallow fields, sedge meadows, and along waterways in wooded areas. During the breeding season, red-winged blackbirds are primarily insectivorous, probing wet soil and vegetation with their bills to extract a variety of invertebrate prey. Owing to the patchy distribution of suitable breeding habitat, numerous nests can often be found within each male's territory.

American robins (*Turdus migratorius*) are migrant songbirds that breed in forests, woodlands, and gardens in riparian and residential areas where lawns or other short grass areas are interspersed with shrubs and trees (Vanderhoff et al. 2014). Robins are the most abundant and widely distributed thrush in the U.S., but breeding densities have rarely been estimated in Missouri forests and woodlands. Robins generally forage on the ground and in low vegetation on invertebrates and fruits. They typically nest at low to moderate heights in trees (Vanderhoff et al. 2014).

Eastern towhees (*Pipilo erythrophthalmus*) are migrant songbirds that breed along forest edges, overgrown fields, woodlands as well as scrubby lots in residential areas. Towhees are ground foragers, scratching in deep leaf litter under dense shrub cover for invertebrate prey. Towhees also forage on seeds, fruits, and flower buds during the spring. Towhee nests are built on the ground or in low vegetation.

Eastern bluebirds (*Sialia sialis*) are migrant songbirds found across eastern North America. Eastern bluebirds are common in open areas in agricultural fields, suburban parks, pastures, backyards, as well as open forested areas. Throughout the year, they primarily feed on invertebrate prey (caterpillars, beetles, crickets, grasshoppers, and spiders) captured on the ground though winter diets often include berries. Owing to their habit as secondary cavity nesters, Eastern bluebirds are readily attracted to man-made nest boxes erected in suitable habitat.

Resident species

Carolina wrens (*Thryothorus ludovicianus*) are common, year-round residents and generalists occurring in a wide range of overgrown, vegetated habitats such as brushy thickets, shrubby/wooded residential areas, brushy suburban yards, and abandoned buildings. Carolina wrens are high trophic songbirds foraging primarily on invertebrate prey (variety of insects and spiders) captured on the ground or on low vegetation. This species has also been known to capture and consume small reptiles. While Carolina Wrens are typically open-cavity nesters, they are versatile and will readily use man-made nest boxes erected in suitable habitat.

Northern cardinals (*Cardinalis cardinalis*) are a common year round resident and generalist occurring across areas with shrubs and small trees including old fields, forest edges, second growth woodlands, open woodland and savanna, hedge rows, and urban and suburban areas (Halkin and Linville 1999). Northern Cardinals nest in low shrubs and trees and forage on the ground and in low shrubs and trees on invertebrates, seeds, and fruits (Halkin and Linville 1999).

Game and invasive species

Mourning doves (*Zenaida macroura*) are the most widespread and abundant game birds in North America. Mourning doves are migratory ground foragers found in open grasslands, agricultural fields, residential yards, road sides, and woodland edges. Seeds make up nearly 100% of their diet. This species

nests in elevated dense foliage, but will also build nests on the ground or in man-made structures such as planters or gutters.

European starlings (*Sturnus vulgaris*) are the most abundant songbirds in North America. They are considered resident to short-distance migrants and remain year round. True generalists, starlings will forage on a wide variety of prey items (seeds, fruits, grains, livestock feed), but prefer to forage on insects and other invertebrates when available. Starlings are ground foragers, probing the ground with their bills to locate insects in the soil in open areas. Starlings are cavity nesters and are typically associated with habitats near human activity—mowed lawns, city streets, abandoned houses, and/or agricultural fields.

Methods

Tissue collection

Adult birds will be captured either at their nests or in foraging areas using mist nets, funnel traps, and/or nest box traps during May, June, and July 2016, 2017, and 2018 from four or five sites. Adult birds will be banded with a FWS aluminum band, basic morphometrics determined, and blood samples will be collected by venipuncture.

Avian blood is most frequently used as a sample matrix in studies evaluating exposure to metals such as Pb. The sampling of blood (<1% of body mass) in wild birds is non-lethal and minimally invasive with no evidence of significant short- or long-term effects on adult survival, reproductive success, body condition, or behavior (Sheldon et al. 2008). Upon capture, a small blood sample will be collected using a small gauge needle (26-28 gauge) to puncture the cutaneous ulnar vein. Blood will be collected as appropriate for each analysis (Pb, ALAD, PCV). Basic morphometric data will be gathered (mass, wing cord, etc.) from adult and nestling birds and each bird will be fitted with an appropriately sized federal bird band prior to release. All nestlings in nest boxes will be banded to allow for the possibility of detection in subsequent years. All birds will be released from the capture site.

In accordance with the Ornithological Council's *Guidelines to the Use of Wild Birds in Research* (Fair et al. 2010) and Federal and State permitting guidelines, the volume of blood collected from an individual must not exceed 1% of its body mass. For example, adult Eastern Bluebirds often weigh approximately 30g which means collection of a maximum blood volume of 300 μ L. However, blood volume collected from any individual is highly dependent on a variety of physiological (e.g. stress, vein diameter) and environmental (e.g. ambient temperature) variables. Target species in this protocol range from 12-80g (with the exception of the Mourning Dove that can reach 120g). Nestling birds present an additional challenge as their age and smaller size will limit the volume of blood that can be collected. To ensure enough blood is available for Pb analysis (200-500 μ L), blood samples will be collected from all nestlings in each brood to be analyzed as a "pooled sample" giving a single concentration for each nest. Similarly, for small species (defined as those weighing <50g) blood samples will be pooled for the adult male and female at each focal nest. For larger species (>50g), efforts will be made to collect the maximum allowed blood volume for Pb analysis at the level of the individual.

Destructive sampling of adult birds will be restricted to a sub-set of individuals of the following species: Mourning doves, European starlings, American robins, and Northern cardinals to add to existing

databases. Birds will be euthanized after capture in funnel traps or mist nets and blood collection at the conclusion of the breeding season in each year. Birds will be euthanized via inhalant (e.g., carbon dioxide or monoxide) or by placing the bird's face in tightly sealed vial with a cotton ball in it that has been soaked in isoflurane. In either case, euthanasia is rapid and these methods are suggested as most humane in field settings by the American Veterinary Association (Fair et al. 2010). Kidney and liver samples will be harvested from 10-20 euthanized individuals per species (40 to 80 birds total) between 2016 and 2018. Each organ will be measured (mass, size in three dimensions, as well as organ mass relative to body mass) digitally imaged, and examined for gross abnormalities prior to preparation for Pb and histopathological analyses.

All tissue collection will occur under appropriate Federal Bird Banding (R. Brasso 23903, sub-permittees M. Roach and K. Hixson) and MO State Collection permits (R. Brasso #16685) as well as an Institutional Animal Care and Use Committee (IACUC) permit from Southeast Missouri State University.

Assessment of biochemical, physiological, and pathological effects of Pb exposure

Lead concentration in blood is one of the most frequently used markers for assessing Pb exposure in the avian community (Burger and Gochfeld 1997, Johnson et al. 2007, Hansen et al. 2011, Beyer et al. 2013). Blood Pb levels reflect short-term (recent) dietary exposure either through direct ingestion of soil or from consuming contaminated prey items and indicate toxicosis (Buekers et al. 2009, Franson and Pain 2011, Beyer et al. 2013). Evaluating blood and tissue exposure as well as histopathology and biomarker analysis can provide insight into the presence and effects of acute and chronic exposure.

Commonly used blood indicators of Pb exposure and toxicity are activity of red blood cell delta-aminolevulinic acid dehydratase (ALAD) and the concentration of erythrocyte metabolite protoporphyrin (Buekers et al. 2009, Hansen et al. 2011, Beyer et al. 2013, Haig et al. 2014). An inhibition of >50% of the ALAD enzyme in birds as a result of Pb exposure can be defined as "injury" under the CERCLA NRDAR regulations . (Beyer et al. 2013). Lead and the inhibition of ALAD can lead to the creation of reactive oxygen species (ROS), anti-oxidant imbalance and oxidative stress can also be used as biochemical, hematological, and tissue (liver) biomarkers of Pb exposure (Martinez-Haro et al. 2011). Under baseline conditions, ROS production and defense systems are in balance. However, measuring just one side of this equation is inadequate for accurate assessment of oxidative stress (Monaghan 2009, Selman 2012). The outcome of oxidative stress i.e. the level of impaired function because of damage to lipids, proteins and DNA is of greatest consequence to the organism and therefore the most significant insights into oxidative stress are gained from assessments of redox status, protective/repair mechanisms, and actual damage to key biological molecules (Monaghan 2009; Selman 2012). IOS can be measured in blood and/or in other tissues, such as liver. Although levels in blood reflect more immediate effects of exposure on the individual, damage within organ tissue, gives better insights into long-term processes (Selman 2012).

Liver tissue will be analyzed for indicators of oxidative status: total sulfhydryl (TSH), total glutathione (TotGSH), reduced glutathione (GSH), protein bound sulfhydryl (PBSH; TSH minus GSH), oxidized glutathione (GSSG; $[\text{TotGSH}-\text{GSH}]/2$), and the ratio of GSSG to GSH (GSSG:GSH). In addition, measures of outcomes of oxidative stress, including damage to lipids (thiobarbituric acid reactive substances-TBARS) and DNA (8-hydroxy-deoxy-guanosine – 8-OH-dG) will be analyzed in liver. In the kidney, the presence of gross lesions, number of renal intranuclear inclusion bodies, and degree of nephrosis will be used as measures of Pb toxicity (Beyer et al. 1988).

For this study, we plan to:

- 1) Assess Pb concentrations in blood, liver, and kidney.
- 2) Measure ALAD activity in blood samples of target species in contaminated and reference sites (target sample size = 10-20 individuals per species each from contaminated and reference sites, 40 to 80 birds total over three years).
- 3) Conduct oxidative stress assays on avian liver samples (target sample size = 10-20 individuals each from contaminated and reference sites, 40 to 80 birds total over three years).
- 4) Assess histopathologic changes in liver and kidney (target sample size = 10-20 individuals each from contaminated and reference sites, 40 to 80 birds total over three years).

Pb concentrations in blood will be compared to Pb concentrations in body tissues. The relations between Pb concentrations and biochemical, physiological, histopathological, and/or reproductive endpoints will be examined.

Assessment of reproduction effects from Pb exposure

Nest monitoring (open-cup nesting species)

We will monitor the reproductive success of nesting birds for the target species during May, June, and July 2016, 2017, and 2018 at four to six sites, including reference. Nests will be located through systematic searching and observation of parental behavior. Each nest will be designated with a unique identification number, and nest flags will be placed at least 5 m away to facilitate re-locating nests. Nests will be monitored every 1-3 days until they fledge or fail. The following information for each nest visit will be recorded: study site, nest ID, UTM's, date, start time, end time, stage (building, laying, incubating, nestling), number of host eggs/nestlings; and number of cowbird eggs/nestlings. Wing chord and tarsus length and mass of nestlings will be recorded when they are 5-6 days old.

Habitat and landscape information will be collected for each nest to control for these factors in our data analysis. Basic habitat measurements at each nest found will be collected soon after it fledges or fails. Date, coordinates, nest plant species, nest plant diameter at breast height (DBH in cm), nest plant height (m), nest height (m), point level canopy cover, percent ground cover of herbaceous vegetation, woody vegetation, and litter within a 5 m radius, small woody stem density within a 5 m radius, and basal area within an 11.3 m radius will be recorded. The United States Geological Survey National Land Cover Dataset in ArcMap Geographic Information System will be used to determine percent cover of deciduous forest, evergreen forest, and open land in a 1 km and 5 km radius around each nest.

Nest Boxes

In addition to following reproduction in natural nests, nest boxes will be erected to recruit breeding populations of Eastern bluebirds and Carolina wrens to the study sites to allow for more efficient tissue sampling of adult and nestling birds as well as monitoring of reproductive success. At least 10-20 nest boxes will be erected in suitable habitat for these target species at selected contaminated sites as well as the reference site. To recruit eastern bluebirds, nest boxes will be placed facing open fields and grassy areas. Bluebirds forage on the ground in grassy areas, but like to have shrubs or small trees near the box for perching. We will target areas within each site, such as along forest edges to erect these boxes. Boxes set for Carolina wrens will be placed deeper in forested areas as they forage in leaf litter on the forest floor, often avoiding open grassy areas. The number of boxes erected per site will be determined by the size of each site and territoriality of the target species. Each nest box will be designed following the standard Eastern/Western bluebird nestbox design (North American Bluebird Society, <http://www.nabluebirdsociety.org/nestboxes/eastwestbox.htm>) and will be fitted with a predator guard to reduce predation. Nest boxes will be monitored every 3-4 days during the breeding season to track nesting success through fledging.

The following information for each nest visit will be recorded: study site, nest ID, UTM coordinates, date, start time, end time, stage (building, laying, incubating, nestling), contents (number of host eggs/nestlings; number of cowbird eggs/nestlings), and comments (describing specific behaviors observed such as percent nest built, parent incubating or feeding, or nestling age). In addition, wing chord and tarsus lengths and weights of nestlings will be measured when young are 5-6 days old.

From each nest (natural or nest box), the adult male and female will be targeted for capture once during the chick rearing period to collect a blood sample for Pb analysis; blood will also be collected from nestling birds on the final, pre-fledge nest visit.

For this study, we plan to:

- 1) Measure Pb levels in tissues of breeding adult and nestling birds in conjunction with soil lead level at each study site,
- 2) Compare clutch size, number of young that hatch, number of young that fledge, and nesting success with soil lead level at nest sites and between study sites,
- 3) Compare clutch size, number of young that hatch, number of young that fledge, and nesting success with lead levels in blood from parents and nestlings,
- 4) Clutch size, number of young that hatch, number of young that fledge, and nesting success compared with biochemical, physiological, and/or pathological effects detected in the maternal and paternal adults from each focal nest.

Quantification of lead in blood and tissues

Blood and kidney and liver tissue portions designated for Pb analysis will be collected into pre-cleaned and labeled containers, and kept frozen until processing and analysis. The samples will be lyophilized

and digested in nitric acid and/or hydrogen peroxide. Concentrations of total recoverable Pb in the samples will be measured by inductively coupled plasma-mass spectrometry (ICP-MS) and reported on a dry mass basis.

ALAD and Oxidative Stress Assays

The activity of ALAD will be quantified in whole blood using the method of Burch and Siegel (1971) as modified by Pain (1987). The details of sample collection and storage, and the assay method are described in the attached standard operating procedure. Hematocrit needs to be determined on a fresh blood sample, and requires 50 to 75 μl of whole blood. The remainder of sample is frozen and subsequently assayed for ALAD activity. This assay has been scaled down to permit quantification of ALAD activity in 25 μl of whole blood (target volume of frozen whole blood >100 μl to permit duplicate or triplicate determinations).

IOS will be measured as follows. Frozen liver samples will be thawed on ice and homogenized on ice in 2X PBS (pH 7.4; Fisher BioReagents, Waltham, MA, USA) at 200 $\mu\text{g}/\mu\text{l}$. The homogenate will be centrifuged at 10,000 x g for 10 min at 4°C and aliquots of the supernatant transferred to tubes and frozen at -80°C until analysis. For GSH, GSSG and GSSG/GSH analysis, liver supernatant will be thawed and diluted in 1X PBS and analyzed using the DetectX® Glutathione Fluorescent Detection Kit (Arbor Assays, Ann Arbor, MI, USA) following the manufacturer's protocol. TSH will be determined in thawed supernatant that had been diluted in 1X PBS using the Measure-iT™ Thiol Assay Kit (Life Technologies - Molecular Probes, Inc., Eugene, OR, USA). Protein bound sulfhydryl (PBSH; TSH minus GSH), oxidized glutathione (GSSG; [TotGSH-GSH]/2), and the ratio of GSSG to GSH (GSSG:GSH) will be calculated using the measured endpoints.

For determination of TBARS, sample supernatant will be thawed on ice and diluted in 1X PBS. TBARS levels will be determined using the QuantiChrom™ TBARS Assay Kit (Bioassay Systems, Hayward, CA, USA) following the manufacturer's instructions. DNA damage will be assessed by ELISA. DNA will be purified from thawed liver samples using the QiaAMP DNA Mini Kit (Qiagen, Gaithersburg, MD) following the manufacturer's protocol. DNA concentration and purity will be assessed on a NanoDrop™ 8000 spectrophotometer (Thermo Scientific, Wilmington, DE). Normalized samples will be denatured and digested by nuclease P1 (Sigma Aldrich, St. Louis, MO). Concentrations of 8-OH-dG will be determined by ELISA using the OxiSelect™ Oxidative DNA Damage ELISA Kit (Cell Biolabs, Inc. San Diego, CA), following the manufacturer's protocol. The standards will be fit using a 4-Parameter Logistic curve. The reporting limit of this assay is 2 pg/ug DNA. Intra-assay variability of duplicates is generally <10%.

Terrestrial invertebrate collection

Common earthworms and/or other soil-dwelling invertebrate prey items of target songbird species will be collected for Pb analysis in the first year. Worms will be sampled at each site used in the present study by digging with trowels in the top 15 cm of soil. Five composite samples of invertebrates will be collected from each site (for a total of 25-30 samples) representing a range of soil concentrations and placed in plastic baggies and frozen prior to submission to the laboratory. Each sample will be a minimum of one

gram, but ideally as much as 10 grams, depending on availability. Samples will be affixed with a unique label and recorded in a chain-of-custody. Locational data and other field observations will be recorded in a field log. Prey samples will be lyophilized, homogenized, acid-digested, and analyzed for Pb concentrations using ICP-MS.

Soil Sampling

To assess lead exposure of target species within their approximate home range, soil samples will be collected during the breeding season. Approximately 25 soil samples will be collected in the first year per nesting site from the top 10 cm of soil from the area represented by the breeding season home range of any given species (generally one hectare or less). Samples will be analyzed using a portable X-ray Fluorescence meter, with 10% of samples being submitted to the U.S. Geological Survey-Columbia Environmental Research Center's (USGS-CERC) laboratory for confirmatory analyses. Soil samples will be analyzed for Pb, Zn, Cd, Co, Ni, and Cu. Soil samples will be affixed with a unique label, recorded in a chain of custody and hand-carried to the laboratory accompanied by the chain of custody form. Locational and other site specific information will be recorded in a field log.

Quality Assurance/Quality Control (QA/QC)

The chain-of-custody form will accompany all samples to their respective laboratories.

Good Laboratory Practices – We will depend on four main standard operating procedures for the field work: mist netting and baited traps, collecting blood, collecting, and dissecting birds. All soil samples submitted for chemical analyses will have a minimum of one duplicate sample for every ten samples collected. We will be utilizing three separate USGS laboratories for analyses:

1. U.S. Geological Survey-Columbia Environmental Research Center's (USGS-CERC) for metals analyses of soil, earthworms, and bird blood and tissues.
2. USGS Patuxent Wildlife Research Center (PWRC) for analysis of blood ALAD activity using standard operating procedures of the PWRC and measures of oxidative stress and damage indicators in liver; and
3. USGS National Wildlife Health Center for pathological analysis of tissues.

General QA/QC procedures of each lab are described below:

USGS-CERC

All analytical measurements performed at USGS-CERC will be completed in accordance with USGS-CERC's Quality Assurance Policy Plan (QAPP). Quality controls (QC) established at USGS-CERC for each analytical method (e.g., ICP-MS, acid digestion, etc.) will be adhered to during the contaminant analysis process. These controls ensure that sampling and handling procedures are appropriate and that analytical results are accurate and precise. Quality processes will include, but are not limited to, the following: laboratory replication; comparison and calibration against known reference materials; proper maintenance and calibration of equipment and instrumentation; second source calibration verification; accurate sample tracking and custody; assessments for interferences, matrix effects, and losses on

preparation; and other considerations of Good Laboratory Practice (GLP). All equipment used in these studies is routinely inspected and preventative maintenance actions are performed as needed. Homogenized samples not consumed by analyses will remain in storage in a dessicator under a nitrogen atmosphere or in a freezer for the duration of time specified by the project coordinator. Data and data packets will be reviewed for accuracy and completeness, uniquely labeled and cross-referenced, and will be stored at USGS-CERC until transfer to the National Archives in Kansas City, MO. Copies of the computerized data files will be maintained in the project file and ultimately archived on backup tape at USGS-CERC.

USGS-Patuxent

The delta-aminolevulinic acid dehydratase assay will be conducted in accordance with USGS-PWRC Standard Operating Procedure CTR-20 (copy attached). Analysis of IOS will be performed using methods validated at PWRC by determination of detection and reporting limits, assay sensitivity, working range, dilutional parallelism, spiking recovery (accuracy), intra-assay variability, and inter-assay variability. All assay plates will include blanks and two or more reference samples for monitoring inter-assay variability. Samples will be analyzed in duplicate and variability between duplicates maintained at <10%.

USGS-National Wildlife Health Center

Liver and kidney samples preserved in 10% neutral-buffered formalin will be processed into paraffin blocks and slides at Wisconsin Veterinary Diagnostic Laboratory in Madison, WI. Liver and kidney will be stained with hematoxylin and eosin stain and kidney will be stained with Fite's acid fast and Ziehl-Neelsen acid fast stains (see attached Standard Operating Procedures). A board-certified veterinary pathologist blinded to the origin and Pb status of the tissues will evaluate liver and kidney samples for microscopic evidence of lead toxicity.

Work Schedule: The study is scheduled to obtain bird reproduction data over three breeding seasons, ideally starting in March 2016. It is possible that three years of data will not be gathered at all sites, as case needs, access, and field conditions dictate.

Expected Products: At least one peer-reviewed paper and a report to the trustees.

Reporting Schedule: Preliminary data will be produced in February prior to the beginning of the ensuing field season. A draft final report will be produced in December 2018.

Records Management: An official archive for each study plan is maintained by the Research Manager and administrative staff. Samples will be collected under chain of custody and all records and correspondence will be preserved for the trustees. Due to the possibility of litigation, files will be kept indefinitely.

Data Analyses

All data compiled related to tissue and soil metal concentrations, ALAD inhibition, measures of oxidative damage and the rate of tissue pathologies observed, and reproductive metrics will be statistically compared to baseline conditions as determined by data collected from the reference site. In addition, data from previous rates of metals concentrations and ALAD from Beyer et al. 2013 observed in reference and study sites will provide advantageous comparisons. Soil and earthworm tissue data will be used to confirm exposure from a given site and model expected responses from habitats with similar soil concentrations.

Analysis of Reproductive Success

Individual nests will serve as the experimental unit and potential response variables are clutch size, number of young hatched, number of young fledged, and nest success (nests that fledge at least one nestling are considered successful). We will use linear models to relate the response variables to soil, parent, and nestling Pb levels. We will fit logistic-exposure models (Shaffer and Thompson 2007) to predict nest survival as a function of covariates and to calculate model-based estimates of nest survival. Generalized linear models will be fit with a Poisson or Gaussian distribution to predict clutch size, number of young hatched, and number of young fledged as a function of the covariates (Peak and Thompson 2014, Cox et al 2013). In addition we will include potential “nuisance” covariates to control for other temporal (e.g. year, day of year, nest age or stage) and habitat effects (e.g. percent forest cover in landscape, habitat type, nest height) so these are not confounded with the effects of the covariates of interest (Reidy et al. 2009, Cox et al 2013, Peak and Thompson 2014). We will fit models for all species combined but include a random intercept term to account for variation in responses by different bird species. An information-theoretic approach (Burnham and Anderson 2002) will be used to evaluate support for the main effects of interest when added to a model containing the nuisance covariates. These approaches will allow us to evaluate effect(s) of soil Pb on bird Pb levels and/ reproductive performance as well as demonstrate the change in the response variable across the range of observed Pb levels (Shaffer and Thompson 2007).

References

- Allert, AL., J.F. Fairchild, R.J. DiStefano, C.J. Schmitt, W.G. Brumbaugh, and J.M. Besser. 2008. Ecological effects of lead mining on Ozark streams: In-situ toxicity to woodland crayfish (*Orconectes hylas*). *Ecotoxicology and Environmental Safety* 72(4):1207-1219.
- Besser, J.M., W.G. Brumbaugh, D.K. Hardesty, J.P. Hughes, and C.G. Ingersoll. 2009. Assessment of metal-contaminated sediments from the Southeast Missouri (SEMO) mining district using sediment toxicity tests with amphipods and freshwater mussels. U.S. Geological Survey, Natural Resource Damage Assessment and Restoration Program Administrative Report 08-NRDAR-02.

- Beyer W. N. Connor EE, and Gerould S. 1994. Estimates of soil ingestion by wildlife. *J Wildl Manage* 58:375–382
- Beyer WN, Spann JW, Sileo L, Franson JC. 1988. Lead poisoning in six captive avian species. *Archives of Environmental Contamination and Toxicology* 17:121–130.
- Beyer WN, Franson, JC, French JB, May T, Rattner BA, Shearn-Bochsler, VI, Warner SE, Weber JS, Mosby DE. 2013. Toxic exposure of songbirds to lead in the Southeast Missouri Lead Mining District, *Archives of Environmental Contamination and Toxicology* 10.1007/s00244-013-9923-3.
- Beyer WN, Chen Y, Henry P, May T, Mosby D, Rattner BA, Shearn-Bochsler VI, Sprague D, Weber J. 2014. Toxicity of Pb-Contaminated soil to Japanese Quail (*Coturnix japonica*) and the use of the blood–dietary Pb slope in risk assessment. *Integrated Environmental Assessment and Management* 10: 22-29.
- Buekers J, Redeker ES, Smolders E. 2009. Lead toxicity to wildlife: Derivation of a critical blood concentration for wildlife monitoring based on literature data. *Science of the Total Environment* 407: 3431-3438.
- Burch H, Siegel A 1971. Improved method for measurement of delta-aminolevulinic acid dehydratase activity in human erythrocytes. *Clinical Chemistry* 17:1038-1041
- Burger J, Gochfeld M. 1997. Age differences in metals in the blood of herring (*Larus argentatus*) and Franklin's (*Larus pipixcan*) gulls. *Archives of Environmental Contamination and Toxicology* 33:436–440.
- Burnham KP, Anderson DR. 2002. *Model Selection and Multimodel Inference: A Practical Information-theoretic Approach*. Springer Verlag, New York, New York, USA.
- Carey M, Burhans DE, Nelson DA. 2008. Field Sparrow (*Spizella pusilla*), The Birds of North America Online (A. Poole, Ed.). Ithaca: Cornell Lab of Ornithology; Retrieved from the Birds of North America Online: <http://bna.birds.cornell.edu/bna/species/103>
- Cox WA, Thompson III FR, Reidy J, Faaborg J. 2013. Temperature can interact with landscape factors to affect songbird productivity. *Global Change Biology* doi: 10.1111/gcb.12117.
- Evans M, Gow E, Roth RR, Johnson MS, Underwood TJ. 2011. Wood Thrush (*Hylocichla mustelina*), The Birds of North America Online (A. Poole, Ed.). Ithaca: Cornell Lab of Ornithology; Retrieved from the Birds of North America Online:<http://bna.birds.cornell.edu/bna/species/246>
- Fair JM, Paul E, Jones J, Clark AB, Davie C, Kaiser G. 2010. Guidelines to the use of wild birds in research. The Ornithological Council, Washington, DC. http://www.nmnh.si.edu/BIRDNET/documents/guidelines/Guidelines_August2010.pdf

- Franson JC, Pain D. 2011. Lead in birds. In: Beyer WN, Meador J, editors, *Environmental contaminants in biota: Interpreting tissue concentrations*. Boca Raton (FL): CRC. p 563–594.
- Haig SM, D’Elia J, Eagles-Smith C, Fair JM, Gervais J, Herring G, Rivers JW, Schulz JH. 2014. The persistent problem of lead poisoning in birds from ammunition and fishing tackle. *Condor* 116: 408-428.
- Halkin SL, Linville SU. 1999. Northern Cardinal (*Cardinalis cardinalis*), The Birds of North America Online (A. Poole, Ed.). Ithaca: Cornell Lab of Ornithology; Retrieved from the Birds of North America Online: <http://bna.birds.cornell.edu/bna/species/440>
- Hansen J, Audet D, Spears B, Healy K, Brazzle R, Hoffman D, Dailey A, Beyer W. 2011. Lead exposure and poisoning of songbirds using the Coeur d’Alene River Basin, Idaho, USA. *Integrated Environmental Assessment and Management* 7:587–595.
- Johnson M, Wickwire W, Quinn M Jr, Ziolkowski D Jr, Burmistrov D, Menzie C, Geraghty C, Minnich M, Parsons P. 2007. Are songbirds at risk from lead at small arms ranges? An application of the spatially explicit exposure model. *Environmental Toxicology and Chemistry* 26:2215–2225.
- Kendrick SW, Porneluzi PA, Thompson III FR, Morris DL, Haslerig JM, Faaborg J. 2015. Stand-Level Bird Response to Experimental Forest Management in the Missouri Ozarks. *Journal of Wildlife Management* 79:50–59.
- Martinez-Haro M, Green AJ, Mateo R. 2011. Effects of lead exposure on oxidative stress biomarkers and plasma biochemistry in waterbirds in the field. *Environmental Research* 111: 530-538.
- Mattsson Brady J, Master TL, Mulvihill RS, Robinson WD. 2009. Louisiana Waterthrush (*Parkesia motacilla*), The Birds of North America Online (A. Poole, Ed.). Ithaca: Cornell Lab of Ornithology; Retrieved from the Birds of North America Online:<http://bna.birds.cornell.edu/bna/species/151>.
- McDonald MV. 2013. Kentucky Warbler (*Geothlypis formosa*), The Birds of North America Online (A. Poole, Ed.). Ithaca: Cornell Lab of Ornithology; Retrieved from the Birds of North America Online: <http://bna.birds.cornell.edu/bna/species/324>.
- Monaghan, P, Metcalfe, NB, Torres, R, 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecol. Lett* 12, 75-92.
- Niethammer, K.R., Atkinson, R.D., Baskett, T.S., and Samson F.B. 1985. Metals in riparian wildlife of the lead mining district of Southeast Missouri. *Arch. Environ. Contam. Toxicol.* 14, 213-223.
- Pain D 1987. Hematological parameters as predictors of blood lead and indicators of lead poisoning in the black duck. *Environmental Pollution* 60:67-81

- Payne, R.B. 2006. Indigo Bunting (*Passerina cyanea*), The Birds of North America Online (A. Poole, Ed.). Ithaca: Cornell Lab of Ornithology; Retrieved from the Birds of North America Online:<http://bna.birds.cornell.edu/bna/species/004>
- Peak RG, Thompson, III FR. 2014. Seasonal productivity and nest survival of Golden-cheeked Warblers vary with forest type and edge density. *Condor* 116:549-559.
- Porneluzi P, Van Horn MA, Donovan TM. 2011. Ovenbird (*Seiurus aurocapilla*), The Birds of North America Online (A. Poole, Ed.). Ithaca: Cornell Lab of Ornithology; Retrieved from the Birds of North America Online: <http://bna.birds.cornell.edu/bna/species/088>
- Reidy JL, Thompson III FR, Peak RG. 2009. Factors affecting Golden-cheeked Warbler Nest Survival in Urban and Rural Landscapes. *Journal of Wildlife Management* 73:407-413.
- Roux, K.E. and Marra, P.P. 2007. The presence and impact of environmental lead in passerine birds along an urban to rural land use gradient. *Arch. Environ. Contam. Toxicol.* 53, 261-268.
- Schmitt, C.J. 2007. Effects of Metals from Lead-Zinc Mining on Aquatic Organisms Inhabiting Streams Draining Mining Districts in Missouri, Kansas, and Oklahoma, 1980-2007. USGS. July 26.
- Selman, C, Blount, JD, Nussey, DH, Speakman, JR, 2012. Oxidative damage, ageing, and life-history evolution: where now? *Trends Ecol Evol* 27, 570-577.
- Shaffer TL, Thompson III FR. 2007. Making meaningful estimates of nest survival with model-based methods. *Studies in Avian Biology* 34:84-95.
- Sheldon LD, Chin EH, Gill SA, Schmaltz G, Newman AEM, Soma KK. 2008. Effects of blood collection on wild birds: an update. *Journal of Avian Biology* 39: 369-378.
- Stratus Consulting. 2014. Associating Soil Lead with Adverse Effects on Songbirds in the Southeast Missouri Mining District. June 2014.
- Struckhoff, M., E. Stroh, and K.W. Grabner. 2013. Effects of mining-associated lead and zinc soil contamination on native floristic quality. *Journal of Environmental Management* 119: 20-28.
- Vanderhoff N, Sallabanks R, James FC. 2014. American Robin (*Turdus migratorius*), The Birds of North America Online (A. Poole, Ed.). Ithaca: Cornell Lab of Ornithology; Retrieved from the Birds of North America Online: <http://bna.birds.cornell.edu/bna/species/462>