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Biomarkers of metals exposure in fish from lead-zinc mining areas of Southeastern Missouri, USA

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Abstract

The potential effects of proposed lead-zinc mining in an ecologically sensitive area were assessed by studying a nearby mining district that has been exploited for about 30 y under contemporary environmental regulations and with modern technology. Blood and liver samples representing fish of three species (largescale stoneroller, *Campostoma oligolepis*, $n = 91$; longear sunfish, *Lepomis megalotis*, $n = 105$; and northern hog sucker, *Hypentelium nigricans*, $n = 20$) from 16 sites representing a range of conditions relative to mining activities were collected. Samples were analyzed for metals (also reported in a companion paper) and for biomarkers of metals exposure [erythrocyte δ-aminolevulinic acid dehydratase (ALA-D) activity; concentrations of zinc protoporphyrin (ZPP), iron, and hemoglobin (Hb) in blood; and hepatic metallothionein (MT) gene expression and lipid peroxidation]. Blood lead concentrations were significantly higher and ALA-D activity significantly lower in all species at sites nearest to active lead-zinc mines and in a stream contaminated by historical mining than at reference or downstream sites. ALA-D activity was also negatively correlated with blood lead concentrations in all three species but not with other metals. Iron and Hb concentrations were positively correlated in all three species, but were not correlated with any other metals in blood or liver in any species. MT gene expression was positively correlated with liver zinc concentrations, but neither MT nor lipid peroxidase differences among fish grouped according to lead concentrations were statistically significant. ZPP was not detected by hematofluorometry in most fish, but fish with detectable ZPP were from sites affected by mining. Collectively, these results confirm that metals are released to streams from active lead-zinc mining sites and are accumulated by fish.
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1. Introduction

The extensive lead (Pb) deposits of southern Missouri were first discovered by early French explorers of the Mississippi River valley. Subsequently discovered ores containing variable amounts of Pb, zinc (Zn), copper (Cu), cadmium (Cd), cobalt (Co), nickel (Ni), silver, and other metals have been mined at varying levels of intensity for more than 300 y. Mining currently occurs only in the "New Lead Belt" (NLB), which exploits a geologic formation known as Viburnum Trend. In contrast to previous large-scale Pb–Zn mining, the NLB was developed during the 1960s and has operated under environmental regulations and with the most efficient extraction technologies available (Jennett and Callier, 1977; Wixson and Jennett, 1975; Wixson, 1978). By the 1980s, two decades of exploitation had depleted the Viburnum Trend and mining activity in the NLB declined. Continuing

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exploration revealed additional potentially exploitable deposits southwest of the Viburnum Trend, within the boundaries of the Mark Twain National Forest. This area is in an environmentally sensitive part of southern Missouri that hosts recreationally significant aquatic resources including springs, caves, natural areas, a National Park, and a Federally designated Scenic River that could be threatened by mining. In response to public concerns, a multi-year interdisciplinary investigation was initiated to evaluate potential environmental consequences of expanded Pb-Zn mining in the exploration area (Imes, 2002). The study described here, which represents part of the larger investigation, was focused in the NLB as a model for the potential consequences of expanded mining elsewhere in southern Missouri.

The primary objective of our study was to document metals concentrations and biomarkers of metals exposure in fish from streams representing a wide range of conditions related to contemporary Pb–Zn mining in the Missouri Ozarks. This was achieved by collecting blood and liver samples of fish from the exploration area and other reference areas and from sites located at various distances from NLB mines, analyzing them for metals and biomarkers of metals exposure, and comparing the results to pertinent data from the scientific literature. Secondary objectives included the refinement of biomarker methods for use with small fish and the documentation of metals concentrations and biomarker responses in a species for which they had not been evaluated previously. The use of small fish was necessary because many NLB mines and there associated mills and tailings disposal facilities are located near headwater streams that are too small to support large fish in the numbers necessary for metals and biomarker analyses by traditional methods (e.g., Schmitt et al., 1984, 1993). The metals analyses, which are reported in a companion paper (Schmitt et al., 2007), indicated that blood and liver Pb concentrations were elevated in fish from sites near NLB mines, but that mining-related trends in the concentrations of Cd and Zn were less evident. Elevated blood and liver concentrations of Co and Ni in fish from one site in the NLB were also reported. In this paper we report the results of the biomarker analyses performed on the fish analyzed for metals by Schmitt et al. (2007).

2. Methods of study

2.1. Study design

Fish of two species, largescale stoneroller (*Campostoma oligolepis;* henceforth stoneroller) and longear sunfish (*Lepomis megalotis;* sunfish), were collected from each site. Northern hog sucker (*Hypentelium nigricans;* hog sucker), a species collected in previous studies (Schmitt et al., 1984, 1993), were retained for analysis when captured incidentally to facilitate temporal and inter-species comparisons. Sites 1–14, in the Black and Meramec River systems, were selected to represent the range of conditions present in the NLB relative to mining; they included reference sites ($n = 3$) located upstream of all mining activity and sites situated 1.4–49.8 km downstream of NLB mines and ore processing facilities (Schmitt et al., 2007). Sunfish were not present in the immediate vicinity of Sites 6 and 14 but were obtained 2–5 km downstream of the other species (Schmitt et al., 2007). A site on the Eleven Point River (Site 15) was selected to represent existing conditions in the exploration area and also served as an additional reference site. The Big River (Site 16), which has been contaminated by mine tailings from historical mining in the Old Lead Belt (e.g., Gale et al., 2004; Schmitt et al., 1984), was also sampled. Sites 2, 5, 9, 14, and 16 were sampled for blood metals and biomarkers by previous studies (Dwyer et al., 1988; Schmitt et al., 1984, 1993, 2005) and were used for temporal and inter-species comparisons. All sites were sampled in September 2001 except Site 16, which was sampled in early December 2001. More complete descriptions of the study area and the fish collection sites are presented by Schmitt et al. (2007).

2.2. Biomarkers

Oxidative damage in hepatic tissues, which can result from exposure to a variety of metals (Farag et al., 1995), was assessed by measuring fluorescent products of lipid peroxidation (FPLPs). The FPLPs represent the interaction of polyunsaturated fatty acid peroxidation products with phospholipids and amino groups of membrane proteins (Dillard and Tappel, 1984). They tend to be long-lived and remain at the sites of oxidative damage (Mezzetti et al., 1999). The FPLPs react with proteins to alter the structure of membranes and form fluorophores, which were measured fluorometrically (Farag et al., 1995).

Metallothioneins (MTs) are ubiquitous low molecular weight proteins and polypeptides of extremely high metal and sulfur content that are involved in the intracellular fixation and regulation of the essential trace elements Zn and Cu. They can also mitigate the harmful effects of toxic metals such as Pb, Cd, and mercury (Kägi and Schäffer, 1988). The induction of MT synthesis, which we quantified by measuring MT mRNA expression with reverse-transcriptase polymerase chain reaction (RT-PCR), represents a sensitive biomarker of metals exposure (Tom et al., 2004).

The biochemical effects of Pb were evaluated at several points in the heme biosynthetic pathway; we measured the activity of the enzyme δ-aminolevulinic acid dehydratase (ALA-D; EC 4.2.1.24) and concentrations of hemoglobin (Hb), iron (Fe), and zinc protoporphyrin (ZPP) in blood. The condensation of two molecules of δ-aminolevulinic acid (ALA) to one molecule of porphobilinogen (PBG), which is catalyzed by ALA-D, occurs early in the heme biosynthetic pathway. Lead inhibits ALA-D stoichiometrically (Chisolm et al., 1985; Kelada et al., 2001) by displacing Zn at the metal binding site (Warren et al., 1998), which inhibits the enzyme by changing its quaternary structure. Erythrocyte ALA-D inhibition is a well documented biomarker of Pb exposure in many vertebrates, including humans (e.g., Blus et al., 1991; Goldstein et al., 1975; Hodson, 1976; Schmitt et al., 2005). The terminal step in the heme biosynthetic pathway is the insertion of Fe^{2+} into protoporphyrin IX, which is catalyzed by the mitochondrial enzyme heme synthetase (ferrochelatase, EC 4.99.1.1; Joselow, 1980). Ferrochelatase inhibition causes the accumulation of protoporphyrin IX, which may also result from Pb-impaired Fe delivery or utilization (Joselow, 1980; Labbé et al., 1999; Sakai, 2000). Protoporphyrin IX chelates free Zn^{2+} to become zinc protoporphyrin (ZPP), which can be measured by hematofluorometry (Labbé et al., 1999; Lee et al., 2001; Gurer-Orhan et al., 2004). Although widely used in human medicine, the measurement of ZPP and other porphyrins as a consequence of Pb exposure in fish has received little study (e.g., Hodson et al., 1984; Theodakoris et al., 1992). Heme homeostasis can also be affected by other metals present in the ores of the NLB, including Cd, Ni, Cu, and Co (e.g., Maines, 1980; Maines and Sinclair, 1977; Taylor, 1990).

2.3. ZPP pilot studies

We conducted pilot studies with laboratory-raised fish of several species to determine whether ZPP could be measured by hematofluorometry and to optimize field and laboratory procedures. In the first study (Study 1), ZPP was measured in preserved erythrocytes of common carp (*Cyprinus*

carpio, $n = 3$) and bluegill (*Lepomis macrochirus*, $n = 6$). Blood (1–3 mL) was obtained by caudal veinipuncture with a heparinized (6 IU/μL) needle and syringe and transferred immediately to chilled heparinized Vacutainers® (BD Diagnostics, Franklin Lakes, NJ), chilled on ice, then refrigerated (4 °C). Erythrocytes were preserved within 8 h as follows: samples were centrifuged for 2 min @3500 rpm, the supernatants were aspirated, and a 1:1 volume of saline solution (9 g/L NaCl) was added. The erythrocytes were re-suspended by gentle rocking and the procedure was repeated three times. After aspiration of the final saline wash, an equal volume of 40% glycerol solution in citrate buffer was added. The preserved samples were analyzed with three different hematofluorometers to evaluate consistency among instruments.

In the second study (Study 2), whole blood (1–3 mL) was obtained as described from bluegill ($n = 10$) and lake sturgeon (*Acipenser oxyrynchus*, $n = 16$), transferred to heparinized Vacutainers, and refrigerated (4 °C). Samples were analyzed by hematofluorometry over 7 d to evaluate the effects of storage time.

2.4. Field procedures

Most fish were collected by DC electrofishing and by seining. Sunfish were also collected by hook-and-line. The collection target was six (each) adult stoneroller (75–150 mm total length, 3–20 g) and juvenile sunfish (100–150 mm, 30–60 g) samples at each site. Hog sucker were larger (100–150 mm, 40–650 g). Most fish were analyzed individually; however, because the sunfish and stoneroller at several sites were small, some were analyzed as composites with a "sample" representing the number of fish (typically 1–3) necessary to provide approximately 400 μL of blood. All fish were held alive in plastic containers filled with aerated stream water for ⩽2 h following capture.

Blood was collected from stoneroller and sunfish with a chilled (0 °C), heparinized microcapillary tube (75 μL for stoneroller and small sunfish, 370 μL for larger sunfish) after severing the caudal peduncle with a razor blade. Blood was obtained from hog sucker by caudal veinipuncture using a heparinized (6 IU/mL) needle and syringe. The blood was dispensed immediately into a chilled 2-mL cryogenic tube from which 200 μL was transferred with a heparinized microcapillary tube to a pre-weighed, acid-cleaned 10-mL borosilicate glass tube with a Teflon® cap, which was then frozen immediately in dry ice for metals analysis (including Fe). One drop was next dispensed onto each of two clean pieces of Parafilm M® (American National Can, Menasha, WI) and analyzed immediately for Hb with a HemoCue® (HemoCue AB, Ängelholm, Sweden) portable blood photometer (Schmitt et al., 2005). Replicate field measurements of Hb were consistent in all three species; coefficients of variation (CVs) were ⩽10% in 89 of 105 (85%) sunfish, 88 of 94 (94%) stoneroller, and 19 of 20 (95%) hog sucker. Hb is therefore reported as the mean of the two observations for each sample (g/dL).

The remaining blood was dispensed into a 5-mL cryogenic vial and chilled (0 °C, for ZPP and ALA-D analysis). Following blood collection the fish was euthanized by cervical dislocation, measured (total length, mm), and weighed (g). The abdominal cavity was opened by dissection and a liver sample (ca. 0.5 g) was obtained and divided into three approximately equal sub-samples for metals and biomarker analyses. These samples were placed in acid-cleaned 0.5-mL cryogenic vials and frozen immediately in dry ice. All contact surfaces and dissecting instruments were thoroughly cleaned with laboratory detergent, de-ionized H_2O, and acetone between samples. At the end of each day the chilled blood samples were assayed for ZPP, then frozen immediately in dry ice. Upon return from the field the metals samples were stored frozen at −20 °C and the biomarker samples at −80 °C until analyzed.

2.5. Laboratory methods

2.5.1. Erythrocyte zinc protoporphyrin (ZPP)

ZPP in whole blood and preserved erythrocytes was measured directly in units of μmol ZPP/mol heme (μmol/mol) based on fluorescence (415 nm excitation, 596 nm emission) using one of several Aviv Model 206D hematofluorometers (Aviv Instrument, Lakewood, NJ). The instruments were calibrated at the beginning and end of each measurement session using procedures and reference materials obtained from the manufacturer (Aviv Biomedical, 2001). One drop (20 μL) of whole blood or preserved erythrocytes was placed on a glass coverslip (25 mm^2) with a clean polyethylene pipette tip and stirred vigorously for 10 s. ZPP was measured 3–6 times at 60-s intervals, with 10 s of stirring between measurements. Duplicate coverslips were prepared and read from at least 10% of each group of samples; the unweighted mean for each fish is reported. The limit of detection (LOD) was 5.0×10^{-12} g ZPP per drop of blood (nominally 1 μmol/mol; Aviv Biomedical, 2001).

2.5.2. ALA-D activity

Erythrocyte ALA-D activity in 25-μL subsamples of homogenized whole blood was assayed in 96-well microtiter plates as described by Schmitt et al. (2005). Each sample was analyzed in triplicate; the arithmetic mean of the three observations is reported. For quality control (QC) purposes one triplicate sample per plate was randomly selected for duplicate analysis in a separate location on the plate. Concentrations of Hb in each well were also measured with the HemoCue as described previously. Enzyme activity in each well was computed as nmol PBG/μL blood/h using the sample absorbance reading and the parameters (slope and y-intercept) from the regression of the PBG standard curve. Mean ALA-D activity was also standardized to both field- and laboratory-determined Hb concentrations and reported as nmol PBG/mg Hb/h. The LODs and limits of quantitation (LOQs) for the method were calculated as described by Keith et al. (1983) using the daily assay method blanks. The LODs and LOQs were consistent among plates; the mean LOD was 0.043 ± 0.001 (SD) absorbance units and the mean LOQ was 0.053 ± 0.002 absorbance units. The ALA-D measurements were also very repeatable; >95% of the triplicate (well) measurements made on each sample had CVs of <20%, and duplicate samples had CVs of <10% in all three species.

2.5.3. Hepatic lipid peroxidation

A total of 30 liver samples (15 each stoneroller and sunfish) were analyzed for FPLPs with a fluorometric assay modified from Farag et al. (1995) and Dillard and Tappel (1984). Five fish of each species representing "low", "medium", and "high" blood Pb concentrations were selected (low = 0.02–0.07 μg/g dw in sunfish, 0.02–0.05 μg/g dw in stoneroller, from Sites 3, 10, and 15; medium = 0.25–0.44 μg/g dw in sunfish, 0.15–0.26 μg/g dw in stoneroller, from Sites 1, 5, and 6; high = 1.42–3.80 μg/g dw in sunfish, 1.65–3.30 μg/g dw in stoneroller, from Sites 14 and 16). Approximately 200 mg of frozen liver tissue from each fish was homogenized in 0.5 mL of ice-cold dH_2O at medium speed for 15 s in an Omni tissue homogenizer (Omni International, Marietta, GA). The homogenate and a 0.5 mL ice-cold dH_2O rinse were transferred to a 15-mL glass centrifuge tube, 2 mL of 2:1 chloroform:methanol were added, and the mixture was vortexed for 1 min. Ice-cold dH_2O (1 mL) was then added and the sample was vortexed and centrifuged (2 min at $1200 \times g$). Chloroform was removed, 100 μL of methanol was added, and the sample was again vortexed. Samples were irradiated at 250 nm in a UV light box for 5 min to remove the fluorescence contribution of compounds such as retinol. Aliquots (200-μL) of each sample were analyzed in triplicate in 96-well plates (360 nm excitation; 460 nm emission) with a Cytofluor® Model 2300 (Millipore, Billerica, MA). A quinine sulfate standard curve was prepared (0.000–0.002 μg/mL quinine sulfate) for each set of samples for standardization of relative fluorescence values per unit of tissue mass. The error associated with the slope of the routine quinine sulfate standard curves was <1% and CVs for procedural blanks averaged ⩽5% for the duration of the study. The LODs and LOQs for the method, calculated as described for ALA-D, were consistent among plates; the mean LOD was 37.5 ± 2.27 (SD) fluorescence units, and the mean LOQ was 44.9 ± 5.72 fluorescence units. Hepatic lipid peroxidation, as indicated by relative fluorescence, exceeded the method LOD and LOQ in all samples analyzed, and >97% of the triplicate (wells) measurements made on each sample had an associated CV of <5%.

2.5.4. Metallothionein (MT) expression

Hepatic MT mRNA was measured in stoneroller liver samples from a reference site (Site 10, Sinking Creek; $n = 6$) and Site 16 (Big River, $n = 6$). Samples were analyzed by RT-PCR using the methods of McClain et al. (2003) and Roberts and Oris (2004) with DNA primers synthesized from fathead minnow (*Pimephales promelas*). Total oligonucleotide was isolated by acid guanidinium thiocyanate phenol-chloroform extraction using Tri-Reagent (Sigma, St. Louis, MO, USA; manufacturer's protocol) and was quantified spectrophotometrically at 260 nm. Reverse transcription was carried out using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia, Uppsala, Sweden). The PCR reactions contained 25 μL total volume [5 μL of reverse transcription product, 2.5 μL of 10X PCR Buffer, 1 μL of 5 mM DNTPs, 0.5 μL of 15 μM oligonucleotide primer, 1 μL of Taq polymerase (2.5 units), and 15 μL of DNAase-free water]. Amplification occurred over 24 cycles (94 °C for 30 s, 55.5 °C for 30 s, and 72 °C for 30 s). Amplification products were electrophoresed in 1.5% LE Agarose (Fisher Biotech, Fairlawn, NJ) and stained with ethidium bromide for visualization and quantitation using Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD). The optical density of each sample's MT band was obtained and standardized on a sample-by-sample basis to the optical density of an 18S rRNA band using commercially available primers (Ambion, Austin, TX). The ratio of MT optical density to 18S optical density was used as a measure of relative gene expression.

2.5.5. Blood moisture content and Fe

Frozen blood samples were freeze-dried, digested with HNO_3 and H_2O_2, and analyzed for Fe by inductively coupled plasma mass spectrometry (ICPMS) as described by Schmitt et al. (2005, 2007). Moisture content was determined from weight loss during lyophilization. Iron was analyzed and reported as both dry-weight (dw) and wet-weight (ww) concentrations, the latter computed from the moisture content of each sample. The LODs for Fe, calculated assuming a digestion weight of 50 mg of dry blood, were 1.0–40 μg/g dw.

2.6. Data set composition and statistical analyses

Release 9.1 of the Statistical Analysis System (SAS Institute, Cary, NC) was used for all statistical analyses. A total of 219 samples were included in the univariate analyses; 94 samples representing all 16 stations were stoneroller (only one sample was obtained from Site 7), 105 samples from 16 sites were sunfish, and 20 samples from Sites 4, 7, 8, 11–13, and 16 were hog sucker. Several samples could not be analyzed for Hb or ALA-D due to clotting, and three sunfish samples representing Sites 1 and 5 were excluded from regression and correlation analyses because of suspiciously high blood or liver Zn concentrations indicative of possible external contamination (Schmitt et al., 2007). Both dw and ww blood Fe concentrations were analyzed for differences among sites and species, but only the ww concentrations were used in correlation and regression analyses because of the association of Fe with Hb, the latter determined in the field on whole blood. Censored values (<LOD) were replaced with 50% of the LOD for statistical computations and graphing, and all data representing elemental and Hb concentrations and ALA-D activity were $\log_{10}$-transformed prior to statistical analysis. Individual variables were analyzed statistically using analysis-of-covariance (ANCOVA) and analysis-of-variance (ANOVA). In these analyses, differences among sites were tested within each species with Fisher's protected LSD test. Sites were also grouped according to their distance from mining (Schmitt et al., 2007): "reference" (no upstream mining-Sites 3, 10, 11, and 15); "near mine" (<10 km downstream from an NLB mine-Sites 2, 4, 6, 7, 12, and 14); and "downstream" (>10 km downstream from an NLB mine-Sites 1, 5, 8, 9, and 13). Differences among groups of sites were tested as planned non-orthogonal contrasts using single degree-of-freedom *F*-tests. Results of the ZPP pilot studies were analyzed by ANCOVA and mixed-model ANOVA to evaluate the effects of fish species, instruments, individual fish, replicate samples, and storage time on ZPP concentrations. The biomarker data reported here were combined with the metals data reported by Schmitt et al. (2007) and examined statistically with Pearson correlation coefficients, simple linear (least-squares) regression, and stepwise multiple linear regression. In the latter, the forward selection method was used and variables were allowed into the models only if they significantly ($P<0.05$) reduced the unexplained sum-of-squares after accounting for all other factors already included (i.e., the Type-II sums-of-squares were used). Molar concentrations of Hb ([Hb]) and Fe ([Fe]) in blood were computed on the basis of the approximate molecular weight of common carp Hb (65.88 kD; Grujic-Injac et al., 1980) and the atomic weight of Fe (55.85) and analyzed using simple linear regression and geometric mean (functional) regression (Ricker, 1973).

3. Results

Blood Pb concentrations were significantly greater in fish of all three species from sites located downstream of mines than at reference sites (Table 1). Concentrations in all three species were greatest at Site 16 (Big River) and at sites located nearest to NLB mines (Table 1). Concentrations of Pb in blood and liver were highly correlated in all three species (Schmitt et al., 2007). Concentrations of Cd in blood and liver also differed significantly among sites, but mining-related trends were less apparent than for Pb, and Zn differences were even less apparent (Schmitt et al., 2007). The biomarker results reflected the differing metals concentrations.

3.1. ALA-D activity

Among-species differences in un-standardized ALA-D activity were not statistically significant ($P>0.05$) when considered across all sites, nor were differences in ALA-D activity standardized to either field- or laboratory-measured Hb (ALA-D/Hb; data not shown). However, significant

Table 1
Activity of the enzyme δ-aminolevulinic acid dehydratase [ALA-D, nm porphobilinogen (PBG)/mL blood/h], ALA-D activity normalized to laboratory-determined hemoglobin (Hb) concentrations (nm PBG/mg Hb/h), field-measured Hb concentrations (g/dL), and concentrations of lead (Pb, μg/g dry weight) and iron (Fe, μg/g wet weight) in the blood of three fish species

Species, site, and type[a]	*n*/df	Blood Pb	ALA-D	ALA-D/Hb	Hb	Blood Fe
Hog sucker[b]						
4 (Bee Fork; M)	2	2.14±0.82 b	1.7±0.1 a	2.6±0.4 cde	9.1±0.5 a	293±3 a
7 (Sweetwater Creek; M)	2	3.20±1.90 b	1.2±0.2 a	3.8±0.1 a	5.5±1.0 ab	135±27 bc
12 (Strother Creek; M)	3	1.37±0.10 b	1.3±0.2 a	2.4±0.1 ed	7.4±0.2 a	217±7 ab
8 (Black R. @ Lesterville; D)	4	0.20± <0.01 c	1.3±0.1 a	2.9±0.3 bcd	8.8±1.0 a	250±34 a
13 (Neals Creek; D)	3	0.36±0.03 c	1.5±0.2 a	3.4±0.2 ab	7.7±0.8 a	232±16 ab

Table 1 (*continued*)

Species, site, and type[a]	*n*/df	Blood Pb	ALA-D	ALA-D/Hb	Hb	Blood Fe
11 (Middle Fork @ Redmondville; R)	3	0.28±0.04 c	1.8±0.3 a	3.4±0.1 abc	8.3±0.5 a	241±14 a
16 (Big R.)	3	6.63±0.87 a	0.6±0.2 b	2.2±0.1 e	4.1±1.3 b	125±37 c
ANOVA-*F*	6, 13[c]	44.00 **	4.73 **	5.70 **	2.98 *	3.43 *
R^2	20	0.95	0.69	0.72	0.58	0.61
Sunfish[d]						
2 (West Fork @ West Fork; M)	10	0.44±0.05 c	0.9±0.1f fg	2.0±0.2 g	7.1±0.4 abcd	172±9 abcde
4 (Bee Fork; M)	8	1.13±0.90 b	0.8±0.1 g	1.9±0.1 g	6.2±0.3 def	175±9 abcde
6 (Logan Creek @ Corridon; M)	6	0.32±0.04 de	1.4±0.1 abc	3.1±0.1 bc	7.6±0.2 a	167±9 cde
7 (Sweetwater Creek; M)	6	1.32±0.30 b	1.1±0.1 cdef	2.5±0.2 def	7.5±0.4 ab	165±17 de
12 (Strother Creek; M)	6	0.62±0.14 c	0.8±0.1 fg	2.3±0.3 efg	5.2±0.3 g	157±8 de
14 (Courtois Creek; M)	6	0.64±0.19 cf	1.0±0.1 efg	2.7±0.2 cde	6.1±0.3 def	178±7 abcd
1 (Black R. @ Sutton Bluff; D)	7	0.16±0.02 f	0.9±0.1 efg	2.0±0.1 fg	6.2±0.2 cdef	173±5 abcde
5 (Logan Creek @ Ellington; D)	6	0.39±0.10 cde	1.4±0.2 abcd	3.1±0.2 bcd	7.2±0.4 abc	175±11 abcde
8 (Black R. @ Lesterville; D)	6	0.09±0.10 g	1.6±0.2 abc	3.5±0.2 ab	6.4±0.3 cde	197±3 ab
9 (Middle Fork @ Black; D)	6	0.17±0.03 f	1.6±0.1 ab	3.6±0.3 ab	6.0±0.3 efg	172±11 abcde
13 (Neals Creek; D)	6	0.18±0.04 f	1.1±0.1 defg	2.6±0.3 cdef	6.2±0.4 cdef	194±8 abc
3 (West Fork @ Greeley; R)	6	0.09±0.02 g	1.1±0.2 efg	2.8±0.2 cde	6.0±0.3 efg	152±8 e
10 (Sinking Creek; R)	7	0.09±0.03 g	1.2±0.1 bcde	2.6±0.2 cde	6.5±0.2 bcde	201±9 a
11 (Middle Fork @ Redmondville; R)	6[e]	0.38±0.07 cde	1.0±0.1 defg	3.1±0.4 bcd	5.4±0.3 gf	170±8 bcde
15 (Eleven Point R.; R/EA)	6	0.24±0.04 ef	2.0±0.3 a	4.0±0.1 a	6.3±0.2 cde	194±8 abc
16 (Big R.)	7	2.65±0.33 a	0.8±0.1 g	2.1±0.2 fg	5.7±0.4 efg	171±10 abcde
ANOVA-*F*	15, 89[c,f]	29.56 **	6.09 **	8.34 **	4.50 **	1.97 *
R^2	105[f]	0.83	0.51	0.59	0.43	0.25
Stoneroller[g]						
2 (West Fork @ West Fork; M)	7	0.33±0.02 efg	1.0±0.1 bc	2.0±0.2 bcd	8.0±0.4 abc	229±9 bc
4 (Bee Fork; M)	6[h]	0.98±0.08 bc	1.0±0.1 ab	1.9±0.1 bcde	8.0±0.2 ab	265±9 ab
6 (Logan Creek @ Corridon; M)	3[i]	0.75±0.11 bcd	1.0±0.1 ab	2.2±0.1 abcd	6.6±0.3 abcd	223±11 c
7 (Sweetwater Creek; M)	1[j]	na[k]	0.7 bcde	1.7 de	2.3 e	na[k]
12 (Strother Creek; M)	6	0.62±0.05 cd	0.6±0.2 e	1.6±0.3 e	7.5±0.2 abc	219±11 c
14 (Courtois Creek; M)	6[l]	2.25±0.33 a	0.6±0.1 cde	1.7±0.1 de	7.5±0.5 abc	220±11 c
1 (West Fork @ Sutton Bluff; D)	7	0.19±0.01 gh	1.0±0.1 ab	2.3±0.1 abc	6.5±0.2 bcd	203±8 c
5 (Logan Creek @ Ellington; D)	6	0.12±0.02 ij	1.7±0.1 a	2.8±0.2 a	6.7±0.4 abcd	215±7 c
8 (Black R. @ Lesterville; D)	6	0.15±0.02 hi	1.1±0.1 ab	1.9±0.1 cde	8.0±0.5 abc	231±13 bc
9 (Middle Fork @ Black; D)	6	0.30±0.05 fg	1.0±0.2 bcd	2.2±0.2 abcd	5.9±0.5 d	207±21 c
13 (Neals Creek; D)	6	0.52±0.06 de	0.5±0.1 de	1.7±0.1 de	6.4±0.5 cd	202±8 c
3 (West Fork @ West Fork; R)	6	0.12±0.04 ij	1.0±0.1 b	2.2±0.1 abcd	7.1±0.2 abcd	218±11 c
10 (Sinking Creek; R)	7	0.03±0.01 k	0.9±0.1 bcd	2.5±0.1 ab	7.8±0.4 abc	214±7 c
11 (Middle Fork @ Redmondville; R)	6	0.35±0.02 ef	0.9±0.2 bcde	2.1±0.1 abcd	7.3±0.5 abcd	219±11 c
15 (Eleven Point R.; R/EA)	6	0.12±0.05 j	1.3±0.3 ab	2.3±0.1 abc	7.8±0.6 abc	286±19 a
16 (Big R.)	7[m]	1.39±0.25 ab	1.1±0.2 ab	2.0±0.3 cde	8.5±0.8 a	270±12 a
ANOVA-*F*	15, 73[c,n]	37.43 **	2.94 **	3.23 **	4.91 **	4.10 **
R^2	89[o]	0.87	0.38	0.40	0.49	0.43

Shown are arithmetic site means±SE, number of observations (*n*), and results of one-way analysis-of-variance (ANOVA) as *F*-values (** $P<0.01$, * $P<0.05$, ns $P>0.05$), degrees-of-freedom (df), and coefficients of determination (R^2). Within species, means followed by the same letter (ranked alphabetically from highest to lowest) are not significantly different ($P>0.05$). Blood Pb data from Schmitt et al. (2007).

[a]M, <10 km downstream of New Lead Belt mine; D, >10 km downstream; R, upstream of all mining (reference); EA, exploration area.

[b]Northern hog sucker, *Hypentelium nigricans*.

[c]df.

[d]Longear sunfish, *Lepomis megalotis*.

[e]$n=5$ for ALA-D and ALA-D/Hb.

[f]$n=104$, df = 15, 88 for ALA-D and ALA-D/Hb.

[g]Largescale stoneroller, *Campostoma oligolepis*.

[h]$n=7$ for ALA-D, ALA-D/Hb, and Hb.

[i]$n=4$ for ALA-D, ALA-D/Hb, and Hb.

[j]$n=0$ for blood Pb and Fe; nd = not determined.

[k]na = not analyzed.

[l]$n=5$ for ALA-D and ALA-D/Hb.

[m]$n=6$ for ALA-D and ALA-D/Hb.

[n]df = 15, 78 for Hb and 14, 76 for blood Pb and Fe.

[o]$n=84$ for Hb, 91 for blood Fe.

species × site interaction for ALA-D indicated among-species differences at some sites. Overall trends for ALA-D activity standardized to field- and laboratory-measured Hb were similar, but the laboratory-standardized values were slightly less variable. Consequently, ANCOVA explained 69% of the total variability in the laboratory-standardized values and 66% in the field-standardized values. We therefore report only the un-standardized (ALA-D) and laboratory Hb-standardized (ALA-D/Hb) values.

Both ALA-D and ALA-D/Hb differed significantly ($P<0.01$) among sites in all three species (Table 1). In hog sucker, ALA-D was significantly ($P<0.05$) lower at Site 16 (Big River) than at all others, but the other sites did not differ significantly from each other (Table 1). Among-site differences in hog sucker were more evident for ALA-D/Hb; Site 16 was lowest, but activity at Site 7 (Sweetwater Creek), which is 2.6 km downstream from a mine (Schmitt et al., 2007), was among the greatest (Table 1). Consequently, differences between sites <10 km downstream from NLB mines, >10 km downstream, and reference sites were not significant ($P>0.05$) in hog sucker, but were significant ($P<0.01$) in sunfish and stoneroller. In general, and although there were exceptions and overlap within groups of sites, ALA-D activity was typically greatest at reference sites (including Site 15, in the exploration area) and at sites >10 km downstream of NLB mines. Conversely, activity was generally lowest at sites near NLB mines and at Site 16 (Big River; Table 1).

ALA-D activity was negatively correlated with blood Pb in all three species; statistically significant ($P\leqslant 0.01$) log-log linear regressions explained 31% of the variation in un-standardized ALA-D activity in hog sucker, 7% in stoneroller, and 13% in sunfish (Fig. 1). Fish of all three species from sites near NLB mines and the Big River had greater blood Pb concentrations and less ALA-D activity than those from reference sites and sites >10 km downstream from mines, which had lower blood Pb concentrations and greater ALA-D activity (Fig. 1).

Multiple regression explained a greater percentage of the variation in ALA-D relationships than simple linear regression in all three species. Models that included Hb, either as an independent variable or by incorporating ALA-D/Hb as the dependent variable, and other variables such as blood Zn, blood Cd, and blood Fe in addition to blood Pb were statistically significant ($P<0.01$) and explained as much as 96% of the variation in hog sucker, 51% in sunfish, and 86% in stoneroller (Table 2). The models for hog sucker also contained negative terms for total length, indicating a decline in ALA-D activity with fish size (and also increasing blood Pb); however, the sample size for hog sucker was small relative to the number of statistically significant terms in the model (Table 2).

3.2. Blood Fe and hemoglobin

Concentrations of Hb were 1.4–11.7 g/dL in hog sucker, 4.1–8.8 g/dL in sunfish, and 0.8–6.0 g/dL in stoneroller.

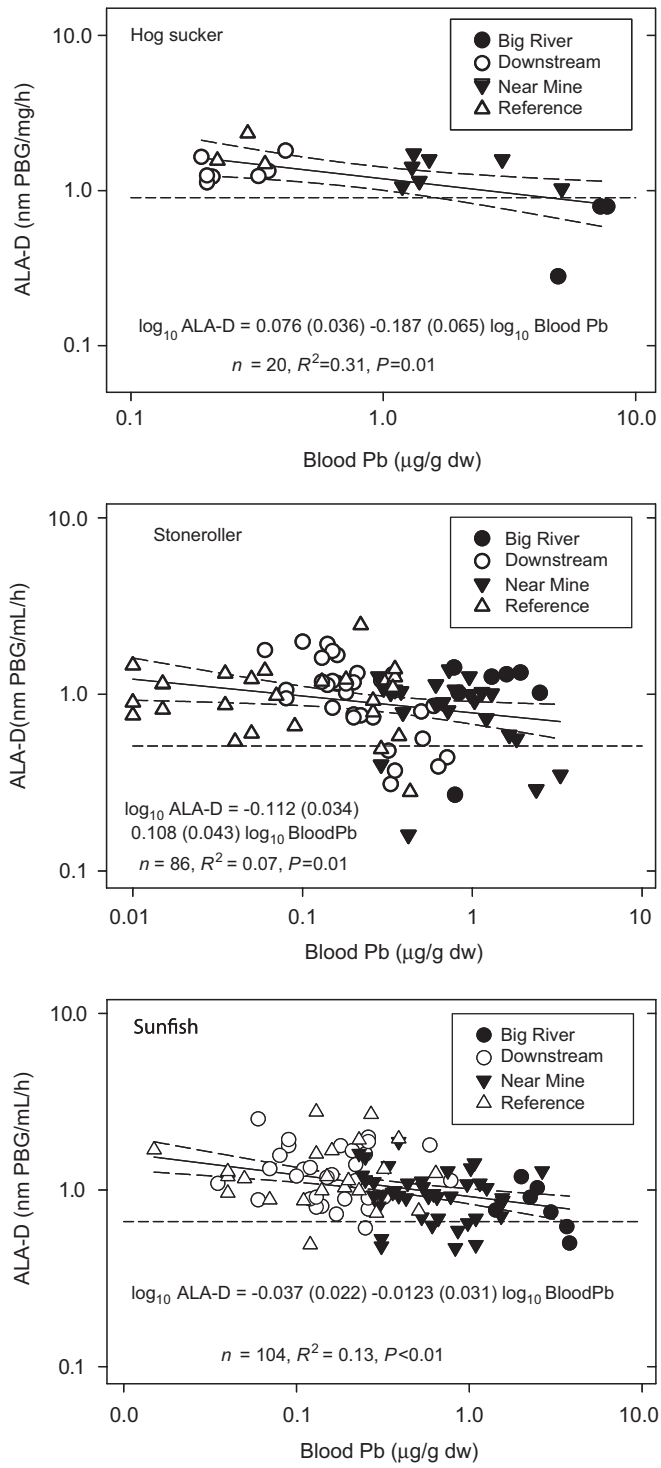

Fig. 1. Activity of δ-aminolevulinic acid dehydratase (ALA-D) and blood lead (Blood Pb, from Schmitt et al., 2007) concentrations in three species of fish from sites <10 km downstream from mining-related facilities in the New Lead Belt (Near Mines), >10 km downstream (Downstream), no upstream mining (Reference), and the Big River. Also shown for each species are the least-squares regressions (solid lines) and 95% confidence intervals (long-dashed lines) between these variables across all sites (standard errors in parentheses), and the value representing 50% of the unweighted mean ALA-D activity at reference sites (short-dashed lines; 0.9 nm PBG/mL/h for hog sucker, 0.5 nm PBG/mL/h for stoneroller, and 0.7 nm PBG/mL/h for sunfish.

Table 2
Statistically significant (**$P<0.01$; *$P<0.05$) functional and multiple linear regression models describing relations between wet-weight concentrations of hemoglobin as measured in the field (Hb_f) and laboratory (Hb_l); total length (TL, mm); concentrations of lead (Pb), cadmium (Cd), zinc (Zn), and iron (Fe) in blood, liver, or both (all μg/g dry weight; from Schmitt et al., 2007); and activity of the enzyme δ-aminolevulinic acid dehydratase (ALA-D) in the blood of three species of fish, with ALA-D as un-standardized values (nM PBG/mL/h) and standardized to Hb_f and Hb_l (nM PBG/mg/h)

Species and model	F (df)	R^2
Hog sucker[a]		
$[Fe] = -0.0002\ (0.0003) + 3.8562\ (0.3011)\ [Hb_f]$[b]	146.03 (1, 18)**	0.89
$\log_{10}$ ALA − D = −4.6932(1.4733) + 1.2276(0.3815) $\log_{10}$ blood Fe + 0.9614(0.2492) $\log_{10}$ blood Zn − 0.0822(0.0318) $\log_{10}$ blood Pb + 0.5265(0.1645)$\log_{10}$ Hb_l − 0.4022(0.1539) $\log_{10}$ TL	39.36 (5, 14)**	0.93
$\log_{10}$ALA − D/Hb_f = −0.2642(0.5296) + 0.6558(0.2290) $\log_{10}$ blood Zn + 0.1344(0.0592)$\log_{10}$ liver Zn − 0.3672(0.1462) $\log_{10}$ TL	4.97 (3, 16)*	0.48
$\log_{10}$ ALA − D/Hb_l = −3.4835(1.4919) + 1.2577(0.4240) $\log_{10}$ blood Fe + 1.0822(0.2093)$\log_{10}$ blood Zn − 0.1422(0.0253) $\log_{10}$ blood Pb + 0.1035(0.0253)$\log_{10}$ blood Cd − 0.4156(0.1864) $\log_{10}$$Hb_f$ − 0.6807(0.1372)$\log_{10}$ TL	12.49 (6, 13)**	0.85
Sunfish[c]		
$[Fe] = 0.0003\ (0.0003) + 3.1749\ (0.2620)\ [Hb_f]$[b]	43.89 (1, 103)**	0.30
$\log_{10}$ ALA − D = 0.0781(0.1803) − 0.2562(0.1036) $\log_{10}$ blood Zn − 0.1049(0.0212) $\log_{10}$ blood Pb + 1.0411(0.1257) $\log_{10}$$Hb_f$	34.62 (3, 100)**	0.51
$\log_{10}$ ALA − D/Hb_f = 2.8472(1.1030) − 0.9637(0.3555) $\log_{10}$ blood Fe − 0.0528(0.0240) $\log_{10}$blood Cd − 0.0890(0.0282) $\log_{10}$ blood Pb + 0.8521(0.0289) $\log_{10}$$Hb_l$	14.12 (4, 99)**	0.36
$\log_{10}$ ALA − D/Hb_l = 0.8012(0.1768) − 0.2612(0.1035) $\log_{10}$ blood Zn − 0.1055(0.0211) $\log_{10}$ blood Pb_d	14.48 (2, 101)**	0.22
Stoneroller[d]		
$[Fe] = 0.0004\ (0.0003) + 3.5466\ (0.2888)\ [Hb_f]$[b]	61.70 (1, 89)**	0.41
$\log_{10}$ ALA − D = −3.0968(1.0645) + 0.7985(0.3287) $\log_{10}$ blood Fe − 0.0751(0.0170) $\log_{10}$ blood Pb + 1.2352(0.0585)$\log_{10}$$Hb_l$	163.71 (3, 82)**	0.86
$\log_{10}$ ALA − D/Hb_f = −0.3465(0.0299) − 0.0837(0.0219) $\log_{10}$ blood Pb + 1.1580(0.0754) $\log_{10}$ Hb_l	131.60 (2, 83)**	0.76
$\log_{10}$ ALA − D/Hb_l = −0.0714(0.1020) + 0.0485(0.0245) $\log_{10}$ blood Cd − 0.0813(0.0168) $\log_{10}$ blood Pb + 0.2559(0.1192)$\log_{10}$$Hb_f$	9.93 (3, 82)**	0.27

Shown for each model are intercepts and regression coefficients (with standard errors), F-values and degrees of freedom (df), and coefficients of determination (R^2).
[a]Northern hog sucker, *Hypentelium nigricans*.
[b]Functional (geometric mean) regression (Ricker, 1973).
[c]Longear sunfish, *Lepomis megalotis*.
[d]Largescale stoneroller, *Campostoma oligolepis*.

Among-species differences in Hb concentrations were not statistically significant (ANOVA, $P>0.05$), but concentrations were not consistent across all sites, as indicated by significant species × site interaction ($P<0.05$). Differences among sites were statistically significant in all three species (Table 1), but there was considerable variability within sites. Consequently, and although some groups of sites differed significantly from others with respect to mining influence, there were no clearly evident mining-related trends. The lone exceptions were hog sucker from Site 16

(Big River), in which Hb concentrations were significantly lower ($P<0.05$) than at all sites except Site 7 (Sweetwater Creek), which is near an NLB mine (Fig. 2; Table 1).

Blood Fe concentrations (ww and dw) differed significantly ($P<0.01$) among species; concentrations were typically greater in stoneroller than in hog sucker or sunfish; the species means were 1735 μg/g dw (228 μg/g ww) for stoneroller, 1646 μg/g dw (215 μg/g ww) for hog sucker, and 1400 μg/g dw (177 μg/g ww) for sunfish. Among-site differences in dw blood Fe concentrations were statistically significant ($F_{6,\ 13}=3.49$, $P<0.05$) in hog sucker but not in sunfish or stoneroller ($F=1.13$–1.62, $P>0.05$); however, among-site differences in ww blood Fe concentrations were significant in all three species (Table 1). Concentrations were 50.1–322 μg/g (ww) in hog sucker, 87.8–230 μg/g in sunfish, and 128–337 μg/g in stoneroller. The results were also similar to those for Hb in that although blood Fe differences among sites were statistically significant, patterns related to mining influences were not generally evident. The exceptions were low blood Fe concentrations in the same fish from Site 16 and Site 7 identified as having low Hb relative to other sites (Fig. 2; Table 1).

Concentrations of Hb and Fe were positively correlated in all three species. Statistically significant ($P<0.01$) linear regressions between [Hb] and [Fe] with small intercepts and regression coefficients of 1.7–3.6 explained 89% of the variation in hog sucker, 41% in sunfish, and 30% in stoneroller (Fig. 2). The regression coefficients for the functional regressions, which account for variation in both variables (Ricker, 1973), ranged from 3.1 in sunfish to 4.0 in hog sucker (Table 2). The expected value is 4.0 based on the tetrameric structure of Hb (Schmitt et al., 2005). Blood Fe concentrations in several stonerollers from sites <10 km downstream from mines were lower than expected based on Hb measurements (Fig. 2), but neither Hb nor blood Fe were significantly correlated with blood Pb in either sunfish or stoneroller ($P>0.05$; data not shown). In contrast, both blood Fe and Hb were negatively correlated with blood Pb in hog sucker ($r=-0.43$ to -0.57, $P<0.05$, $n=20$) due to the low Hb and Fe concentrations and correspondingly high blood Pb concentrations in several fish from the Big River (Fig. 2).

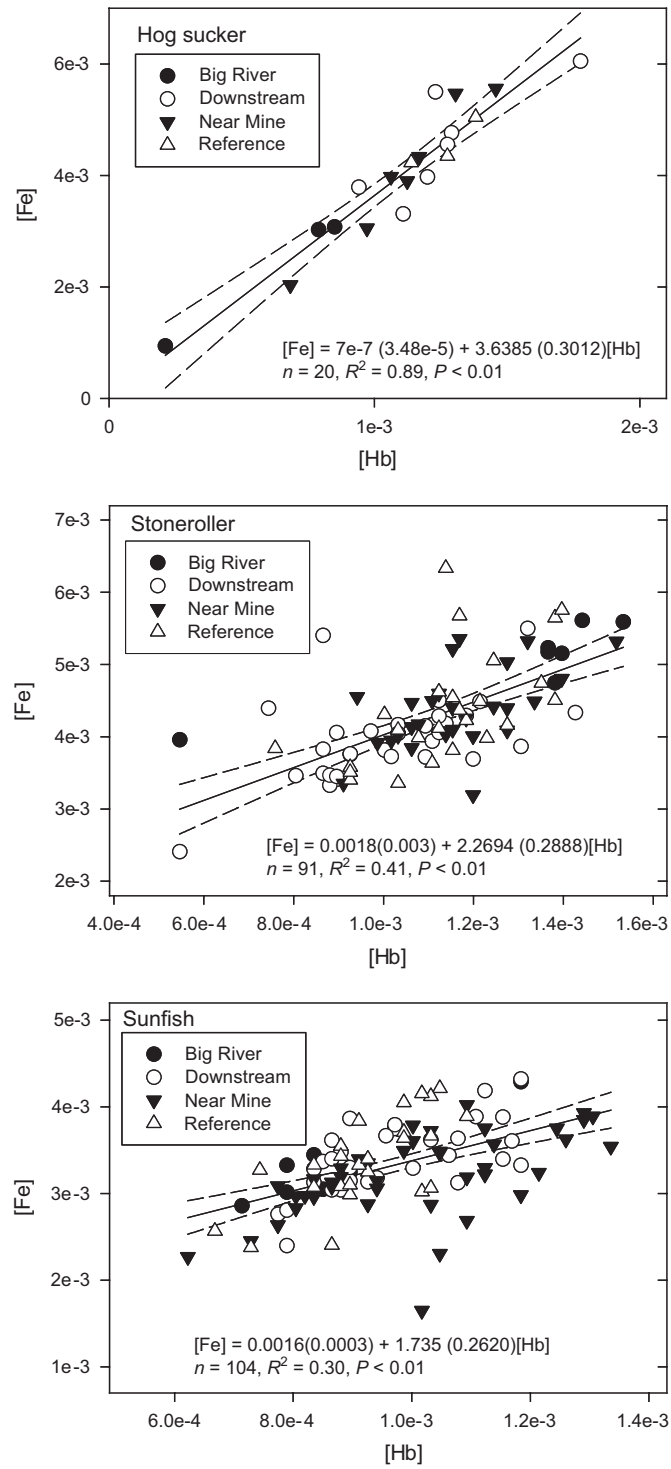

Fig. 2. Molar concentrations of iron [Fe] and hemoglobin [Hb] in the blood of three species of fish. Also shown are the linear regressions (solid lines) and 95% confidence intervals (dashed lines) between these variables across all three species (standard errors in parentheses).

3.3. Zinc protoporphyrin

3.3.1. Pilot studies

Replicate measurements of the samples from Study 1 were consistent; with one exception, all within-fish CVs were between 0% and 10%. ANOVA explained >99% of the total ZPP variation in preserved common carp and bluegill erythrocytes; differences among fish were significant ($F_{8,\ 16}=31.0$, $P<0.01$) as were those between instruments ($F_{2,\ 16}=31.0$, $P<0.01$) and species ($F_{1,\ 16}=5075.3$, $P<0.01$). Concentrations were 10–23 μmol/mol (mean = 17.9 μmol/mol) in bluegill and 4–8 μmol/mol (mean = 5.7 μmol/mol) in common carp (data not shown). Differences among instruments were also statistically significant ($P<0.01$), but the unweighted instrument means (13.1, 13.6, and 14.6 μmol/mol) differed by <10% and differences were not consistent among fish, as indicated by significant fish × instrument interaction ($F_{16,\ 26}=2.8$, $P<0.01$).

In Study 2, ANOVA also explained >99% of the total ZPP variation in whole blood from bluegill and lake sturgeon. Differences between species were significant ($F_{1,\ 25} = 7433.0$, $P < 0.01$) as were those among individual fish of the same species ($F_{22,\ 25} = 92.6$, $P < 0.01$), but differences among replicate coverslips from the same fish were not ($F_{22,\ 352} = 0.8$, $P > 0.05$; data not shown). Day-0 mean concentrations in bluegill were 28.9–53.9 μmol/mol (overall mean = 35.1 μmol/mol) and 6.6–18.9 μmol/mol (mean = 11.2 μmol/mol) in lake sturgeon. Concentrations in the refrigerated samples increased over time in both species; the linear term for days post-collection was significant ($F_{2,\ 25} = 666.6$, $P < 0.01$), and the rate of increase was greater for bluegill (8.32 μmol/mol/d) than lake sturgeon (1.31 μmol/mol/d). As percentages of the Day-0 means, the daily rates of increase were 24% in bluegill and 12% in lake sturgeon. Coefficients of variation (based on all measurements of the same fish on a given day) were 2.7–5.7% in bluegill (mean = 3.9%) and 4.3–23.6% (mean = 10.3%) in lake sturgeon. Collectively, the results of the pilot studies indicated that 3–6 measurements of one coverslip would adequately characterize the ZPP concentration of each fish, but that samples should be analyzed within 24–36 h of collection. These findings were incorporated into the field protocol.

3.3.2. Field study

We detected ZPP in only 26 of 85 stoneroller (31%) from Sites 9–16; 15 of 104 sunfish (14%) from Sites 3, 4, 6, 8, 10, 14, and 16; and two of 19 hog sucker (11%) from Sites 3 and 16 (Fig. 3). Overall, greatest ZPP concentrations were in fish from Site 7 (Logan Creek), Site 8 (Sweetwater Creek), and Site 16 (Big River; Fig. 3). Within-site variability was considerable (Fig. 3), but CVs for individual fish (among measurements) of all three species were generally <10%. In samples with measurable ZPP, concentrations were 1–71 μmol/mol in sunfish, 2–89 μmol/mol in stoneroller, and 37–135 μmol/mol in hog sucker (Fig. 3). The hog sucker and stoneroller maxima were in samples from Site 16, but the two greatest sunfish concentrations were from Site 8, which is <10 km below an NLB mine (Fig. 3). The hog sucker from Site 16 with high ZPP was also among the fish from the Big River with low Hb and blood Fe concentrations (Fig. 2). The ZPP field data were not analyzed statistically due to the large percentage of censored (i.e., <LOD) values.

3.4. Hepatic lipid peroxidation

Blood and liver concentrations of Pb and Zn and of Cd in liver differed significantly between species in the fish selected for FPLP analyses (Table 3). Fish with the highest Pb concentrations did not necessarily have the highest Cd or Zn concentrations. Blood and liver Pb concentrations also differed significantly ($P < 0.01$) among the groups of fish selected on the basis of Pb concentrations (low, medium, high) for FPLP analysis. Liver Pb concentrations were 0.01–3.41 μg/g dw in individual sunfish and 0.04–7.79 μg/g in stoneroller (Table 3). Blood and liver Cd differences among groups of fish were also significant in both species, but were less evident than those for Pb (Table 3). Neither blood nor liver Zn differences were statistically significant in either species ($P > 0.05$; Table 3).

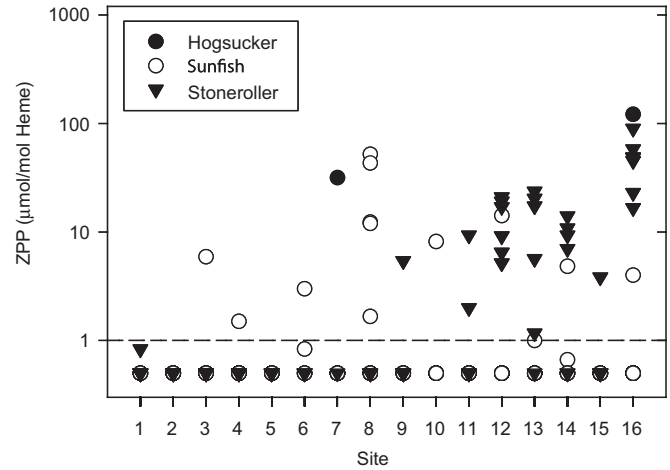

Fig. 3. Concentrations of zinc protoporphyrin (ZPP) in the blood of three species of fish from sites in the New Lead Belt (1–14), the Exploration Area (15), and the Big River (16). The dashed line represents the approximate limit-of-detection (1.0 μmol/mol heme).

Hepatic lipid peroxidation (as FPLPs) differed significantly ($P < 0.01$) between species, with values for stoneroller typically 3-fold greater than those for sunfish (Table 3). However, differences among high-, medium-, and low-Pb exposure fish of the same species were not significant ($P > 0.05$; Table 3). Correlation and regression analyses also did not detect statistically significant relations between FPLPs and metals concentrations in either blood or liver of individual fish ($P > 0.05$).

3.5. Metallothionein induction

Blood and liver Pb concentrations also differed significantly ($P < 0.01$) between sites in the stoneroller selected for MT analysis by RT-PCR, as did blood Cd concentrations (Table 4). Differences for liver Cd and Zn were less evident; they only approached statistical significance ($P = 0.09$–0.10), and mean concentrations of both were greater in fish from Site 10 (Sinking Creek, a reference site) than from Site 16 (Big River; Table 4). Maximum concentrations were 2.98 μg/g Pb (Site 16), 1.89 μg/g Cd (Site 10), and 93.3 μg/g Zn (also Site 10). Estimates of hepatic MT mRNA varied greatly among fish at both sites; CVs were >50% and differences between sites were not statistically significant ($P > 0.05$; Table 4). Nevertheless, hepatic MT mRNA and liver Zn were positively correlated; a log-log linear regression between these variables was marginally significant ($P = 0.06$) and explained 31% of the variation in MT mRNA (Fig. 4). No other correlations approached statistical significance.

Table 3
Arithmetic mean ± SE concentrations of lead (Pb), cadmium, and zinc in blood and liver (all μg/g dry-weight, from Schmitt et al., 2007) and fluorescent products of lipid peroxidation (FPLPs, fluorescence standardized to quinaldine sulfate) in liver of fish of two species characterized as being low, medium, or high in blood Pb

Species and relative Pb	*n*/df	Blood			Liver			
		Lead	Cadmium	Zinc	Lead	Cadmium	Zinc	FPLPs
Sunfish[a]								
Low	5	0.04 ± 0.01 a	0.018 ± 0.006 a	55.0 ± 5.5 a	0.04 ± 0.01 a	1.650 ± 0.233 a	91.7 ± 8.8 a	1.024 ± 0.062 a
Medium	5	0.32 ± 0.04 b	0.026 ± 0.015 a	40.4 ± 1.7 a	0.39 ± 0.10 b	4.490 ± 0.742 b	96.2 ± 3.3 a	1.016 ± 0.124 a
High	5	2.82 ± 0.45 c	0.030 ± 0.006 a	54.5 ± 4.0 a	1.95 ± 0.39 c	11.472 ± 1.926 c	66.5 ± 4.6 a	1.164 ± 0.115 a
Stoneroller[b]								
Low	5	0.03 ± 0.01 a	0.010 ± 0.001 a	49.6 ± 1.6 a	0.13 ± 0.05 a	0.792 ± 0.230 a	93.7 ± 2.7 a	3.546 ± 0.368 a
Medium	5	0.19 ± 0.02 b	0.037 ± 0.009 b	68.5 ± 2.2 b	0.32 ± 0.10 b	1.296 ± 0.286 b	97.2 ± 9.4 a	3.196 ± 0.384 a
High	5	2.33 ± 0.29 c	0.025 ± 0.005 a	62.2 ± 6.6 a	5.03 ± 0.80 c	3.376 ± 2.096 c	116.6 ± 15.2 a	3.082 ± 0.451 a
ANOVA-*F*								
Species (S)	1, 24[c]	7.05*	0.19 ns	9.74**	6.43*	23.41**	7.73**	84.53**
Pb	2, 24[c]	376.79**	4.12*	1.10 ns	7.43*	9.70**	0.79 ns	0.23 ns
S × Pb	2, 24[c]	0.65 ns	1.35 ns	9.10**	4.08*	0.67 ns	6.50**	0.53 ns
R^2	30	0.97	0.32	0.56	0.90	0.65	0.48	0.78

Also shown are results of two-way analysis-of-variance (ANOVA) as *F*-values (**$P<0.01$; *$P<0.05$), degrees-of-freedom (df), and coefficients of determination (R^2). Within each group of three means, values followed by the same letter are not significantly different ($P>0.05$).
[a]Longear sunfish, *Lepomis megalotis*.
[b]Largescale stoneroller, *Campostoma oligolepis*.
[c]df.

Table 4
Arithmetic mean ± SE concentrations of lead, cadmium, and zinc in blood and liver (all μg/g dry-weight, from Schmitt et al., 2007) and metallothionein (MT) induction in liver of largescale stoneroller[a] from two sites.

Site	*n*/df	Blood			Liver			
		Lead	Cadmium	Zinc	Lead	Cadmium	Zinc	MT
10 (Sinking Creek)	6	0.03 ± 0.01	0.010 ± 0.001	49.5 ± 2.5	0.12 ± 0.04	1.018 ± 0.237	88.6 ± 0.2	0.914 ± 0.190
20 (Big River)	6	1.35 ± 0.29	0.021 ± 0.004	83.2 ± 6.5	2.35 ± 0.21	0.508 ± 0.077	82.1 ± 2.8	0.855 ± 0.184
ANOVA-*F*	1, 10[b]	190.75**	14.47**	25.52**	111.9**	3.33 ns[c]	3.50 ns[b]	0.05 ns
R^2	11	0.95	0.59	0.72	0.92	0.25	0.26	<0.01

Also shown are the results of analysis-of-variance (ANOVA) as *F*-values (**$P<0.01$; *$P<0.05$), degrees-of-freedom (df), and coefficients of determination (R^2).
[a]*Campostoma oligolepis*.
[b]df.
[c]$P = 0.09$–0.10.

4. Discussion

Threshold blood Pb concentrations associated with effects on heme synthesis in fish, as indicated by reduced ALA-D activity, vary among species. In most species (including hog sucker and sunfish), reduced ALA-D activity has been reported at blood-Pb concentrations exceeding about 0.5 mg/L (about 4 μg/g dw assuming 87.5% moisture), but varying indirectly with Zn burden (Dwyer et al., 1988; Schmitt et al., 1984, 1993, 2002). In channel catfish (*Ictalurus punctatus*) the threshold may be only 0.1 mg/L (0.8 μg/g; Schmitt et al., 2005). Blood Pb concentrations in most of our samples were <1.0 μg/g dw, yet statistically significant negative correlations between ALA-D activity and blood Pb were evident in all three of the species we investigated (Fig. 1). Collectively, and despite the fact that ALA-D inhibition is not equally sensitive to Pb in all species (Campana et al., 2003; Schmitt et al., 2005), our findings indicate that the blood Pb threshold for ALA-D inhibition may be <1.0 μg/g dw in many fishes. In humans, ALA-D is polymorphic (e.g., Hu et al., 2001). Although the existence of multiple ALA-Ds has not been investigated or reported in fish, their occurrence and relative abundance could at least partly explain differences in Pb sensitivity among species.

The ores of the Viburnum Trend are enriched with a variety of metals, but only Pb, Zn, and Cu are recovered commercially in substantial quantities from NLB mines

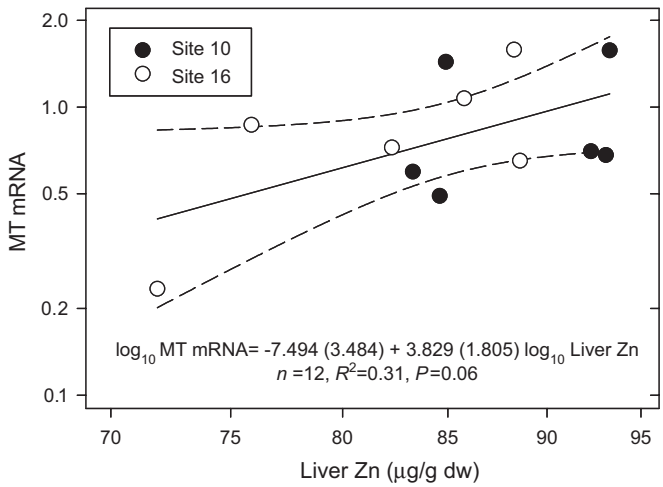

Fig. 4. Metallothionein (MT) rRNA and liver zinc concentrations (Liver Zn; from Schmitt et al., 2007) in stoneroller from two sites. Also shown is the least-squares regression (solid line) and 95% confidence interval (dashed line) between these variables.

(e.g., Wixson and Jennett, 1975). Small amounts of Co are recovered incidentally (Shedd, 2005), and solid wastes containing Co and Ni are generated by the facility on Strother Creek (Doe Run Company, 2003). Although elevated concentrations of these latter metals have not been reported previously in fish from the NLB, Schmitt et al. (2007) detected elevated concentrations of both Ni and Co in blood and liver of hog sucker from Site 12 (Strother Creek) relative to Site 11 (Middle Fork at Redmondville, a reference site) and Site 16 (Big River). Concentrations of Pb, Cd, and Zn were also comparatively high in fish from Site 12 (Schmitt et al., 2007), and ALA-D activity was inhibited in all three species (Table 1); however, Co and Ni were not measured in fish from other sites. Nickel toxicity is additive with that of other metals, including Cd, Cu, Pb, and Zn (Enserink et al., 1991), and it can affect heme metabolism. Erythropoiesis and hemoglobin synthesis in fish can be stimulated by Ni (Alkahem, 1994); it can inhibit ALA-D and possibly also ALA synthetase (ALA-S; δ-aminolevulinate synthase, E.C. 2.3.1.37), and can induce heme oxygenase (HO; E.C. 1.14.99.3l; Maines, 1980). ALA-S is generally perceived as the rate limiting step in heme synthesis, and HO catalyzes the degradation of heme to biliverdin (Maines, 1980). Although inspection of the data (Fig. 1) did not indicate that the points representing fish from Site 12 deviated from the overall trend of the blood Pb:ALA-D relationships, the possible contribution of Ni to the inhibition of ALA-D at some sites cannot be ruled out as a possible cause of the low apparent blood Pb thresholds in the species we investigated. However, evaluating the contribution of Ni would require more data representing a wider range of sites and concentrations.

Cobalt is also an essential element (it is a component of vitamin B_{12}) that can be toxic to fish. Although the mechanism of Co toxicity is unknown, it can act as an antagonist with respect to the toxicity of other metals (Marr et al., 1998). In warm-blooded vertebrates, Co has been shown to stimulate ALA-S and HO (e.g., Maines and Sinclair, 1977; Maines et al., 1976), but its effects on heme homeostatis in fish has not been investigated.

In addition to affecting heme metabolism, Co, Cd, and Ni can also inhibit cytochrome P-450 induction in fish (Maines, 1980; Spaethe and Jollow, 1989; Taylor, 1990; Brüschweiler et al., 1996). The cytochrome P-450 enzymes are involved in the detoxification of xenobiotic organic chemicals and the homeostasis of steroid hormones; their inhibition could therefore influence both the toxicity of organic chemicals and reproduction in fish (Stegeman and Hahn, 1994). Sodium cyanide, along with xanthates, alcohols, and other organic chemicals, are used in the milling process (Jennett and Callier, 1977; Wixson, 1978). These and other organic chemicals may interact with metals to affect heme synthesis and metabolism.

Consistent with previous studies, positive terms for blood Zn were included in some of our statistical models describing ALA-D activity (Table 2). The ameliorative effect of Zn has been attributed to the fact that it is required as a cofactor for ALA-D, and that Zn may be able to displace Pb from the metal binding site of the enzyme (Warren et al., 1998); however, as noted by Schmitt et al. (2002), not all researchers agree on the latter. The lack of a consistent Zn effect in our data may reflect both differences among species and the comparatively narrow range of blood Zn concentrations in our fish relative to other studies. In contrast to Pb and Cd, Zn is an essential element; internal concentrations tend to be tightly regulated by fish (Bury et al., 2003). The range of blood Zn concentrations in our fish was much smaller than that of Pb and Cd, and differences among groups of sites based on their proximity to NLB mines were not statistically significant (Schmitt et al., 2007).

Prolonged exposure of birds and mammals (including humans) to Pb ultimately results in anemia. However, and as noted by Schmitt et al. (2002, 2005), such effects have only been inconsistently documented in fish. Some of our fish from Site 16 (Big River) and Site 7 (Sweetwater Creek) with high blood Pb appeared to be anemic, as indicated by comparatively low Hb and blood Fe concentrations, but the sample size was small and results were inconsistent; i.e., blood Fe and Hb concentrations were also low in some fish with low blood Pb and vice-versa. In general, our findings support the widely held belief that ALA-D is not a rate-limiting step in heme synthesis by fish, and that most environmental Pb concentrations are lower than those necessary to produce anemia and other hematological effects as measured by the methods typically employed. Nevertheless, ALA-D is an extremely sensitive indicator of environmental Pb exposure. In addition, Hb and Fe concentrations, as well as other traditional endpoints used to assess fish blood such as cell counts and hematocrit, are relatively crude measurements (Houston, 1997; Schmitt et al., 2002). More sensitive techniques have demonstrated Cd effects on erythron status in laboratory studies

(Houston, 1997), but to date no thorough study of Pb effects on erythropoiesis in fish has been reported.

Comparisons of ALA-D activity among investigations can be difficult due to species differences, differing acclimation temperatures, activity standardization (to Hb, hematocrit, etc.), and assay-related variables (pH, temperature, etc.; Schmitt et al., 2005). Comparisons are therefore often based on proportional activity relative to reference or control values generated independently within each study. In wildlife, 50% ALA-D inhibition relative to relevant reference or control activity is considered evidence of injury associated with exposure to environmental Pb (US Department of the Interior, 1987), a value that has also been used with fish (e.g., Schmitt et al., 2002, 2005). Mean unstandardized ALA-D activity in reference fish (from Sites 3, 10, 11, and 15) averaged 1.8 nmol PBG/mL/h in hog sucker, 1.3 nmol PBG/mL/h in sunfish, and 1.0 nmol PBG.mL/h in stoneroller; and ALA-D/Hb averaged 3.4 nmol PBG/mg/h in hog sucker, 1.6 nmol PBG/mg/h in sunfish, and 1.1 nmol PBG/mg/h in stoneroller (Table 1). Relative to these values, mean ALA-D activity was inhibited by $\geqslant 50\%$ only in hog sucker from Site 16 (Big River) and stoneroller from Site 13 (Neals Creek; Table 1). Activity was also reduced by $\geqslant 50\%$ in a few individual sunfish and stoneroller from Site 16 and from some sites <10 km downstream from NLB mines (Fig. 1). Mean ALA-D/Hb was not inhibited by $\geqslant 50\%$ relative to reference sites in any taxa from any site (Table 1); however, some ALA-D/Hb differences were confounded with low Hb concentrations (Fig. 2; Table 1). Overall, the degree of inhibition evident in the species we evaluated from the Big River and sites near NLB mines were less than what was reported for common carp and bass by Schmitt et al. (2005), which is consistent with the lower blood Pb concentrations in our fish.

Higher-level biological effects, including but not limited to behavioral changes, have been reported less frequently and associated only indirectly with Pb exposure and ALA-D inhibition in fish. Inhibition may ultimately result in the accumulation of ALA (Kelada et al., 2001), which is structurally similar to γ-aminobutyric acid (GABA). The stimulation of GABA receptors in the nervous system by ALA may be a primary mechanism of Pb-induced neurotoxicity (Kelada et al., 2001; Warren et al., 1998). "Black tail", which represents a grossly observable response to Pb-induced neurotoxicity, was associated with blood-Pb of 1.7 mg/L (about 13.6 μg/g dw) and ALA-D inhibition of 74% relative to controls in laboratory-exposed rainbow trout (*Oncohrynchus mykiss*; Hodson et al., 1979). Blood Pb in our fish did not exceed 7.72 μg/g dw (Fig. 1), but some fish from the Big River analyzed in previous studies did (Schmitt et al., 1984, 1993, 2005). Although black tail has not been reported in wild fish by any scientific investigations, a recent newspaper article reported that it had been observed in fish from the Big River (Rehagen, 2004). Behavioral effects have been induced in fathead minnow exposed to part-per-million concentrations of waterborne Pb (Weber et al., 1991), in mirror carp (*Cyprinus carpio*) at $\leqslant 50$ μg/L in moderately hard water (Shafiq-ur-Rehman, 2003), and in rainbow trout at 29 μg/L in moderately hard water (Burden et al., 1998); growth and ALA-D activity were also affected at higher concentrations in the rainbow trout, and lipid peroxidation was detected in the brains of the mirror carp. In contrast, neither behavioral nor hematological effects were elicited in smallmouth bass (*Micropterus dolomieu*) fingerlings exposed to $\leqslant 405$ μg/L of Pb in moderately hard water for 90 d (Coughlan et al., 1986). Effects on bone strength, which may impair swimming performance and increase vulnerability to predators, were detected in sunfish from the Big River with blood Pb concentrations of about 0.5 mg/L (Dwyer et al., 1988). Stippled erythrocytes and spinal deformities have been induced in common carp exposed to part-per-million concentrations of Pb in the laboratory (Holcombe et al., 1976; Beretić et al., 1980), as have additional sub-lethal effects in other fishes (Johansson-Sjöbeck and Larsson, 1979; Weber et al., 1991). Organism-, population-, and community-level effects were associated with reduced ALA-D activity in two species of catfish (Pimelodidae) inhabiting a tailings-contaminated stream (Moraes et al., 2003). Blood Pb concentrations were not measured in this study, but muscle Pb concentrations in the two species averaged 2.97 μg/g and 7.55 μg/g dw (0.59 μg/g and 1.5 μg/g ww assuming 80% moisture), which are within the range of recently reported fillet concentrations in fish from the Big River (Gale et al., 2004).

Other than ALA-D, the effects of Pb and other metals on porphyrin metabolism in fish has received limited study. Theodakoris et al. (1992) reported increased ZPP concentrations in bluegill exposed to Pb-contaminated sediments, with ZPP increasing over 8 wk of exposure. In contrast, Hodson et al. (1984) detected only low levels of porphyrins by scanning fluorometry in blood extracts from rainbow trout exposed to Pb in the laboratory; ALA-D activity was $>50\%$ inhibited in these fish, which also showed evidence of neurotoxicity (black tails). We detected ZPP by hematofluorometry in all specimens of three species, including bluegill, in our pilot studies. We also detected ZPP fluorescence in some stoneroller, hog sucker, and sunfish from Pb-contaminated sites, but none in other specimens of the same species with equally high blood Pb concentrations (Fig. 3). In addition, ZPP concentrations in bluegill and lake sturgeon tended to increase over time in refrigerated samples, which may represent the fluorescence of bilirubin (Buhrmann et al., 1978) or some other substance.

In mammals, ZPP has been shown to be both more (Simmonds et al., 1995) and less (Schuhmacher et al., 1997) sensitive than ALA-D inhibition to Pb. In birds and in the American toad (*Bufo arenarum*), ferrochelatase inhibition results in the accumulation of free erythrocyte protoporphyrin (FEP), and FEP measurement by hematofluorometry has been used as a biomarker of Pb exposure (Anderson and Havera, 1985; Arrieta et al., 2004; Blus et al., 1991; Franson et al., 1986; Roscoe et al., 1979). In the American

toad, FEP is more sensitive to Pb than ALA-D (Arrieta et al., 2004), but the general consensus among avian toxicologists is that FEP accumulation is less sensitive than ALA-D (Pain, 1989; Beyer et al., 2000). The limited data available (ours and previous studies) indicate that ZPP is also less sensitive than ALA-D in fish, but substantially more data and information on porphyrin biochemistry in fish is needed. A particular shortcoming is the lack of information on possible effects of other metals such as Co and Ni, which have been shown to substitute for Fe in protoporphyrin IX in mammals (Rosenberg, 1993).

Hepatic lipid peroxidation, as indicated by the measurement of FPLPs, differed by about 3-fold in the two species we analyzed, but no differences related to metals were detected (Table 3). Using similar methods, Farag et al. (1995, 2003) detected significantly ($P<0.05$) greater hepatic lipid peroxidation in brown trout (*Salmo trutta*) and rainbow trout from mining-contaminated sites on the Clark Fork River and in the Boulder River system of Montana than at reference sites. Liver Cd concentrations averaged about 2.5 μg/g dw and liver Pb about 1.0 μg/g dw in brown trout from the Clark Fork site, which are substantially lower than the concentrations in our stoneroller. Liver Zn concentrations in the Clark Fork fish were not measured, but they contained elevated concentrations of Cu and arsenic (Farag et al., 1995). Maximum liver Cd concentrations in our stoneroller and sunfish were substantially lower than those in the rainbow trout from the Boulder River site, which were about 100 μg/g dw. Our liver Zn maxima were somewhat lower than those from the Boulder River (Farag et al., 2003, which were about 200 μg/g dw, but maximum liver Pb concentrations in our fish of both species selected as "high Pb" (maxima 3.41–7.79 μg/g dw) were substantially greater (maxima <1.0 μg/g dw; Farag et al., 2003). The Boulder River fish, like those from the Clark Fork, had also been exposed to comparatively high Cu concentrations (Farag et al., 2003). Collectively, these results indicate that metals other than Pb caused the lipid peroxidation reported by Farag et al. (1995, 2003).

The results of other investigations also indicate that lipid peroxidation is less sensitive to Pb than to other metals. In a laboratory study, Campana et al. (2003) detected only small effects on hepatic lipid peroxidation in European (Lusitanian) toadfish (*Halobatrachus didactylus*) following interperitoneal (ip) injection of Pb even though liver Pb concentrations reached 300 μg/g ww (1429 μg/g dw assuming 79% moisture; Harrison and Klaverkamp, 1990). As noted previously, Pb concentrations in fish from the tailings-contaminated reaches of the Big River are among the greatest on record (e.g., Gale et al., 2004; Schmitt et al., 1984, 1993, 2005, 2007), and a human consumption advisory is in effect (Missouri Department of Health and Senior Services, 2006). Nevertheless, concentrations of other mining-derived metals in fish are greater elsewhere (e.g., Schmitt et al., 1993, 2007; Farag et al., 2003). Copper is among the metals recovered from mines in the NLB (Wixson and Jennett, 1975; Wixson, 1978), but neither previous nor ongoing studies have reported elevated concentrations in NLB streams (Schmitt et al., 1993; Petersen et al., 1998; Besser et al., 2003). Based on a review of extant literature, Campana et al. (2003) hypothesized that in contrast to Cu, neither Pb nor Cd can change their oxidation states, a requirement of the redox cycling necessary for lipid peroxidation. Collectively, these findings indicate that hepatic lipid peroxidation in fish may not be as sensitive to Pb as ALA-D, but that it may have potential as a biomarker of exposure to other metals in NLB fish.

Hepatic MT mRNA induction, which we measured by RT-PCR, was correlated only with liver Zn. Within-site variation was comparatively high (Fig. 4), and differences among sites were not statistically significant (Table 4). As noted, the range of metal concentrations spanned by the fish and sites selected for these analyses may not have been sufficient for the detection of among-site differences in MT induction, and the sample size was small (Table 4). Campana et al. (2003) detected increased MT protein concentrations in European toadfish 7 d after interperitoneal injection of Pb, by which time liver Pb concentrations had plateaued at about 300 μg/g ww (1429 μg/g dw assuming 79% moisture). These concentrations are >10-fold higher than liver Pb concentrations in our fish (Schmitt et al., 2007). Campana et al. (2003) also noted that the time course for induction may be temperature related, and that both the timing and intensity of the MT response may differ among metals. They hypothesized that Pb was a weak MT inducer relative to other metals because of its comparatively low (compared with other metals) sulfhydryl binding affinity. Farag et al. (1995) reported an increase in hepatic MT protein of about 3-fold relative to reference sites in brown trout from a contaminated site on the Clark Fork, and Farag et al. (2003) detected as much as 30-fold greater MT protein concentrations in the livers of rainbow trout from mining-contaminated streams in the Boulder River system. Given that Pb is a weak inducer of MT (Campana et al., 2003), these responses may reflect the comparatively greater concentrations of metals other than Pb to which the Montana fish had been exposed (Farag et al., 1995, 2003), as also hypothesized for lipid peroxidation. The induction of MT can also be affected by a wide range of factors such as fish age, gender, size, and reproductive status (Lacorn et al., 2001). Although we attempted to standardize fish size, we did not control for these other factors, which probably contributed to the variation in our data (Fig. 4).

Another factor to consider when using MT as a biomarker is the method used to measure it relative to the exposure history of the organisms. Traditional methods typically determine the amount or concentration of MT present in the tissues (e.g., Hogstrand and Haux, 1990; Lacorn et al., 2001; Campana et al., 2003; Farag et al., 2003) or the amount of Cd that can be bound by MT or other proteins (e.g., Hamilton et al., 1987; Cope et al., 1994; Kamman et al., 1997). These methods document the

cumulative, long-term exposure history of the animals. In contrast, RT-PCR responds to active or recent MT synthesis (Norey et al., 1990). Consequently, MT mRNA might not be detected in resident fish that have acclimated to contemporary exposure conditions. RT-PCR has been used to detect MT induction in caged, un-acclimated fish held in metals-contaminated streams (McClain et al., 2003; Roberts et al., 2005). These findings indicate that RT-PCR might be better suited to use with transplanted, naïve fish, in which extraneous (and potentially confounding) variables such as genetic diversity, exposure/acclimation history, age, reproductive status, nutrition, size, and gender can also be controlled; and that measurement of metallothionein concentrations by traditional methods is better for documenting the long-term exposure of wild fish. Studies incorporating these methods at sites representing a wider range of metals concentrations may ultimately demonstrate both metallothionein and lipid peroxidase differences among NLB sites and stronger concentration-dependent relationships.

As a final consideration, multiple MT isoforms have been documented in fish and other organisms (Klaverkamp et al., 1984; Carginale et al., 1998; Lacorn et al., 2001; Muto et al., 1999), and not all isoforms respond to metals. Lacorn et al. (2001) reported that only one of two MT isoforms in dab (*Limanda limanda*) was induced by metals, the other by rising temperature. Furthermore, expression of the cDNAs specific to different MT isoforms has been shown to be differentially regulated by exposure to different metals (Carginale et al., 1998; Ren et al., 2000). Lacorn et al. (2001) therefore questioned the use of MT as a biomarker of exposure without first confirming that the isoform being measured responds to metals.

5. Conclusions

We documented elevated blood metals concentrations and negative statistical relations between blood Pb concentrations and ALA-D activity in stoneroller, sunfish, and hog sucker. Greatest Pb concentrations and lowest ALA-D activity in all three species were in fish from the Big River and at sites <10 km downstream from NLB mines. Conversely, lowest Pb concentrations and greatest ALA-D activity were in fish from reference sites and the exploration area. Consistent with previous findings, and with some exceptions, blood Pb concentrations in NLB fish exceeded reference concentrations but were lower than those from the Big River and other historical mining areas in the US and elsewhere, as would be expected given the modern technology and environmental regulation of the NLB. Nevertheless, ALA-D activity was inhibited in fish of all species from sites near mines, indicating that Pb is both bioavailable and biochemically active in streams draining the NLB. It is generally believed that because the conversion of ALA to PBG is not the rate-limiting step in heme synthesis, the inhibition of ALA-D does not by itself lead to anemia. However, we observed limited evidence of anemia (i.e., lower Hb and blood Fe concentrations) in some fish from mining-affected sites, which differs from previous findings. As noted by Schmitt et al. (2005), the use of more sensitive indicators (e.g., Houston, 1997) may eventually demonstrate a relationship between ALA-D activity and heme status in fish. In addition, the influence of Ni and Co from mining in the NLB on porphyrin metabolism in fish is unknown.

Trends for Cd and Zn were less evident than those for Pb. Although greatest concentrations of Cd and Zn were in fish from sites <10 km downstream from NLB mines, there was considerable variability within and among sites. Brumbaugh et al. (2005) noted similar variability for Cd and Zn relative to Pb in fish from mining areas in Oklahoma, and suggested that these differences reflected differing long-term exposure histories and accumulation patterns. Although liver Zn and MT induction were correlated, our findings indicate that RT-PCR may not be the best way to document long-term exposure and MT effects. We also failed to detect effects on hepatic lipid peroxidation, which indicates that both MT and hepatic lipid peroxidation are less sensitive to Pb than to other metals. However, the biomarkers for this study were selected based on the well known effects of Pb on heme synthesis and of several metals on MT induction and lipid peroxidation. Previous studies in the NLB (i.e., Wixson, 1978; Schmitt et al., 1993; Petersen et al., 1998) had not reported elevated Ni or Co concentrations, which became evident during the course of our investigation and subsequent studies (Besser et al., 2003; Schmitt et al., 2007). Ongoing studies in the NLB are therefore examining for ecological effects attributable to metals beyond Pb, Zn, and Cd (including Ni and Co), at a wider range of sites. Future biomarker studies, if conducted, should incorporate a battery of biomarkers that have been shown to respond to a variety of mining-associated metals. In addition to the endpoints we used, these should include histopathological evaluations to document macrophage aggregate numbers, hyperplasia, and other lesions in the kidney; histopathological lesions in the gill, brain, and liver; and reproductive and developmental biomarkers, including gonadal histopathology (e.g., Farag et al., 1999, 2003; Campana et al., 2003; Pereira et al., 1993). Both caged and wild fish should be studied to document both short- and long-term exposures (e.g., Theodakoris et al., 1992; McClain et al., 2003; Roberts et al., 2005) and the effects of milling reagents should be investigated.

Lastly, it is important to recognize that the environmental effects of mining vary temporally in response to many factors. In addition to natural factors, these include the variable mineral composition of ores, mining intensity changes related to global market forces, and improvements in extraction and effluent treatment technology. Mining intensity in the NLB waned during the 1990s, but elevated concentrations of metals and their effects in fish were nevertheless evident at most sites located near NLB mines. Mining and exploration activity in southern Missouri has

recently begun to increase in response to rising demand for Pb and other metals. Continued investigation and monitoring of streams in this region is therefore warranted.

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Disclaimer

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