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Laser Ablation ICP-MS Co-Localization of Mercury and Immune Response in Fish

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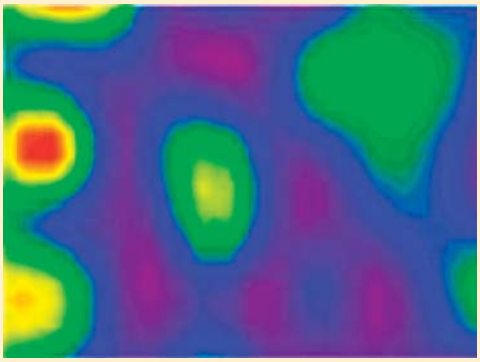
Supporting Information

ABSTRACT: Mercury (Hg) contamination is a global issue with implications for both ecosystem and human health. In this study, we use a new approach to link Hg exposure to health effects in spotted gar (*Lepisosteus oculatus*) from Caddo Lake (TX/LA). Previous field studies have reported elevated incidences of macrophage centers in liver, kidney, and spleen of fish with high concentrations of Hg. Macrophage centers are aggregates of specialized white blood cells that form as an immune response to tissue damage, and are considered a general biomarker of contaminant toxicity. We found elevated incidences of macrophage centers in liver of spotted gar and used a new technology for ecotoxicology studies, laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS), to colocalize aggregates and Hg deposits within the tissue architecture. We conclude that Hg compromises the health of spotted gar in our study and, perhaps, other fish exposed to elevated concentrations of Hg.



INTRODUCTION

Since the industrial revolution, anthropogenic releases of Hg have contaminated ecosystems worldwide. Because of its long residence time in the atmosphere, Hg can be transported far from sources and deposited in even the most remote ecosystems.[1] In aquatic ecosystems, the methylation of Hg and its subsequent biomagnification in food webs results in high concentrations of Hg in fish. Hg poses health risks to piscivorous wildlife and humans as well as fish themselves.[2,3] In locations solely impacted by atmospheric deposition—constituting the great majority of waterbodies—concentrations of Hg (in axial muscle) in fishes are often less than 2 μg/g wet wt.[4] Laboratory studies indicate that whole-body Hg concentrations in fish exceeding 0.3 μg/g wet wt (equivalent to 0.5 μg/g wet wt in axial muscle) may result in effects that are sublethal.[5,6] Thus, many wild fish appear at risk of toxic effects from Hg exposure.

Current assessments of effects of Hg on wild fish rely on statistical approaches. The simplest approach is to calculate a hazard quotient, by dividing the concentration of Hg in a fish population of interest (generally a summary statistic, e.g., geometric mean) by a threshold concentration above which effects are predicted to occur. A value >1 indicates the population is at risk of toxic effects. Sandheinrich et al.,[7] for example, used this approach to estimate that 44% of walleye (*Sander vitreus*) populations in the Great Lakes region of North America are at risk of Hg toxicity. Hazard quotients are used for screening-level assessments, however, and do not indicate actual effects. Another approach is to test for changes in anatomy or physiology, especially of "biomarkers", in fish exposed to elevated concentrations of Hg. A biomarker is a contaminant-induced change in anatomy or physiology of an organism and, ideally, should be validated by a laboratory experiment.[8] For example, sex steroid hormones are reproductive biomarkers of Hg in fish, as illustrated in a lab experiment in which fathead minnows (*Pimephales promelas*) exposed to elevated concentrations of dietary methylmercury (MeHg) had suppressed levels of plasma testosterone and 17-ß estradiol, and the result on reproduction was reduced spawning

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success.[9] Field studies have subsequently shown significant negative correlations between Hg concentrations in axial muscle and sex steroid hormones levels in plasma of fish,[10,11] but environmental conditions that vary among sites (and may influence fish physiology), as well as the presence of other contaminants, precludes the conclusion that Hg is the cause of suppressed hormone levels. Even in the extreme case of Clay Lake (Ontario, Canada), where a chloralkali plant resulted in northern pike (*Esox lucius*) with Hg concentrations of 6−16 μg/g wet wt (in axial muscle), Lockhart et al.[12] could not attribute changes in physiology of northern pike, compared to a reference site, to Hg. With the exception of Minamata Bay, no study has conclusively linked environmental exposure and toxic effects of Hg in fish.

In this study, we use a new approach to link Hg exposure to health effects in spotted gar (*Lepisosteus oculatus*) from Caddo Lake (TX/LA). Previous field studies have reported elevated incidences of macrophage centers in liver, kidney, and spleen of fish with high concentrations of Hg.[13−16] Macrophage centers are aggregates of specialized white blood cells that form in fish as an immune response to tissue damage, and are considered a general biomarker of contaminant toxicity.[17] Hg is known to alter cellular calcium homeostasis and this effect leads to the well documented mercury mediated apoptosis and necrosis.[18] Increased intracellular calcium has been linked to macrophage activation.[19,20] We hypothesize that Hg-mediated areas of cell death resulted in increased macrophage activity, thus explaining why MA and Hg were colocalized (as shown by LA-ICP-MS) in gar liver.

EXPERIMENTAL SECTION

Study Sites and Sampling. Spotted gar (n = 10) were captured from wetland and open-water habitats of Caddo Lake (TX/LA). Sampling sites and the limnology of the lake have been described elsewhere.[21−23] Briefly, Caddo Lake and its associated wetlands cover approximately 10,850 ha and are composed of cypress swamps, marshes, bottomland hardwood forests, grasslands, and pine forests, much of which remain in a relatively undisturbed condition.[24,25] The western portion of the lake is shallow (many areas <1 m) and characterized by wetland habitat dominated by bald cypress (*Taxodium distichum*) and water elm (*Planara aquatica*), and other aquatic vegetation including fanwort (*Cabomba caroliniana*), common waterweed (*Elodea* sp.), yellow pond-lily (*Nuphar lutea*), and invasive water hyacinth (*Eichhornia crassipes*).[26] In contrast, the eastern portion of the lake is comprised primarily of open-water habitat with an average depth of 1.4 m.[27] Fish from Caddo Lake contain some of the highest Hg concentrations recorded in Texas[28] and mercury contamination in Caddo Lake is of particular concern because the lake supports high biodiversity, including rare and threatened species[24,29] which may be negatively impacted by Hg exposure. The most probable source of Hg loading into Caddo Lake is atmospheric deposition.[30] The primary anthropogenic sources of Hg in the region are coal-burning power plants;[31] however, a substantial proportion of the Hg deposited in the region may originate from sources outside of North America.[32] Fish and grass shrimp (*Palaeomonetes kadiakensis*) in the western, wetland portion of the lake contain higher concentrations of Hg than those in the eastern, open-water portion of the lake.[21,22] The observed differences in Hg accumulation is most likely due to differences in the MeHg availability between the two habitats, with the wetland being more conducive to Hg methylation or more efficient at the incorporation of MeHg into the base of the food chain.[21,22] Although fish from all locations in Caddo Lake contain elevated levels of Hg, in this study the wetland site was considered "contaminated" and the open-water site was considered to be a "reference" site. Spotted gar were targeted for this study because they were abundant at the reference and contaminated site and known from previous studies to have elevated concentrations of Hg in their tissues.[22] Gar were euthanized immediately after capture, total length and wet weight were recorded, and samples of axial muscle and liver tissue were collected. Subsamples of tissues for Hg determination (muscle and liver) and color analysis (liver only) were placed in individual plastic bags and frozen at −20 °C. Subsamples of liver tissue for histopathology and LA-ICP-MS were fixed in neutral buffered formalin and later transferred to 70% ethanol for storage.

Hg Analysis. Samples were thawed, dried in a 60 °C oven, and homogenized to a flour-like consistency with a ball-mill grinder. Total Hg was analyzed in the homogenized samples with a direct Hg analyzer (DMA-80, Milestone Inc. Monroe, CT). Quality assurance included reference and duplicate samples, as previously described.[21,22] Reference materials were within certified ranges, and the mean relative percent difference of duplicate samples was 3.63%.

Liver Color. Liver color measurements were carried out as previously described by Drevnick et al.[13] Briefly, a 100 mg piece of liver was homogenized in 1 mL deionized water, and 0.2 mL chloroform was added. The mixture was centrifuged for 15 min at 12 000 rpm. An aliquot of the supernatant was transferred to a microplate well and measured for absorbance at 400 nm on a BioTek Synergy 2 spectrophotometer (Winooski, VT, USA). Each liver was analyzed in duplicate. A mean percent difference <20% between replicates was deemed acceptable.

Macrophage Aggregates. Livers from individual gar were viewed for analysis of MA as previously described by Drevnick et al.[13] Livers were randomly assigned a unique number to facilitate blind study, embedded in paraffin, sectioned, and mounted on glass slides.[33] For each individual gar, three unstained sections were viewed with fluorescence microscopy (excitation filter 355−425 nm, suppression at 460 nm)[13] at 200X magnification. A representative field from each section was photographed, and a digital grid (squares 25 μm × 25 μm) was overlaid on the image with Adobe Photoshop (Adobe Systems Inc., San Jose, California). Fifty grid squares (out of a total of 130 per field) were selected with a random number generator. Presence or absence of MA was noted and recorded in each of the selected grid squares. The proportion of grid squares containing MA out of the total 50 assessed was used as a quantitative measure of the extent of liver damage. The average score of the three sections was used in the data analysis.[13]

LA-ICP-MS. Microscope slides created for the pathological portion of the study were used to investigate the distribution of mercury in spotted gar liver sections by LA-ICP-MS. A microscope slide, with unstained, paraffin-embedded liver tissue, was placed into the chamber of a 213 nm Nd:YAG laser ablation source (New Wave Research, Fremont, CA). A CCD camera enabled zooming and scanning of the slide to locate liver tissue. Five areas of normal liver tissue and five MA were chosen at random and ablated from liver sections from seven individual gar (the three individuals not analyzed had MA scores of zero).

A 15 μm beam diameter and 100 μm raster spacing enabled adequate sampling of MA and decreased the risk of interferences and resampling of previously ablated material. A Varian 820 ICP-MS coupled to the laser source was used to monitor ^{202}Hg and ^{78}Se isotopes. Paraffin near the edge of the liver sections was also ablated, and confirmed a negligible contribution of ^{78}Se and ^{202}Hg. The mass detector collected data for 100 s each run, the first 30 s of which was laser warm-up. Mean isotope counts for the first 30 s were used as an estimate of background noise and subtracted from the mean counts for the remainder of the run to calculate a signal. After ablation, slides were viewed again under fluorescence microscopy to ensure lipofuscin containing MA were targeted. Table S1 in the Supporting Information describes the instrument parameters used for analysis.

Statistical Analyses. Statistics were performed with JMP 4 Statistical Analysis Software (SAS Institute, Cary, NCA). Data were transformed, if necessary, to meet the assumptions of tests. One-tailed student's t tests were used to compare differences in variables between habitat types. Least-squares regression models were used to describe relationships between variables. A paired t test was used to compare the mean isotope signals (^{202}Hg or ^{78}Se) between normal tissue and MA. Significance of statistical tests was determined with a type I error (α) of 0.05. In addition, Psi-Plot (Poly Software International, Pearl River, NY) was used to create a 3D surface contour describing the relative Hg concentration in a gar liver section.

RESULTS AND DISCUSSION

Hg Concentrations. A complete presentation of Hg concentrations throughout the Caddo Lake food web is presented in Chumchal et al.[23] In spotted gar sampled for this study, Hg concentrations (mean ± SE) in axial muscle were higher from wetland habitats (0.657 ± 0.091 μg/g wet wt) than from open-water habitats (0.239 ± 0.069 μg/g wet wt) of Caddo Lake (p = 0.0032). Chumchal and Hambright[22] reported for Caddo Lake that Hg concentrations are higher in food webs from wetland than from open-water habitats. Hg inputs likely do not differ between the two habitat types, as the principal source of Hg to the system is atmospheric deposition.[30] Rather, Hg methylation may be enhanced in wetland habitats[34] and, if so, would explain why fish in general (several species) are more contaminated in wetlands than in open-waters of Caddo Lake.[22] For spotted gar, Hg concentrations are also a function of size (total length) and age,[22] but neither of these factors can explain differences in Hg accumulation between habitat types. Gar sampled were from a narrow size range (total length: range = 45−60 cm; mean ± SE = 50.3 ± 2.57 cm from wetland, 55.6 ± 1.85 cm open-water). Gar ages were not measured for this study, but data from Chumchal and Hambright[22] indicate there are no differences in age at size between habitat types.

Spotted gar from wetland habitats have Hg concentrations known to be toxic to fish. Recent analyses of the available data for Hg toxicity in fish indicate that effects are likely to occur at concentrations in axial muscle exceeding 0.5 μg/g wet wt.[5,6] Concentrations in five of the six gar from wetland habitats exceeded this value (hazard quotient of mean concentration >1). In contrast, all four of the gar from open-water habitats had concentrations lower than the threshold value (hazard quotient <1). Based on this simple hazard evaluation, we would expect to find evidence of Hg toxicity in gar from wetland habitats (treatment), but not from open-water habitats (reference).

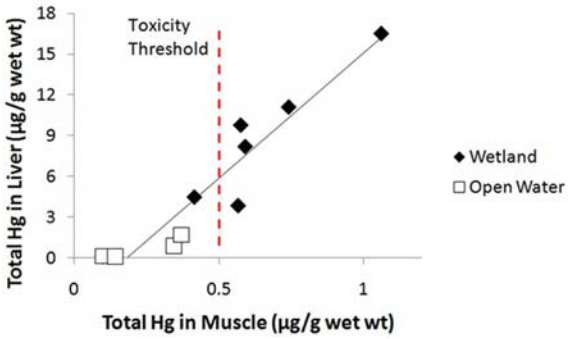

Figure 1. Total mercury concentration (μg/g wet wt) in spotted gar muscle and liver from open-water and wetland habitats.

Hg concentrations (mean ± SE) in liver of spotted gar were higher from wetland habitats (9.01 ± 1.90 μg/g wet wt) than from open-water habitats (0.728 ± 0.376 μg/g wet wt) of Caddo Lake (p = 0.0034 ; Figure 1). Hg concentrations in liver were greater than in muscle tissue of gar from both habitats. Chumchal et al.[23] also reported higher Hg concentrations in spotted gar liver than in muscle. Though only total Hg was measured in this study, Chumchal et al.,[23] analyzed MeHg and total Hg in spotted gar muscle and liver from Caddo Lake. Hg found in the muscle tissue was primarily in the organic form, which is consistent with previous report.[35,36] However, in the liver tissue, the majority of Hg was present in the inorganic form.[23] Elevated inorganic Hg concentrations in the livers of gar could be due to (1) a dietary source or (2) the way Hg is metabolized, that is, demethylation, which has not been well described in fish. Drevnick et al.[13] reported similarly low MeHg and high inorganic Hg concentrations in the livers of northern pike. In that study, the authors speculate that the high inorganic Hg content in the livers was not due to demethylation, but rather to the inorganic Hg content of larval odonates, a common prey item of northern pike in that ecosystem. MeHg concentrations were similar in pike muscle and liver suggesting MeHg was not being converted to inorganic Hg. Conversely, from Chumchal et al.,[23] MeHg comprised the majority of total Hg in gar muscle, but only a small percentage of the total Hg in gar livers, suggesting that demethylation could be occurring.

At the cellular level, MeHg is transported into the liver where it can be conjugated to glutathione and excreted in the bile.[37,38] Inorganic Hg, however, is complexed in the liver with selenium into large polyatomic ions.[39,40] These ions are then associated with binding proteins such as selenoprotein P resulting in an accumulation of inorganic Hg in the liver relative to other tissues.[41−43] Demethylation has been speculated to occur in birds[39] and fish[44] but little direct cellular evidence or delineated mechanism has been shown. Mercury speciation data from these two studies is mentioned here because of the possible link to liver damage observed in spotted gar from this study. Hepatic demethylation in fish should be the focus of future studies along with inorganic Hg's role in liver toxicity.

Biomarkers in Liver Tissue. Liver color (as measured by absorbance; λ=400 nm) was significantly different between habitat types. Gar from the wetland habitat had significantly darker livers than those from open-water (p < 0.0001). Liver coloration in spotted gar was positively correlated with total Hg in the muscle (p = 0.01; r^2 = 0.58) and total Hg in the liver (p = 0.013; r^2=0.56) (Figure 2).

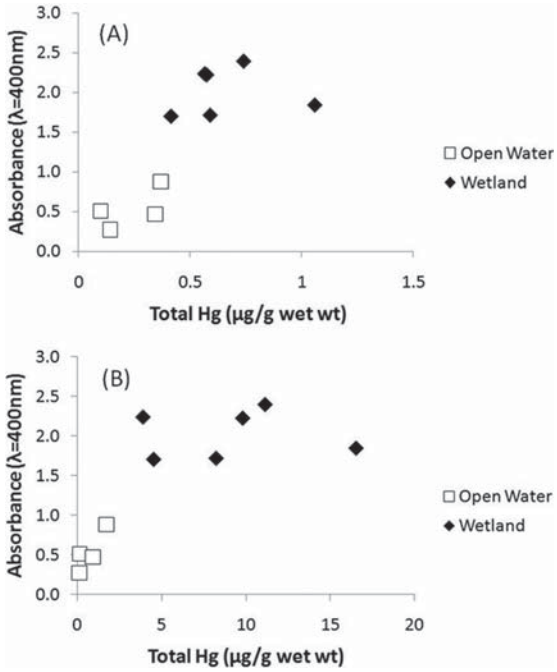

Figure 2. Relationship between liver color (λ = 400 nm) and total mercury concentration (μg/g wet wt) in the muscle (A) and liver (B), of spotted gar from open-water and wetland habitats.

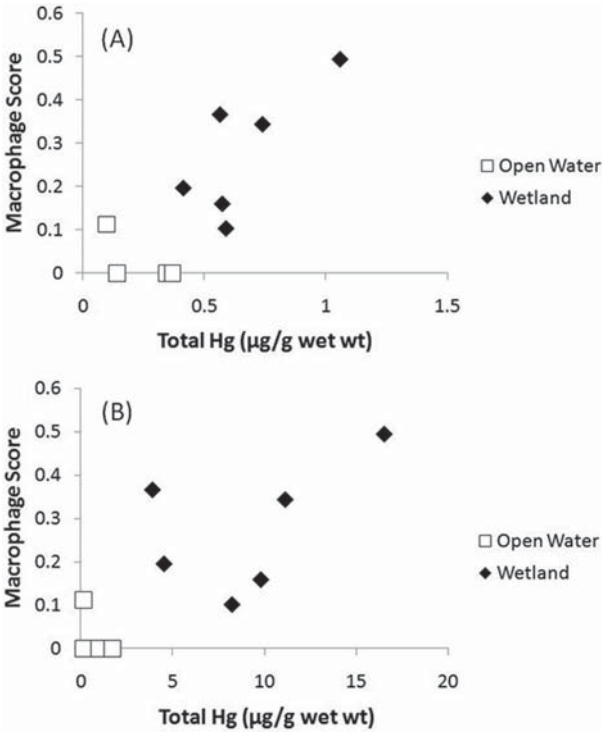

Figure 3. Relationship between mean hepatic macrophage score and total mercury concentration (μg/g wet wt) in muscle (A) and liver (B), of spotted gar from open-water and wetland habitat types.

During histologic examination of gar livers, we identified darkly pigmented deposits as macrophage aggregates, containing melanin and lipofuscin. Melanin is a pigment derived from tyrosine which can scavenge free radicals, bind metal ions, and is produced by hematopoietic cells in fish. Lipofuscin pigment is composed of oxidized lipids and proteins and is a sign of cell membrane or mitochondrial damage due to chronic oxidation.[45] Drevnick et al.[13] identified lipofuscin as the pigment responsible for the majority of variation in pike liver color. In spotted gar from Caddo Lake liver color and the percent of MA were positively correlated in gar liver (p = 0.013; r^2 = 0.56). The presence of lipofusicn in MA could account for some of the variation in gar liver color.

Both recent field- and laboratory- based studies have shown positive correlations between Hg exposure and MA in wild fish.[15,16] Other studies have found evidence of increased MA in fish from other types of contaminated environments.[46,47] Changes in both MA and lipofusicn have been associated with aging,[45,48] and this was not controlled for in the present study. However, Drevnick et al.[13] measured age using scales and determined it not to be a significant factor in the occurrence of liver damage in Isle Royale northern pike. In that study, age was used as a covariate to statistically show that Hg concentration was the best predictor of liver damage in pike. Also, from Chumchal et al.,[23] there does not appear to be any differences in age at size of spotted gar between habitat types of Caddo Lake. Thus, sampling gar of equal size, as was done in this study, from both sides of the lake would likely result in fish of the same age.

Habitat-specific differences in hepatic MA were observed for spotted gar. Spotted gar sampled from wetland habitats had approximately five times higher occurrences of MA than those sampled from open-water areas (p = 0.004). Spotted gar contained hepatic MA with a mean occurrence of 9% in wetland areas, 0.31% in open-water, and a maximum of 22% of the histological grids in a single individual. The occurrence of MA was positively correlated with total Hg in the muscle (p = 0.004; r^2 = 0.67) and total Hg in the liver (p = 0.009; r^2 = 0.59) (Figure 3).

LA-ICP-MS. We determined, using laser ablation ICP-MS, that ^{202}Hg counts were significantly higher in MA than in visibly normal gar liver tissue (p = 0.003). In general, MA had twice as many ^{202}Hg counts as parenchyma (Figures 4 and 5). MA also had significantly higher ^{78}Se than parenchyma (p = 0.031), where counts rarely rose above the estimated background signal. Former studies have employed autometallography (AMG) or electron microscopy (EM) coupled with energy dispersive X-ray analysis (EDAX) in an attempt to show the colocalization of Hg and pathologies, with differing results. Rawson et al.[49] used EDAX and EM to suggest Hg localization in the hepatic lysosomes of bottlenose dolphins (*Tursiops truncatus*), speculating that Hg was the cause of increased lipofuscin deposits. Woshner et al.[50] used AMG to show that in the livers of beluga whales (*Delphinapterus leucas*), Hg was colocalized with stellate macrophages. However, Hg and lipofuscin were rarely colocalized and the authors suggest that diet rather than Hg was the cause of lipofuscin accumulation. Furthermore, Hg was likely found as HgSe in beluga livers,[50] which is widely considered to be the endproduct of Hg detoxification in marine mammals.[50−52] Higher counts of ^{78}Se in gar macrophages warrants further investigation of the identity of Hg compounds, as it is possible that Hg is bound to Se in these immune cells. However, our study, unlike Woshner et al.[50] consistently demonstrates colocalization of Hg and lipofuscin deposits in the liver tissue, suggesting a causative relationship.

MA are important in the immune function of fish, and serve as stores for the endproducts of cellular breakdown.[48,53] Hg is

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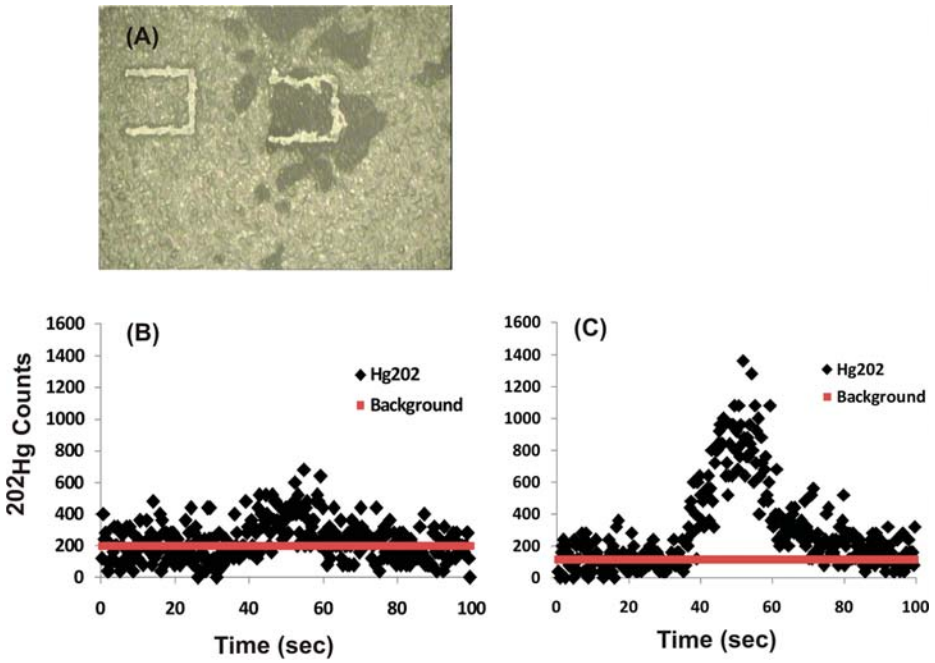

Figure 4. (A) Image of a spotted gar liver section after laser ablation of normal parenchyma and a macrophage aggregate. Dark patches are macrophage aggregates. Lighter patches are normal parenchyma. (B) Graph showing ^{202}Hg counts in the ablated normal parenchyma and (C) macrophage aggregate.

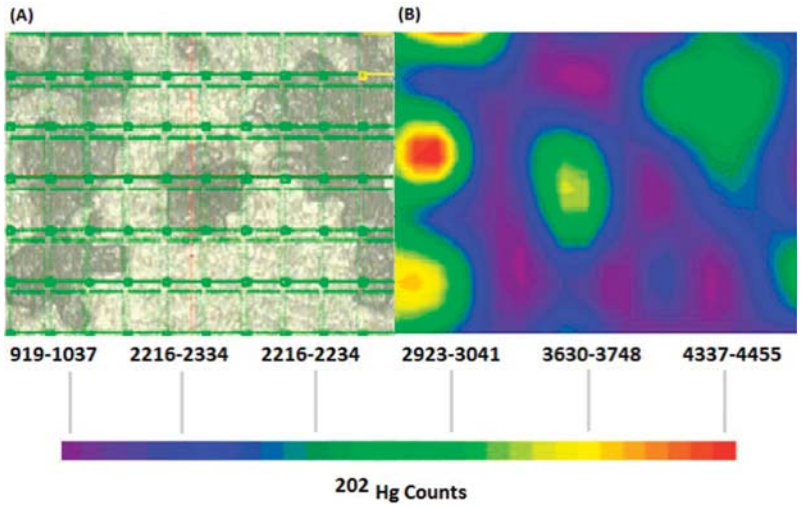

Figure 5. (A) A preablation image of a spotted gar liver section with a superimposed raster pattern. Dark patches are macrophage aggregates. Lighter patches are normal parenchyma. (B) 3D contour image showing the relative distribution of ^{202}Hg after laser ablation of the section shown in (A).

known to increase intracellular levels of calcium leading to apoptosis and necrosis, thus increasing oxidized lipids and proteins.[18] Increases in free calcium in the cell have also been implicated in the activation of macrophage respiratory burst,[19,20] which can produce reactive oxygen species capable of breaking the MeHg bond.[54] We found elevated incidences of macrophage centers in liver of spotted gar and used a new technology for ecotoxicology studies, LA-ICP-MS, to colocalize lesioned areas and Hg deposits within the tissue. We conclude that Hg compromises the health of spotted gar in our study and, perhaps, other fish exposed to elevated concentrations of Hg. Future studies should focus on the importance of these cells in fish Hg metabolism.

The strong correlations between two measures of pathology (MA and color) and Hg content in both the liver and muscle of spotted gar further implicate Hg as the cause of liver damage. The relationship between liver damage and habitat suggests that Hg is the cause, rather than another contaminant. Liver discoloration and MA were elevated in spotted gar from wetland habitats. Previous reports by Chumchal et al.[21] and Chumchal and Hambright[22] have documented higher concentrations of Hg in wetland fish in Caddo Lake than fish sampled from open-water. It is unlikely that other contaminants present in Caddo Lake which might biomagnify would display the same habitat-specific differences observed here; Hg relies on wetlands for methylation to achieve the biomagnifying species whereas contaminants such as

organochlorines do not. We did not measure other inorganic and organic compounds in gar tissues for this study. However, the Texas Commission of Environmental Quality in cooperation with U.S. EPA surveyed contaminants in muscle and whole bodies of a variety of fish species from several sites within Caddo Lake. They found that mercury was present in muscle from all sites and at elevated levels. Zinc, magnesium, iron and manganese were present in fillets and whole fish at normal and expected levels while other metals were not detected. Pesticides, PCBs, and perchlorate were not detected in muscle or whole fish samples.[28]

Implications. Both the hazard quotient and biomarker based approaches suggested that Hg impairs the health of spotted gar from wetland habitats of Caddo Lake. This was supported further, by the novel use of LA-ICP-MS to colocalize Hg and liver damage in these fish. Past studies involving Hg exposure in wild fish, have noted negative correlations between Hg and measures of fish health. Larose et al.[55] described negative relationships between Hg and both, liver antioxidant activity and the hepatosomatic index of wild fish. Drevnick et al.[13] noted decreased lipid reserves in pike livers and decreased condition factor of fish with higher Hg concentrations. Similarly, Lockhart et al.[12] documented decreased fat storage in the liver and emaciation of northern pike from the Hg contaminated Clay Lake. Concentrations of Hg in Caddo Lake spotted gar are within the range of concentrations concluded to impair fish health. Future studies are needed to determine how elevated Hg concentrations are impacting fish health and fitness.

■ ASSOCIATED CONTENT

Supporting Information. One table of LA-ICP-MS parameters. This material is available free of charge via the Internet at http://pubs.acs.org.

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