Mercury toxicity in livers of northern pike (*Esox lucius*) from Isle Royale, USA

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Abstract

Many laboratory studies have documented that mercury can be toxic to fish, but it is largely unknown if mercury is toxic to fish in their natural environments. The objective of our study was to investigate the toxic effects of mercury on northern pike (*Esox lucius*) at Isle Royale, Michigan. In 124 northern pike from eight inland lakes, concentrations of total mercury in skin-on fillets ranged from 0.069 to 0.622 μg/g wet wt. Concentrations of total mercury in livers increased exponentially compared with concentrations in fillets, to a maximum of 3.1 μg/g wet wt. Methylmercury constituted a majority of the mercury in livers with total mercury concentrations <0.5 μg/g wet wt, but declined to 28-51% of the mercury in livers with total mercury concentrations >0.5 μg/g wet wt. Liver color (absorbance at 400 nm) varied among northern pike and was positively related to liver total mercury concentration. The pigment causing variation in liver color was identified as lipofuscin, which results from lipid peroxidation of membranous organelles. An analysis of covariance revealed lipofuscin accumulation was primarily associated with mercury exposure, and this association obscured any normal accumulation from aging. We also documented decreased lipid reserves in livers and poor condition factors of northern pike with high liver total mercury concentrations. Our results suggest (i) northern pike at Isle Royale are experiencing toxicity at concentrations of total mercury common for northern pike and other piscivorous fish elsewhere in North America and (ii) liver color may be useful for indicating mercury exposure and effects in northern pike at Isle Royale and possibly other aquatic ecosystems and other fish species.

Keywords: mercury, methylmercury, northern pike, liver, lipofuscin

1. Introduction

Many laboratory studies have documented that mercury can be toxic to fish. Early studies reported effects on survival and growth, but fish were exposed to inorganic mercury or methylmercury (MeHg) in water at unrealistically high concentrations (Wiener and Spry, 1996). It has since been discovered that nearly all mercury in wild fish is MeHg (Bloom, 1992), and MeHg is accumulated almost entirely via dietary uptake (Hall et al, 1997). There is now a growing body of evidence that suggests reproduction of fish is impaired by exposure to dietary MeHg at concentrations reported for many aquatic food webs in North America (e.g., Matta et al., 2001; Hammerschmidt et al., 2002; Drevnick and Sandheinrich, 2003). We have found that MeHg induces apoptosis of steroidogenic gonadal cells in fish (Drevnick et al., 2006b), resulting in suppressed sex steroid hormone levels that are linked to inhibited reproduction (Drevnick and Sandheinrich, 2003). Apoptosis is often a symptom of oxidative stress (Holden, 2000), and other recent laboratory tests have reported that dietary MeHg causes oxidative stress in fish (Berntssen et al., 2003; Gonzalez et al., 2005), with resultant histopathological effects in liver and other tissues (de Oliveira Ribeiro et al., 2002). It is largely unknown, however, if MeHg has comparable sublethal effects on wild fish in their natural environments.

Most investigations of MeHg toxicity to wild fish populations have focused on ecosystems impacted by direct anthropogenic inputs of mercury. Toxic effects, including liver histopathology, have been associated with very high concentrations of MeHg in wild fish living downstream of mercury cell chlor-alkali plants (Lockhart et al., 1972; Raldúa et al., 2007). Other studies of rivers and reservoirs impacted by industry, military installations, and mining have indicated MeHg may alter reproductive biomarkers in wild fish, but causality is difficult to establish because of spatial and temporal variability and the presence of other contaminants (Hontela et al., 1995; Meinelt et al., 1997; Adams et al., 1999; Friedmann et al., 2002; Hinck et al., 2006; Webb et al., 2006). In contrast, there are few investigations of MeHg toxicity to wild fish in remote aquatic systems (Wiener et al., 2007), where atmospheric deposition is the principal source of mercury (Fitzgerald et al., 1998).

The objective of our study was to investigate the toxic effects of MeHg on a top piscivorous fish species, the northern pike (*Esox lucius*), in lakes of Isle Royale, Michigan. Isle Royale is a protected island ecosystem in Lake Superior that receives mercury almost entirely

from atmospheric deposition (Woodruff et al, 2003). Atmospheric contaminants other than mercury also are deposited to Isle Royale (e.g., organochlorines; Swackhamer and Hites, 1988), but they accumulate to relatively low concentrations in northern pike (Michigan Department of Environmental Quality, 2005) and are not of toxicological concern. Mercury is a concern, however, because atmospherically deposited mercury is efficiently converted to MeHg and biomagnified in aquatic food webs (Gorski et al., 2003), resulting in elevated concentrations in northern pike (Kallemeyn, 2000). Levels of total mercury in skin-on fillets of northern pike, nearly all of which is MeHg (Bloom, 1992), have recently declined at Isle Royale (Drevnick et al., 2007). Even with this decline, we report elevated concentrations of total mercury and MeHg in livers of northern pike and that these mercury levels are linked to oxidative stress, histopathological effects, and poor fish health. These results suggest the health of fish and their ability to reproduce may be affected by concentrations of mercury common in fish across North America.

2. Materials and Methods

2.1. Fish sampling

A potential link between MeHg exposure and sublethal toxic effects was examined for northern pike inhabiting eight Isle Royale lakes: Angleworm, Eva, Intermediate, Richie, Sargent, Shesheeb, Siskiwit, and Wagejo. These study lakes were selected to span a broad gradient of MeHg contamination, based on a survey of fish mercury levels in the mid 1990s (Kallemeyn, 2000). Northern pike (n = 124) were collected by gill net or hook-and-line and sacrificed on site according to protocols approved by the Miami University Institutional Animal Care and Use Committee. The sex of each northern pike was determined, and total length (cm) and wet weight (kg) were measured and used to calculate condition factor:

$$\text{condition factor} = [100{,}000 \times \text{wet weight}] / \text{total length}^3$$

Scales, skin-on fillets, and livers were sampled from northern pike. Scrupulous trace-metal clean techniques (Hammerschmidt et al., 1999) were used during dissection to minimize mercury

contamination. At least two scales from each northern pike were examined to estimate age (Jearld, 1983). Skin-on fillets and a subsample of liver were placed in individual food-grade plastic bags and frozen at -20 ˚C. A separate subsample of liver was preserved in 10% phosphate-buffered formalin for histology.

2.2. Mercury analysis

For total mercury, skin-on fillets and livers were acid digested according to US EPA Method 245.6 (U.S. Environmental Protection Agency, 1991) and analyzed by cold-vapor atomic absorption spectroscopy (Drevnick et al., 2006a). Duplicate samples, spiked samples, and certified reference materials (TORT-2, DORM-2) were digested and analyzed with each batch of samples. Mean relative standard deviation for duplicate samples was 7.5%. Recovery of known additions averaged 92.8%. Mean measured concentrations of reference materials were within (TORT-2) or 5.5% below (DORM-2) the certified ranges.

Ten livers were also selected for analysis of MeHg. Livers were lyophilized, digested with dilute nitric acid (Hammerschmidt and Fitzgerald, 2005), and analyzed by flow-injection gas chromatographic cold-vapor atomic fluorescence spectrometry (Tseng et al., 2004). Three samples were analyzed in duplicate with a mean relative standard deviation of 9.6%.

2.3. Liver Toxicity

During fish dissection, we serendipitously observed differences in liver color among northern pike, and developed a method to quantitatively measure this variation. A 100 mg piece of liver was homogenized in 1 mL water, 0.2 mL chloroform were added, and 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 with a spectrophotometer. It was determined during methods development that 400 nm was the wavelength with maximum absorbance for the supernatant. Each liver was analyzed in duplicate, and the results were deemed acceptable if the relative standard deviation between duplicates was <15%.

Ten formalin-preserved livers (subsamples from the same northern pike that were analyzed for MeHg) were viewed for histopathology and to identify the substance that caused

some livers to appear darkly colored. Livers were randomly assigned a unique random number to facilitate blind study, embedded in paraffin, cut into 6 μm sections, and mounted on glass slides (Presnell and Schreibman, 1997). One slide from each liver was deparaffinized, rehydrated, and stained with hematoxylin and eosin (Presnell and Schreibman, 1997). All slides were randomly assigned to one of two staining racks and stained in the same solution batches. Multiple sections on each stained slide were viewed with light microscopy at 200X magnification for histopathology. An additional unstained slide from each liver was viewed with fluorescence microscopy (excitation filter 355-425nm, suppression at 460nm; Woshner et al., 2002) at 200X magnification to identify lipofuscin, which from previous reports (Woshner et al., 2002; Raldúa et al., 2007) we hypothesized to be the substance that caused some livers to appear darkly colored.

Lipofuscin was quantified on a Zeiss Axio compound fluorescent microscope equipped with an Axiocam High Resolution camera (Carl Zeiss Inc., Berlin, Germany). A representative field of the liver of each individual was photographed at 200X, and a digital grid (squares 25μm x 25μm) was overlaid on the digital 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 lipofuscin was noted and recorded in each of the selected grid squares. The proportion of grid squares containing lipofuscin out of the total 50 assessed was used as a quantitative measure of the extent of liver damage. This process was carried out on three sections for each individual, and the average score of those three sections was used in the data analysis.

2.4. Data analysis

We analyzed data with SPSS for Windows software (version 14.0, SPSS Inc., Chicago, Illinois) and Blossom statistical software (version 2005.05.06, USGS, Fort Collins, Colorado). Least-squares, stepwise multiple, and quantile regression models were used to describe relationships between and among variables. Quantile regression was appropriately used for data with wedge-shaped distributions (Cade and Noon, 2003). The largest quantile for which the slope was statistically significant from zero ($P < 0.05$) was chosen. Analysis of covariance (ANCOVA) was used to test for the effect of liver total mercury concentration on liver color,

with age as a covariate. Except for stepwise multiple regression, data were transformed ($\log_{10}$ transformation for liver color, total mercury concentration in fillet and liver, and liver-to-fillet total mercury ratio; arcsine square root transformation for condition factor, quantitative lipofuscin data, and % MeHg of total mercury) to meet the assumptions of normality and homogeneity of variance. Data were not transformed for stepwise multiple regression for ease of interpretation. A type I error (α) of 0.05 was used to judge the significance of statistical tests.

3. Results and Discussion

3.1. Total mercury and MeHg

Concentrations of total mercury in skin-on fillets of northern pike ranged from 0.069 to 0.622 µg/g wet wt in the eight study lakes (Table 1). This variation is normal within and among fish populations and is due to a myriad of biological, chemical, and physical variables (Wiener et al., 2006). In general for lakes, fish highly contaminated with mercury (>1 µg/g wet wt total mercury in fillets) occur in systems that efficiently methylate inorganic mercury and incorporate the resultant MeHg into the food web (Wiener et al., 2003). Recently at Isle Royale, reduced rates of sulfate deposition have inhibited mercury methylation (Drevnick et al., 2007), and the data we present for advisory lakes represent a significant decline during the past decade in total mercury concentrations in skin-on fillets of northern pike.

Concentrations of total mercury in livers increased exponentially in relation to concentrations in skin-on fillets (least-squares regression, $r^2 = 0.76$, $P < 0.001$, $n = 124$), to a maximum of 3.1 µg/g wet wt (Fig. 1). Similar exponential relationships have been observed in other fish species (Goldstein et al., 1996; Cizdziel et al., 2003). Cizdziel et al. (2003) estimated that the concentration of total mercury in a liver will be less than or similar to that in the fillet when the concentration in the fillet is <0.5 µg/g wet wt. Conversely, when the concentration of total mercury in a fillet is >0.5 µg/g wet wt, the concentration in the liver will be greater than that of the fillet. This prediction largely holds true for our data. It is unknown, however, why this exponential increase occurs. Mercury speciation may be important for understanding the cause.

Methylmercury constituted a majority of the mercury in livers with total mercury concentrations <0.5 µg/g wet wt, but declined to 28-51% of the mercury in livers with total

mercury concentrations >0.5 µg/g wet wt (quantile regression, 99th quantile, $P = 0.042$, $n = 10$; Fig. 1 inset). These latter values are considerably lower than the nearly 100% MeHg reported for fish muscle (Bloom, 1992). Low % MeHg in liver has been reported in another study (Barghigiani et al., 1989) and is hypothesized to be due to *in vivo* demethylation (Cizdziel et al., 2003). However, fish have never been shown to be capable of demethylation (Wiener and Spry, 1996). Further, if demethylation were occurring in northern pike livers of this study, we would expect MeHg concentrations in livers to be less than total mercury concentrations in skin-on fillets. Instead, MeHg concentrations in livers are about the same or slightly greater (slope = 1.2) than total mercury concentrations in skin-on fillets. Rather than demethylation, we hypothesize the low % MeHg in livers is due to the accumulation of inorganic mercury from another source. Fish accumulate inorganic mercury much less efficiently than MeHg (Wiener and Spry, 1996), but macroinvertebrates (e.g., odonate larvae) that contain ~50% of total mercury as inorganic mercury (Hall et al., 1998) are a common prey item of northern pike in the lakes of Isle Royale. Inorganic mercury, as well as all other substances that are digested and pass through the intestinal wall, circulate first via the portal system to the liver. The liver thus has a "first pass" at accumulating inorganic mercury with metal-binding proteins before it circulates to other tissues. Metallothioneins (low molecular weight metal-binding proteins) have a high affinity for inorganic mercury, but not MeHg (Wiener and Spry, 1996), and have been shown in wild fish to be upregulated in liver in response to mercury exposure (Schlenk et al., 1995). Taken together, northern pike at Isle Royale are likely exposed to sufficient amounts of inorganic mercury and able to sequester in the liver what is accumulated through the gut, possibly explaining the high % inorganic mercury and low % MeHg in livers. With age and exposure to more mercury, this sequestration of "excess" inorganic mercury in the liver can also explain why there is more total mercury in livers than in fillets.

3.2. Liver Toxicity

Liver color (absorbance at 400 nm) was related positively to liver total mercury concentration (least-squares regression, $r^2 = 0.30$, $P < 0.001$, $n = 124$). Within a continuous gradient, lightly-colored, pink livers (Fig. 2A) had low total mercury concentrations and darkly-colored, red to brown livers (Fig. 2B) had high total mercury concentrations. Monitoring

programs use liver color as one of many indicators of general fish health (e.g., Schmitt and Dethloff, 2000), but the cause of variation in liver color among fish and its implications are often unknown.

We identified lipofuscin as the major pigment in darkly-colored livers. Lipofuscin was visible with light (Fig. 2C) and fluorescence (Fig. 2D) microscopy, and the quantitative lipofuscin data (Fig. 2E) explained 81% of the variation in liver color (least-squares regression, $r^2 = 0.81$, $P = 0.006$, $n = 7$). Only seven of the ten livers viewed for histopathology were quantified for lipofuscin because livers were overfixed, which made quantification difficult. Lipofuscin is a pigment that results from lipid peroxidation (i.e., oxidative stress) of membranous organelles and also is called the "pigment of aging", as it also accumulates in cells as a result of age-related processes (Porta, 1989). Accordingly, ANCOVA was used to test the influence of liver total mercury concentration on liver color with age as a covariate. In the ANCOVA model, liver total mercury concentration still had a significant effect on liver color ($F_{1,107} = 5.372$, $P = 0.022$) but age did not ($F_{7,107} = 1.758$, $P = 0.103$). Thus, the effect of mercury on lipofuscin was of sufficient magnitude to obscure any normal effect of aging. Indeed, lipofuscin is usually contained within cells (Porta, 1989), but livers from our study also showed lipofuscin granules concentrated in some parts of the extracellular matrix. A similar pattern of lipofuscin accumulation has been reported for other fish species with high concentrations of total mercury in liver (Raldúa et al., 2007). These results suggest a connection between mercury exposure and oxidative stress in Isle Royale northern pike.

We also observed a decrease in lipid reserves within hepatocytes in the livers of northern pike exposed to elevated concentrations of mercury. Hepatocytes took up stain in relation to lipid content. Lightly-colored livers appeared washed out after hematoxylin and eosin staining and had a relative abundance of lipid vacuoles, while darkly-colored livers took up more stain and had fewer lipid vacuoles. Unfortunately, however, we could not reliably quantify lipid content in hepatocytes because of overfixation of livers. de Oliveira Ribeiro et al. (2002) also documented a decrease in lipid reserves in livers of arctic charr (*Salvelinus alpinus*) exposed in the laboratory to a single dose of MeHg, but not in arctic charr exposed to a single dose of dietary inorganic mercury. This suggests that the mercury toxicity we observed in Isle Royale northern pike livers may be due to MeHg and not inorganic Hg. Methylmercury is more toxic than inorganic mercury (National Research Council, 2000), and metallothioneins protect cells

from inorganic mercury but not MeHg (Wiener and Spry, 1996). Although supported, this argument is speculative and more research should be conducted on the roles of MeHg and inorganic mercury in liver toxicity.

Fish health, as suggested by condition factor, was related inversely to total mercury in livers of Isle Royale northern pike (Fig. 3). Accordingly, fish with elevated levels of total mercury in the liver generally had more lipofuscin granules and decreased lipid reserves in the liver, and their overall condition was impaired. As is typical when MeHg limits a biological response (Hammerschmidt et al., 2002; Drevnick and Sandheinrich, 2003; Drevnick et al., 2006b), there is little to no relationship between condition factor and liver total mercury concentration at low concentrations, but as liver total mercury concentrations increase, the maximum values for condition factor decrease (quantile regression, 97^{th} quantile, $P = 0.040$, $n = 124$). Northern pike with the highest concentrations of total mercury in livers and lowest condition factors appeared emaciated. Rather than assigning causality to mercury exposure for low condition factors, Cizdziel et al. (2003) hypothesized that emaciated fish undergoing starvation deplete lipid reserves in the liver for energy, which concentrates mercury in the liver because it is primarily bound to sulfhydryl groups in the remaining protein. This hypothesis is plausible and may explain the decrease in lipid reserves we observed in northern pike livers. However, the decrease in lipid reserves de Oliveira Ribeiro et al. (2002) observed was in response to a single dose of dietary MeHg in arctic charr that were not emaciated or undergoing starvation. Further, oxidative stress and the accumulation of lipid peroxidation products (i.e., lipofuscin) would not be expected in livers from starvation alone (Abele et al., 2007). Our research and that of others (Lockhart et al., 1972; Cizdziel et al., 2003) have documented an inverse relationship between MeHg exposure and condition factor, but again more research is required to assign causality.

3.3. Implications

Northern pike at Isle Royale are experiencing toxicity at concentrations of total mercury common for northern pike and other piscivorous fish elsewhere in North America. From linear regressions of $\log_{10}$-transformed data (Sorensen et al., 1990), calculated concentrations of total mercury in skin-on fillets of legal-sized (61 cm) northern pike in the eight study lakes were 0.115

(Siskiwit), 0.218 (Intermediate), 0.230 (Richie), 0.236 (Angleworm), 0.302 (Sargent), 0.324 (Wagejo), 0.337 (Eva), and 0.472 (Shesheeb) µg/g wet wt. In comparison, Lockhart et al. (2005) reported a mean total mercury concentration of 0.378 µg/g wet wt for fillets from 1,169 northern pike (with a mean total length of 61 cm) from northern Canada. Even greater, Kamman et al. (2005) observed a mean total mercury concentration of 0.645 µg/g wet wt for fillets from 1,065 northern pike (also with a mean total length of 61 cm) from eastern Canada and the northeastern USA. In western North America, concentrations of total mercury in fillets of northern pike are generally lower than elsewhere (Peterson et al., 2007), but still may exceed 1.5 µg/g wet wt (Jewett et al., 2003). Further, in three major surveys (Kamman et al., 2005; Lockhart et al., 2005; Peterson et al., 2007), other fish species consistently had higher fillet total mercury concentrations than northern pike. Taken together, these comparisons indicate fish health may be affected by concentrations of total mercury common in fish across North America.

Along with serving as an indicator of MeHg toxicity, liver color may be used to predict concentrations of total mercury in fillets from northern pike at Isle Royale. Total mercury concentrations in skin-on fillets were significantly correlated to the color of livers (step-wise multiple regression, equation: skin-on fillet total mercury concentration = 0.081 + [0.254 x liver color], $r^2 = 0.511$, $F_{1,122} = 127.357$, $P < 0.001$; Fig. 4). If the regression model is expanded to include total length and wet weight, two variables commonly measured by people who catch and eat fish, nearly 70 % of the variation in total mercury concentrations in skin-on fillets is accounted for (equation: skin-on fillet total mercury concentration = -0.519 + [0.184 x liver color] + [0.015 x total length] + [-0.189 x wet weight], $r^2 = 0.690$, $F_{3,120} = 89.023$, $P < 0.001$). With careful development, this relationship could be used by anglers at Isle Royale to estimate MeHg exposure as they decide lakeside whether to eat their catch.

4. Conclusion

Very recently, Larose et al. (2007) reported a direct link between mercury exposure and oxidative stress in livers of wild fish. We extend that observation by reporting liver toxicity directly resulting from that oxidative stress. We previously linked oxidative stress to decreased hormone production and, consequently, inhibited reproduction in fathead minnows (*Pimephales promelas*) exposed to dietary MeHg (Drevnick et al., 2006b). Thus, mercury exposures

encountered by northern pike and other fishes at Isle Royale and elsewhere in North America may cause liver toxicity as well as inhibit their ability to reproduce (Scheuhammer et al., 2007).

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489 Table 1. Summary data for age, total length, wet weight, and concentrations of total mercury
490 (Hg_T) in skin-on edible fillets and livers of northern pike collected from lakes at Isle Royale,
491 Michigan, 2004-2006. SD represents one standard deviation.

		Fish Characteristics (means)			Hg_T (µg/g wet wt) in edible fillet			Hg_T (µg/g wet wt) in liver		
Lake	*n*	Age (y)	Length (cm)	Weight (kg)	Mean	SD	Range	Mean	SD	Range
Angleworm	12	4.3	51.4	0.80	0.171	0.138	0.077-0.528	0.392	0.859	0.058-3.074
Eva	12	5.2	52.5	0.81	0.262	0.115	0.133-0.463	0.284	0.192	0.092-0.766
Intermediate	20	5.0	52.3	0.85	0.171	0.072	0.096-0.424	0.181	0.224	0.061-1.107
Richie	16	4.9	56.0	0.97	0.200	0.073	0.114-0.346	0.176	0.052	0.115-0.267
Sargent	20	5.7	55.4	1.01	0.242	0.139	0.114-0.622	0.340	0.413	0.090-1.483
Shesheeb	14	5.4	55.0	0.90	0.299	0.119	0.142-0.471	0.221	0.098	0.091-0.394
Siskiwit	11	5.1	67.2	2.14	0.122	0.041	0.069-0.234	0.087	0.029	0.048-0.121
Wagejo	19	5.0	52.7	0.82	0.242	0.127	0.101-0.582	0.216	0.163	0.065-0.617

492

Figure Legends

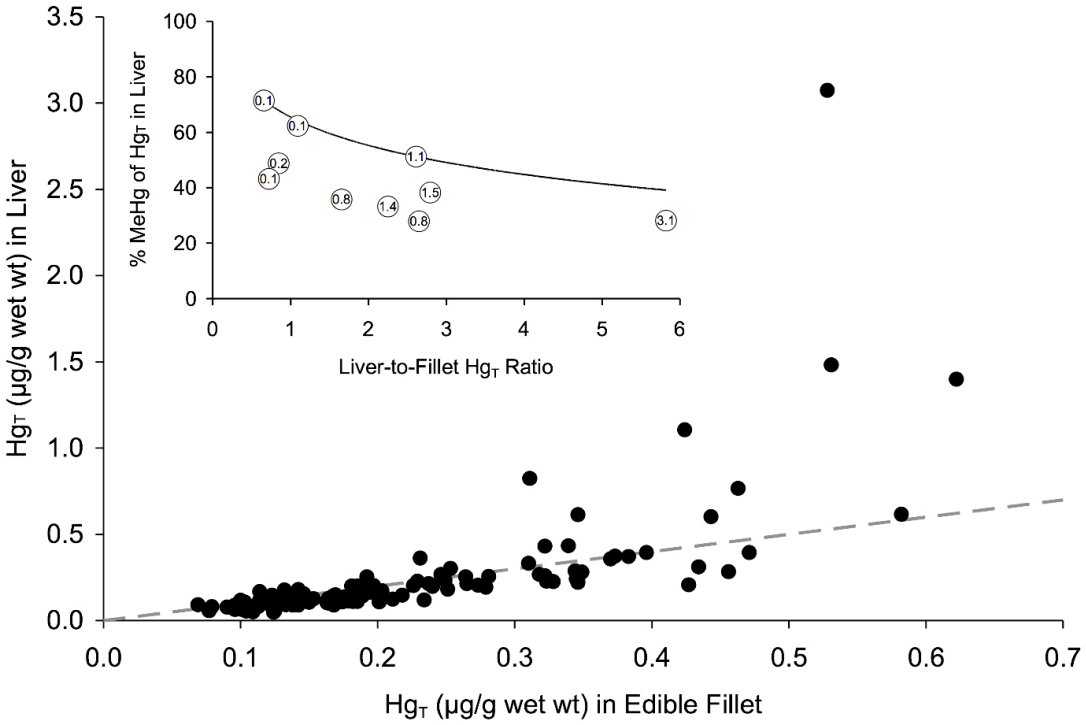
Fig. 1. Relationship between concentrations of total mercury (Hg_T) in skin-on edible fillets and livers of northern pike collected from lakes at Isle Royale, Michigan, 2004-2006. The dashed line represents a 1:1 relationship between variables. Inset graph shows the relationship between liver-to-fillet Hg_T ratio and % methylmercury (MeHg) of Hg_T in livers from ten of the northern pike. The numbers inside the data points represent liver Hg_T concentration. The trend line represents the 99th quantile.

Fig. 2. Mercury toxicity in livers of northern pike from Isle Royale, Michigan. Lightly-colored, pink livers (A) had low total mercury concentrations and darkly-colored, red to brown livers (B) had high total mercury concentrations. Lipofuscin was identified as the pigment in darkly-colored livers, with light (C) and fluorescence (D) microscopy. Arrows indicate lipofuscin granules. The mean proportion of grid squares (see materials and methods for explanation) containing lipofuscin (E) explained 81% of the variation in liver color (least-squares regression, $r^2 = 0.81$, $P = 0.006$, $n = 7$).

Fig. 3. Relationship between total mercury (Hg_T) concentration in livers and condition factor in northern pike from lakes at Isle Royale, Michigan. The trend line represents the 97th quantile.

Fig. 4. Relationship between liver color (as absorbance at 400 nm) and total mercury (Hg_T) concentration in skin-on edible fillets of northern pike from lakes at Isle Royale, Michigan.

515 Figure 1



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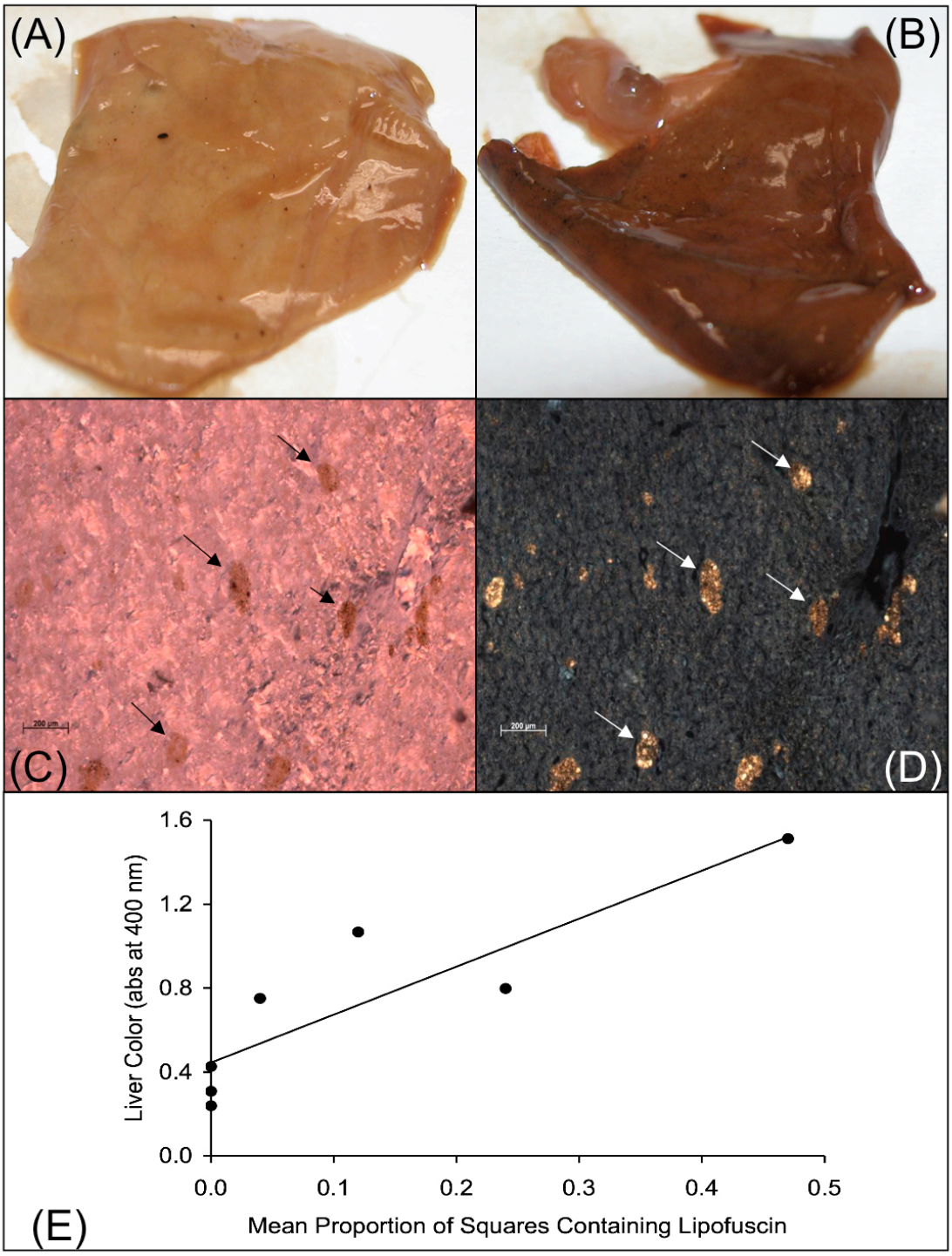
517 Figure 2



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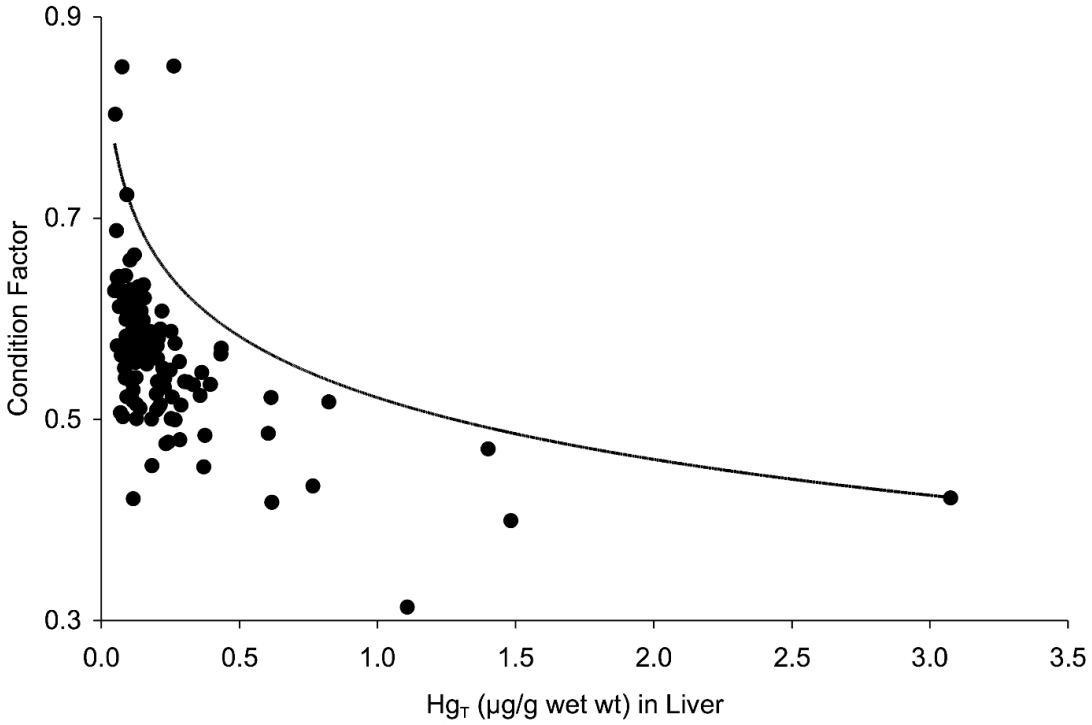

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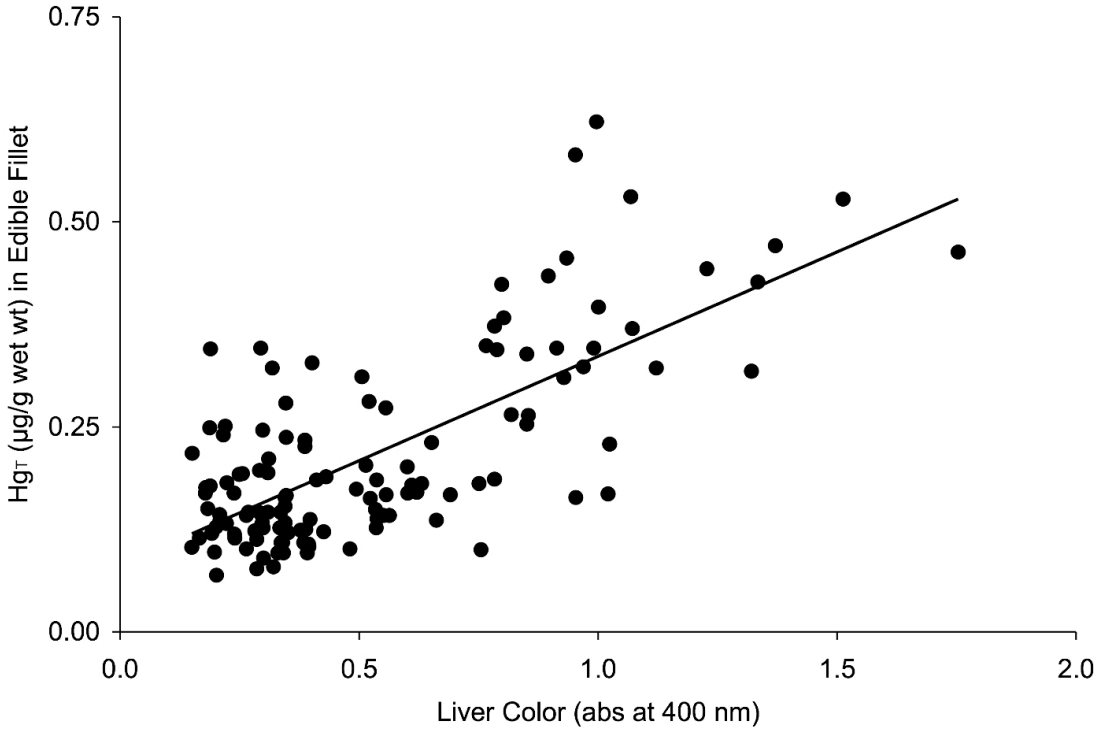
521 Figure 4



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