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ABSTRACT

METHYLMERCURY IN FISH:
ACCUMULATION, TOXICITY, AND TEMPORAL TRENDS
by: Paul E. Drevnick

Mercury contamination of fish is a serious problem, and consideration of policy options for a solution requires answering two questions. (1) What is the evidence that fish are being adversely affected by mercury? (2) How would mercury concentrations in fish respond to reduced anthropogenic emissions of mercury? I performed research integral to answering these questions. First, I studied mercury accumulation in northwest Atlantic sea lamprey, an agnathan fish. I discovered there are fundamental differences in mercury accumulation between sea lamprey and teleosts. All life stages of sea lamprey are exposed to high concentrations of mercury. More research should be directed towards understanding mercury contamination of diverse groups of fishes and its toxicological effects. Second, I studied the toxicological mechanisms of methylmercury in fish. Exposure to methylmercury impairs the reproduction of fish, and previous research has linked impaired reproduction in fish to the suppression of sex steroid hormones. I exposed fathead minnows to dietary methylmercury to test the hypothesis that methylmercury induces apoptosis in steroidogenic cells in fish, thereby interfering with the synthesis of sex steroid hormones. Methylmercury increased the number of apoptotic follicular cells in ovarian follicles, which in turn was related to suppressed 17β-estradiol concentrations in females. These results ultimately suggest apoptosis of steroidogenic cells as a mechanism for the impairment of reproduction in fish exposed to methylmercury. Third, I studied the response of mercury contamination in fish to changing atmospheric pollution. Serendipitously, I discovered a decline during the past decade in mercury concentrations in fish at Isle Royale, USA. Changes in mercury source have not caused the decline, as deposition has remained stable during the past decade. I examined sulfur concentrations in lake sediments to test the hypothesis that declines in sulfate deposition have resulted in less methylation of mercury and thus less mercury in fish. Results indicate mercury concentrations in fish are primarily a function of sulfate deposition. In sum, (1) fish are adversely affected by methylmercury and (2) mercury concentrations in fish will decline in response to reduced anthropogenic emissions of mercury *and* sulfur.

METHYLMERCURY IN FISH:
ACCUMULATION, TOXICITY, AND TEMPORAL TRENDS

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CHAPTER 1
INTRODUCTION

1.1. BACKGROUND

Mercury contamination of fish is a serious environmental problem. Atmospheric transport and deposition of anthropogenic emissions of mercury have contaminated aquatic ecosystems on a global scale (Fitzgerald et al. 1998). In aquatic ecosystems, microbes transform deposited inorganic mercury into a more toxic form, methylmercury (Winfrey and Rudd 1990). Methylmercury biomagnifies in aquatic food webs, resulting in elevated concentrations in fish (Watras and Bloom 1992). Methylmercury is toxic to fish and severely degrades the ecological services that fish provide (Wiener et al. 2003). Consumption of contaminated fish is the major source of methylmercury uptake in wildlife and humans and is associated with negative health effects (National Research Council 2000). At least 41 of 50 states in the U.S. have fish consumption advisories due to mercury contamination (U.S. Environmental Protection Agency 2001).

At the 8th International Conference on Mercury as a Global Pollutant (Madison, WI, August 6-11, 2006), participants identified two questions that are integral to ongoing consideration of policy options for addressing the environmental mercury problem:

(1) What is the evidence that humans, fish, wildlife, and other biota are being adversely affected by exposure to methylmercury (Scheuhammer et al. 2007)?

(2) How would methylmercury concentrations in fish respond to reduced anthropogenic emissions of mercury (Munthe et al. 2007)?

Indeed, the U.S. Environmental Protection Agency (2007) is now considering options to implement the Clean Air Mercury Rule. The Clean Air Mercury Rule was issued in 2005 with the intent to "protect children and pregnant women from the health impacts of mercury" by reducing emissions from coal-fired power plants. The estimated cost of implementing the Clean Air Mercury Rule is $4 billion. There is debate among scientists and economists of the merits of this costly rule because there is much uncertainty about the toxicological effects of methylmercury and little is known of the relationship between anthropogenic emissions of mercury and concentrations of methylmercury in fish (Gayer and Hahn 2005; Zeller and Booth

2005). Our knowledge of mercury in the environment must improve to adequately answer these questions.

Towards answering these questions, this dissertation contributes new knowledge of mercury fate and effects in the environment. I performed novel research about the accumulation, toxicity, and temporal trends of methylmercury in fish to fill gaps in knowledge integral to understanding the environmental mercury problem.

1.2. GAPS IN KNOWLEDGE

Mercury contamination throughout life cycles of diverse groups of fishes

Much of what is known about the accumulation of mercury in fishes comes from analyses of adult teleosts. This is due to a regulatory focus of characterizing the exposure of humans to mercury via consumption of fish. Greater than 99% (by weight) of fish caught and eaten in the U.S. are teleosts (National Marine Fisheries Service 2004). This focus on human health has resulted in little research directed towards understanding mercury contamination throughout life cycles of diverse groups of fishes and its potential toxicological effects (Wiener and Spry 1996). Ancient fishes, such as agnathans and chondrichthyans, have life histories and physiologies that can vary substantially from teleosts. These differences may significantly influence the amount of mercury accumulated (de Pinho et al. 2002). Moreover, mercury is most likely to be toxic to fish that are apex predators (Wiener et al. 2003). Lampreys (Agnatha) and sharks (Chondrichthyes) are common apex predators in marine food webs. Therefore, a comprehensive evaluation of the adverse effects of methylmercury exposure on fish must include knowledge of the ontogenetic dynamics of mercury accumulation in non-teleost fishes.

Toxicological mechanisms of mercury in fish

Exposure to methylmercury impairs the reproduction of fish. Laboratory studies have documented that concentrations of dietary methylmercury encountered by fish in some surface waters of North America result in (1) reduced and delayed spawning, (2) small gonads and less fecundity, and (3) altered reproductive behavior (Matta et al. 2001; Hammerschmidt et al. 2002; Sandheinrich and Miller 2006). Specific mechanisms are unknown, but likely relate to disruption of the hypothalamic-pituitary-gonadal (HPG) axis. The HPG axis regulates fish

reproduction. Hypothalamic secretion of gonadotropin-releasing hormone stimulates pituitary release of gonadotropins FSH and LH, which in turn act in the gonads to stimulate the synthesis of sex steroid hormones. Sex steroid hormones, such as testosterone (T) and 17β-estradiol (E2), are critical for successful reproduction (Fostier et al. 1983). My previous research provided strong evidence that reproductive failure in fathead minnows (*Pimephales promelas*) exposed to dietary methylmercury was related to suppressed levels of T and E2 (Drevnick and Sandheinrich 2003). Research to determine the mechanism suppressing levels of T and E2 is necessary to develop biomarkers that can be used to evaluate the effects of methylmercury exposure on wild fish populations (Chan et al. 2003).

Response of mercury contamination in fish to changing atmospheric pollution

Considerable effort is now being spent to determine how methylmercury concentrations in fish will respond to newly mandated reductions in anthropogenic emissions of mercury. For example, a whole-ecosystem experiment is being conducted in Ontario, Canada, in which mercury deposition to a lake is being manipulated (Hintelmann et al. 2002). However, little data actually link increased mercury deposition to increased methylmercury concentrations in fish (Hammerschmidt and Fitzgerald 2006). Further, factors other than mercury deposition must be important for the bioaccumulation of methylmercury in fish. Swain and Hellwig (1989) reported that, since preindustrial times in Minnesota, USA, mercury deposition has increased by 3X, while concentrations of methylmercury in fish have increased by 10X. Several studies suggest that sulfate, a major component of acid deposition, may have synergistic effects on the amount of methylmercury that eventually bioaccumulates in fish (e.g., Gilmour et al. 1992, Branfireun et al. 1999, Jeremiason et al. 2006). Sulfate deposition has been declining across North America and Europe since the 1970s due to controls on sulfur dioxide releases from anthropogenic sources (Stoddard et al. 1999). Thus, understanding how methylmercury concentrations in fish will respond to reduced anthropogenic emissions of mercury requires the consideration of other factors, especially sulfate.

1.3. OBJECTIVES

The goal of my research was to contribute to the understanding of the environmental mercury problem. I accomplished my goal by addressing the following objectives, which correspond in order to the preceding gaps in knowledge:

Objective 1: Determine the ontogenetic dynamics of mercury accumulation in northwest Atlantic sea lamprey (*Petromyzon marinus*).

Objective 2: Determine the mechanisms for reproductive toxicity in fish exposed to dietary methylmercury.

Objective 3: Determine temporal trends in mercury concentrations in fishes from inland lakes of Isle Royale National Park, USA.

1.4. ORGANIZATION OF DISSERTATION

This dissertation includes six chapters: an introduction (Chapter 1), four "data" chapters (Chapters 2-5), and a conclusion (Chapter 6). Data chapters describe original research and are written as manuscripts for publication. Chapter 2 corresponds to Objective 1, Chapters 3 and 4 correspond to Objective 2, and Chapter 5 corresponds to Objective 3. The concluding chapter includes a synthesis of results, implications for public policy on mercury, and recommendations for future research. References, tables, and figures appear at the end of individual chapters. Format may vary among chapters based on requirements for publication in the target journal.

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CHAPTER 2
ONTOGENETIC DYNAMICS OF MERCURY ACCUMULATION IN NORTHWEST ATLANTIC SEA LAMPREY (*PETROMYZON MARINUS*)[1]

2.1. INTRODUCTION

Much of what is known about accumulation of mercury in fishes comes from analyses of adult teleosts. This is due to a regulatory focus of characterizing the exposure of humans to mercury via consumption of fish. Greater than 99% (by weight) of fish caught and eaten in the U.S., for example, are teleosts (National Marine Fisheries Service 2004). This focus on human health has resulted in little research directed towards understanding mercury contamination throughout life cycles of diverse groups of fishes and its potential toxicological effects (Wiener and Spry 1996).

The ontogenetic dynamics of mercury accumulation in teleosts have been well characterized. Within a given population, concentrations of mercury in teleosts gradually increase with increasing age (e.g., Braune 1987; Gorski et al. 1999). Embryos are primarily exposed to mercury via maternal transfer in proportion to female exposure (Hammerschmidt et al. 1999; Johnston et al. 2001). Larvae, juveniles, and adults accumulate mercury proportional to the mercury content of their food items (Mathers and Johansen 1985), as food is the dominant pathway of mercury uptake in teleosts (Hall et al. 1997). Some teleost species can have pronounced increases in mercury accumulation with age, such as lake trout (*Salvelinus namaycush*), following a change in diet from invertebrates to prey fish (MacCrimmon et al. 1983). In total, a myriad of biological, chemical, and physical variables influence mercury accumulation in teleosts (Wiener et al. 2003).

Little is known about the ontogenetic dynamics of mercury accumulation in non-teleost fishes. Ancient fishes, such as agnathans and chondrichthyans, have life histories and physiologies that can vary substantially from teleosts. This may significantly influence the amount of mercury accumulated (de Pinho et al. 2002).

[1] Note: This chapter has been published in the *Canadian Journal of Fisheries and Aquatic Sciences* (63:1058-1066) with Horgan, M.J., Oris, J.T., and Kynard, B.E. as coauthors.

Sea lamprey (*Petromyzon marinus*) are agnathan fish with a life history that is considerably different from that of teleosts (Hardisty and Potter 1971). Larvae, called ammocoetes, are sedentary filter feeders that live in sediments of freshwater streams for approximately 3-7 years. At the end of the larval stage, ammocoetes metamorphose into parasitic-phase sea lamprey. Metamorphosing animals develop eye, mouth, and gut parts and migrate downstream to the ocean. In the ocean, juveniles (sexually immature) feed as external parasites on fish. Following 1-2 years of feeding, adults (sexually mature) complete the sea lamprey life cycle by migrating upstream in freshwater streams, spawning, and dying. The ontogenetic dynamics of mercury accumulation are not known in this unique life cycle.

The objective of our study was to examine the ontogenetic dynamics of mercury accumulation in Northwest Atlantic sea lamprey. Our results document high mercury concentrations for ammocoetes and highly variable concentrations of mercury in adults. We explain these results by measuring variables (i.e., sediment mercury, stable isotopes) hypothesized to be important for mercury accumulation in sea lamprey. These findings suggest that there are fundamental differences in mercury accumulation between teleosts and ancient fishes and, in particular, that these differences result in the exposure of sea lamprey to high concentrations of mercury.

2.2. MATERIALS AND METHODS

We studied the ontogenetic dynamics of mercury accumulation in sea lamprey from the Connecticut River and its tributaries, Massachusetts, U.S.A. (Figure 2.1). Sea lamprey occur as native, anadromous populations along the Atlantic coasts of Europe and North America. The Connecticut River is believed to have the largest population of sea lamprey in the Northwest Atlantic (Beamish 1980). Indeed, the number of migrating adults has been monitored since 1975 and usually exceeds 20000 individuals per year (Stier and Kynard 1986; U.S. Fish and Wildlife Service 2005).

Ammocoetes, metamorphosing animals, and adults were collected in 2003 and 2004. Ammocoetes were sampled from a canal of the Connecticut River in Turners Falls, Massachusetts, in summer during both years of the study. The canal is used for the generation of electricity; ammocoetes were sampled by hand as water level was drawn down for annual

maintenance on the power station. Metamorphosing animals were likewise collected in a canal of the Connecticut River in Holyoke, Massachusetts, in the autumn of 2004. Adults were collected during their annual migration/spawning in spring with dip nets from the Fort River (tributary of the Connecticut River near Amherst, Massachusetts) in 2003 and from the mainstem of the Connecticut River in Turners Falls in 2004. Samples were placed in food-grade plastic bags, frozen (-20 °C) within a few hours of collection, and transported to Miami University (Oxford, Ohio) for processing.

Sea lamprey were measured for total length and wet weight, and eggs were dissected from adult females. Length-frequency distributions were used to classify ammocoetes into four age groups (Hardisty and Potter 1971). There is some uncertainty in this classification procedure because it has not been validated for sea lamprey. We deliberately selected ammocoetes from the center of the putative age modes to maximize the likelihood of accurate age classification. Ammocoetes from four age groups (1-4) were collected. No young-of-the-year (age 0) ammocoetes were collected, as they may not be present at the sampling site at this time of year. Instead, eggs were used to assess the concentration of mercury in sea lamprey at the beginning of their life cycle. Eggs were dissected from the ovaries of gravid females through a ventral incision anterior to the urogenital opening with stainless steel implements.

Individual sea lamprey were acid-digested and analyzed for total mercury. Eggs and small ammocoetes (ages 1-3) were digested whole. Large larvae (age 4), metamorphosing animals, and adults were homogenized individually with a porcelain mortar and pestle (ammocoetes, metamorphosing animals) or blender (adults) and digested in duplicate. Digestions were performed according to U.S. EPA Method 245.6 (U.S. Environmental Protection Agency 1991). Each digestate was analyzed by cold-vapor atomic absorption spectroscopy with a Varian 220FS SpectrAA and Vapor Generation Accessory. The method detection limit (American Public Health Association et al. 1995) for this procedure was estimated to be 17 ng $Hg \cdot g^{-1}$ wet weight.

To explain the results of mercury analyses, we performed further analyses of variables that were considered important for the accumulation of mercury in ammocoetes (i.e., concentrations of mercury in stream sediment) and adults (i.e., carbon and nitrogen isotope compositions). Ammocoetes live burrowed in sediment in freshwater streams. Their diet consists of biofilm on detritus, algae, and sediment (Sutton and Bowen 1994). Due to the close

association of this life stage with sediment, we hypothesized that concentrations of mercury in ammocoetes would reflect or be positively correlated with concentrations of mercury in the sediment where they live. To test this hypothesis, we collected ammocoetes, by electrofishing, and sediments from three tributaries (Fort, Miller, and Sawmill Rivers) and the canal of the Connecticut River in Turners Falls in the summer of 2004. Concentrations of mercury were determined in ammocoetes (as described above) and sediments and compared among streams. For adults, we hypothesized that the concentration of mercury in an individual would be a function of its trophic status. Mercury concentrations in fish, within a given body of water, increase with increasing trophic status (Cabana et al. 1994; Kidd et al. 1995). We used isotope ratios of nitrogen, an indicator of trophic status (Peterson and Fry 1987), to test this hypothesis. Isotope ratios of carbon were also used to speculate as to the sources (Peterson and Fry 1987) of mercury in adults (e.g., benthic versus pelagic fish).

Sediment samples were acid-digested and analyzed for total mercury. Samples were collected to a depth of three centimeters with a plastic scoop. Samples were placed in polypropylene containers, frozen (-20 °C) within a few hours of collection, and transported to Miami University. Sediments were then dried at room temperature, ground with a porcelain mortar and pestle, and passed through a 2-mm stainless steel sieve (Rada et al. 1993). Samples were then digested in duplicate according to U.S. EPA Method 245.5 (U.S. Environmental Protection Agency 1991). Each digestate was analyzed for total mercury as described above. The method detection limit for this procedure was estimated to be 5 ng $Hg \cdot g^{-1}$ dry weight.

Stable carbon and nitrogen isotope compositions of adult sea lamprey were analyzed at the UC Davis Stable Isotope Facility, Davis, California. Homogenized tissue samples were dried in an oven at 65 °C, ground to a flour-like texture, and packaged (~ 1 mg) into tin capsules. Samples were then combusted and analyzed with an isotope ratio mass spectrometer (PDZ Europa Hydra 20/20). Details of analytical methods and derivation of values for $\delta^{13}C$ and $\delta^{15}N$ have been reported elsewhere (e.g., Cloern et al. 2002).

We ensured the reliability and validity of our data by strict adherence to quality assurance and quality control procedures (American Public Health Association et al. 1995). Sea lamprey and sediments were processed with gloved hands on the inner surface of food-grade plastic bags. Gloves and bags were changed between samples. All implements used in contact with samples were soap (stainless steel only) or acid washed. Glassware used in digestions and mercury

analyses was acid washed. Reagents used in digestions and mercury analyses were suitable for trace metal analysis. The accuracy of mercury determinations was assessed by digesting and analyzing with each analytical batch of samples (1) procedural blanks, (2) calibration standards, (3) replicate subsamples of sea lamprey or sediment, (4) procedurally spiked samples, and (5) certified reference materials from the National Research Council of Canada (TORT-2 and DORM-2 for sea lamprey analyses, PACS-2 for sediment analyses). Method precision (relative standard deviation), estimated from replicate analyses of subsamples, averaged 5% in sea lamprey and 11% in sediment. Mean recovery of spiked samples was 94% and 99% in sea lamprey and sediment, respectively. Mean measured concentrations of total mercury in two of the three reference materials (TORT-2, PACS-2) were within certified ranges. Mean concentration of the third material (DORM-2) was within 2% of the lower limit of the certified range. For stable isotope analysis, accuracy was assessed by analyzing laboratory standards before and after every twelve samples. Measured values were within 2% of the known values for carbon and nitrogen standards. Method precision was again estimated by replicate analyses of sea lamprey. Mean relative standard deviation for carbon and nitrogen isotopes were 0.3% and 3%, respectively.

Data analysis was performed with SPSS for Windows software (version 13.0, SPSS Inc., Chicago, Illinois). Regression models were used to describe relationships between variables and to test our specific hypotheses that (1) concentrations of mercury in ammocoetes would reflect that of the sediment in their nursery streams and (2) the concentration of mercury in an individual adult would be a function of its trophic status. A student's t-test was used to compare mercury concentrations between sexes of adults. Analysis of variance (ANOVA) was used to test for effects of sampling year and age group on mercury concentrations and wet weights in ammocoetes. Analysis of covariance (ANCOVA) was used to again test for effects of sampling year and age group on mercury concentrations in ammocoetes, but with wet weight as a covariate. Assumptions of statistical tests were validated before analysis. A Type I error (α) of 0.05 was used to judge the significance of all statistical tests.

2.3. RESULTS

Concentrations of mercury were lowest in eggs, increased with size in ammocoetes, decreased during metamorphosis, and varied considerably in adults (Figure 2.2). Eggs had a mean (± standard deviation) concentration (for both years) of 84 (± 64) ng Hg·g^{-1} wet weight. Concentrations in eggs were positively correlated to those in females from which they were dissected ($r^2 = 0.935$, $p < 0.001$, $n = 16$; Figure 2.3). Concentrations in eggs were approximately 20% of those in females. Mercury concentrations increased approximately 4-fold from eggs to age-1 ammocoetes. Predictably, ammocoetes exhibited increased concentrations of mercury with wet weight (2003: $r^2 = 0.163$, $p = 0.010$, $n = 40$; 2004: $r^2 = 0.505$, $p < 0.001$, $n = 40$), to a mean concentration of 492 (± 88) ng·g^{-1} wet weight in age-4 ammocoetes. The mean concentration of mercury in metamorphosing animals was 10% less than that in age-4 ammocoetes. Mercury concentrations were highly variable in adults, ranging from 83-942 ng·g^{-1} wet weight. Concentrations in adults were unrelated to sex ($t_{30} = -0.886$, $p = 0.383$), length ($r^2 = 0.071$, $p = 0.141$, $n = 32$), or weight ($r^2 = 0.102$, $p = 0.074$, $n = 32$).

Differences were evident between years and among age groups for concentrations of mercury in ammocoetes at Turners Falls. Mercury concentrations were greater in ammocoetes in 2003 than 2004 (ANOVA; year $F_{1,72} = 20.5$, $p < 0.001$; age $F_{3,72} = 14.8$, $p < 0.001$). These differences may be related to concomitant differences in wet weights of ammocoetes. Wet weights were also greater in 2003 than 2004 (ANOVA; year $F_{1,72} = 12.1$, $p < 0.001$; age $F_{3,72} = 403.6$, $p < 0.001$). As we have shown that mercury concentrations of ammocoetes are significantly correlated to wet weights, we analyzed mercury concentrations again but with wet weight as a covariate. Results (ANCOVA; year $F_{1,71} = 21.0$, $p < 0.001$; age $F_{3,71} = 2.9$, $p = 0.041$) suggest that increased concentrations of mercury in older and larger ammocoetes are primarily a function of wet weights although age still has a significant effect. In fact, post-hoc analysis of the ANCOVA (Tukey HSD; $p < 0.001$) indicates that the significant age effect results from age-4 ammocoetes having higher mercury concentrations than all younger age groups when the effect of size is factored out.

Calculated concentrations of mercury in ammocoetes standardized to a wet weight of one gram were positively correlated to mean mercury concentrations in sediments from the four streams sampled ($r^2 = 0.948$, $p = 0.026$, $n = 4$; Table 2.1). Standardized concentrations were

used to account for the effect of wet weight on mercury concentration in ammocoetes and were calculated with least-squares linear regression models.

Stable isotope enrichments of carbon and nitrogen were each positively correlated to concentrations of mercury in adult sea lamprey ($\delta^{13}C$: $r^2 = 0.546$, $p < 0.001$, $n = 31$; $\delta^{15}N$: $r^2 = 0.303$, $p = 0.001$, $n = 32$; Figure 2.4). One extreme outlier was excluded from the regression analysis between $\delta^{13}C$ and mercury concentration, although the relationship was statistically significant with this value included in the dataset ($r^2 = 0.278$, $p = 0.002$, $n = 32$). Stable isotope values ranged from -19.3‰ to -16.0‰ for $\delta^{13}C$ and from 11.4‰ to 14.3‰ for $\delta^{15}N$.

Body burdens of mercury, defined as the total mass of mercury in an individual sea lamprey, greatly increased from ammocoetes to adults ($r^2 = 0.982$, $p < 0.001$, $n = 122$; Figure 2.5). Burdens were not calculated for eggs because they were not weighed and analyzed individually, but as masses. Even though ammocoetes and metamorphosing animals had relatively high mercury concentrations, they had low mercury burdens because of their small sizes. Adults were much larger and had burdens, on average, two orders of magnitude greater than earlier life stages.

2.4. DISCUSSION

The ontogenetic dynamics of mercury accumulation in sea lamprey are unlike those in teleosts. The reasons for this difference are likely the unique life history and physiology of lampreys and possibly other factors, such as mercury source and speciation. The life history of egg-ammocoete-metamorphosing animal-adult is exclusive to these ancestral vertebrates. Further, many physiological differences between sea lamprey and teleosts are known (Hardisty and Potter 1971). For example, shortly after methylmercury is ingested with food, it passes from the gut to the circulatory system, binding to red blood cells, and is then distributed throughout the body (Oliveira Ribeiro et al. 1999). In red bloods cells of teleosts and other more modern vertebrates, methylmercury is bound to cysteine residues of hemoglobin (Doi 1991). The hemoglobin molecule of sea lamprey is considerably different from that of more modern vertebrates (Hombrados et al. 1983). It is unknown how this affects mercury toxicokinetics, but cysteine residues may be more abundant in proteins of sea lamprey than more modern vertebrates (Nonaka and Takahashi 1992). Cysteine is not only important for the binding of

methylmercury in red blood cells, but is also the predominant biological ligand for methylmercury in cells (Harris et al. 2003). Mercury source and speciation may also contribute to differences in mercury accumulation. The sources and speciation of mercury in sea lamprey are unknown. Food is the most likely source of mercury to sea lamprey, but this has not been shown. Nearly all (> 95%) mercury in teleosts has been reported to be methylmercury (Bloom 1992) from dietary sources (Hall et al. 1997). This predominance of methylmercury occurs because teleosts assimilate methylmercury much more efficiently from food than inorganic mercury, and elimination of methylmercury is much slower than it is for inorganic mercury (Trudel and Rasmussen 1997). It would be worthwhile to determine if dietary methylmercury is also (as for teleosts) the predominant pathway of mercury into sea lamprey.

Whereas concentrations of mercury in eggs were lowest throughout the sea lamprey life cycle, there appeared to be a higher rate of maternal transfer of mercury to eggs compared to teleosts. Concentrations of mercury in eggs were positively correlated to concentrations in females, as has been reported for teleosts (Hammerschmidt et al. 1999; Johnston et al. 2001). However, concentrations in eggs were 20% of that in females. This is a much greater transfer of mercury from female to egg than is known for teleosts (< 3%; Niimi 1983). This disparity may be because sea lamprey are semelparous, whereas the teleosts in which this has been studied are iteroparous. Semelparous species invest substantially more resources in gonadal development than iteroparous species in a given year (Calow 1979), and may transfer more mercury into gametes as a result of this investment.

Ammocoetes reflect the level of mercury contamination in their nursery streams, as evidenced by the strong relationship in mercury concentration between ammocoetes and sediments. We do not suggest that ammocoetes accumulate mercury from the sediments where they burrow, but that concentrations in ammocoetes and sediments are positively correlated because they both reflect the level of contamination within a watershed. The streams sampled had mercury concentrations in sediments representative of relatively uncontaminated watersheds (Gray et al. 2004). Still, concentrations of mercury were high in ammocoetes. Renaud et al. (1998) remarked of this "exceptionally high bioaccumulation capacity" and suggested that ammocoetes may be suitable biomonitors for mercury contamination in streams. Our results support this, but a wider range of contamination should be tested if this is to be useful.

Annual differences in concentrations of mercury in ammocoetes may be related to the amount of food ingested. Snyder and Hendricks (1995) determined that concentrations of mercury in filter-feeding organisms are related to the amount of food consumed. This relation could explain why ammocoetes generally had higher mercury concentrations in the year that they also had higher growth (both higher in 2003 than 2004; growth rate indicated by wet weight). Higher streamflow in 2003 (496 $m^3 \cdot s^{-1}$) than in 2004 (394 $m^3 \cdot s^{-1}$) (U.S. Geological Survey 2005) may have delivered more food to ammocoetes resulting in greater growth and mercury concentrations. This conclusion contrasts bioenergetic models that predict an inverse relationship between teleost mercury concentration and growth rate (Harris and Bodaly 1998). Tests of this "growth dilution" hypothesis have not been supportive, however, and further modeling has shown that concentrations of mercury in fish are much more responsive to changes in the amount of mercury consumed in food than changes in growth (Stafford and Haines 2001).

Two trends in accumulation of mercury in ammocoetes emerged from the data: (1) concentrations of mercury were higher in 2003 than 2004 and (2) age-4 ammocoetes had higher mercury concentrations than younger ammocoetes, after accounting for size. One could analyze the mercury data by cohort rather than by year by comparing, for example, age 1 in 2003 to age 2 in 2004. Three such comparisons are possible, and in two out of three comparisons, the cohort clearly has lower mercury concentrations in the second year. This pattern might suggest loss of mercury with age, however, loss is unlikely as the oldest ammocoetes had the highest mercury concentrations of all age classes. We argue this pattern is more likely driven by different environmental conditions (e.g., streamflow) between the two years, which also affected weight at age. Ammocoetes increase their biomass 2- to 5-fold from one age class to the next, allowing ample opportunity for interannual environmental differences in mercury availability to be expressed in whole body concentrations. This study design cannot distinguish between environmental and ontogenetic mechanisms among age classes, but strongly suggests that the oldest ammocoetes have the highest mercury concentrations.

Mercury concentrations appeared to decrease during metamorphosis. This decrease was not unexpected, given that metamorphosing animals do not feed for an extended period (Hardisty and Potter 1971) and hence do not accumulate mercury from dietary sources. There is some uncertainty in this interpretation, however, because metamorphosing animals were not collected at the same time and place as age-4 ammocoetes. Consequently, we cannot rule out that

metamorphosing animals originated from a different location with less mercury than age-4 ammocoetes. Further, this conclusion is dependent upon the assumptions that all metamorphosing animals were from age 4 and diet was their main source of mercury.

The high variability in concentrations of mercury in adult sea lamprey is uncommon among fishes. In a given population of teleosts, mercury concentrations in adults are generally greater in females than males and increase with size (Wiener et al. 2003). This trend is not the case for the sea lamprey population we studied. Differences are likely due to the feeding ecology and abundance of potential habitats of parasitic sea lamprey.

Parasitism by sea lamprey of numerous food sources at multiple trophic levels in the marine environment likely results in highly variable mercury concentrations in adults. This finding is indicated by the results of stable isotope analyses. The range of enrichment of $\delta^{15}N$ in this study (maximum value minus minimum value) was 2.9‰. Food web studies have shown that $\delta^{15}N$ is enriched by approximately 3.4 ‰ per trophic level (Vander Zanden and Rasmussen 2001). Thus, adult sea lamprey sampled for this study did not feed at a discrete trophic level. Further, a retrospective analysis of data presented by Fry (1988) of the food web structure on Georges Bank, a potential foraging site for sea lamprey in the Northwest Atlantic, reveals that $\delta^{13}C$ values $\geq$ -17.3‰ are characteristic of benthic fishes, whereas $\delta^{13}C$ values < -17.3‰ are characteristic of pelagic fishes. Carbon stable isotope values are also enriched with trophic level by about 0.8‰ (Vander Zanden and Rasmussen 2001); however, we observed a range of 3.3‰ for $\delta^{13}C$. Consequently, adult sea lamprey in our study appear to have fed on fish from both benthic and pelagic habitats. Host attachment and scarring data verify that sea lamprey in the Northwest Atlantic do indeed parasitize many species in at least three trophic levels (Beamish 1980; Halliday 1991). With the knowledge that mercury concentrations vary among potential host species and with trophic level in the marine environment (Atwell et al. 1998), it is reasonable to conclude that it is this nondiscrete feeding that results in highly variable concentrations of mercury in adult sea lamprey. Reports from other taxa also support this conclusion (Atwell et al. 1998; Bearhop et al. 2000).

The concentration of mercury in an individual adult sea lamprey is indeed a function of its food source and trophic status, however, there is still much unexplained variability. Much of this is probably related to the multitude of marine ecosystems that sea lamprey can inhabit. The marine distribution of sea lamprey includes nearshore and offshore areas at shallow, midwater,

and deep depths (Beamish 1980; Halliday 1991). Mercury contamination varies spatially. Further complications include the incomplete knowledge of dispersal by juvenile (from natal streams) and homing by adult (to spawning streams) sea lamprey in the Atlantic Ocean.

Greater than 99% of mercury accumulated during the sea lamprey life cycle is by juvenile/adults from marine sources. The ontogenetic diet shift from filter feeder to parasite results in substantial growth during the latter life stage. In our study, sea lamprey increased in size from approximately 4 g as age-4 ammocoetes to 600 g as adults. Ammocoetes have a diet that is poor in caloric value (Bowen et al. 1995), whereas the diet of juveniles/adults, which is primarily blood, is rich in calories (Farmer et al. 1975). Juveniles/adults thus assimilate more energy and nutrients with less effort and grow to larger sizes (Colinvaux 1978). Blood is a significant reservoir for mercury in fish (Oliveira Ribeiro et al. 1999), and juvenile/adult sea lamprey accumulate their high burdens of mercury because they feed on the blood of fish. Marked increases in mercury accumulation following ontogenetic diet shifts have been reported in other fish as well (MacCrimmon et al. 1983), but not to the extent we measured in sea lamprey.

Concentrations of mercury in adult sea lamprey are high enough to be deemed unsafe for human consumption. Sea lamprey are not commonly eaten by humans in North America, but are regularly used as food in Europe (Collette and Klein-MacPhee 2002). Fifteen of 32 adult sea lamprey in this study exceeded the maximum level set by the European Union for mercury in fishery products (500 $ng \cdot g^{-1}$ wet weight; Commission of the European Communities 2001).

Sea lamprey themselves are exposed to potentially toxic concentrations of mercury. In laboratory studies, teleosts with mercury concentrations as low as 1000 $ng \cdot g^{-1}$ wet weight have displayed behavioral, physiological, and reproductive anomalies (Hammerschmidt et al. 2002; Drevnick and Sandheinrich 2003). Maximum concentrations of mercury in adults of this study approached this value. Adult sea lamprey in the Great Lakes can have mercury concentrations that greatly exceed 1000 $ng \cdot g^{-1}$ wet weight (Evans et al. 1972; MacEachen et al. 2000). This has not been of concern, however, because sea lamprey are an invasive species in the Great Lakes. Sea lamprey in their native range and other lamprey species are generally in need of conservation measures to ensure their survival (Renaud 1997).

In conclusion, there are fundamental differences in mercury accumulation between sea lamprey and teleosts. All life stages of sea lamprey are exposed to relatively high concentrations

of mercury, especially adults. Other ancient fishes may also be exposed to high concentrations of mercury. More research should be directed towards understanding mercury contamination throughout life cycles of diverse groups of fishes and its possible toxicological effects.

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Table 2.1. Results of sea lamprey (*Petromyzon marinus*) ammocoete and sediment sampling from the Connecticut River and tributaries.

	Ammocoete		Sediment	
River	*n*	Hg (ng · g^{-1} wet weight)	*n*	Hg (ng · g^{-1} dry weight)
Connecticut	40	323 (75)	6	10.7 (2.8)
Fort	10	375 (88)	4	11.5 (1.4)
Millers	10	459 (123)	4	18.1 (2.7)
Sawmill	10	485 (167)	4	20.6 (3.1)

Note: *n* represents the number of samples analyzed from each stream. Mercury values for ammocoetes represent calculated concentrations in 1-g individuals. Mercury values in sediments represent mean concentrations. Numbers in parentheses represent standard error.

Figure 2.1. Location of collection sites for sea lamprey (*Petromyzon marinus*) in the Connecticut River and tributaries in Massachusetts, U.S.A. The point of each triangle nearest the stream indicates the collection site.

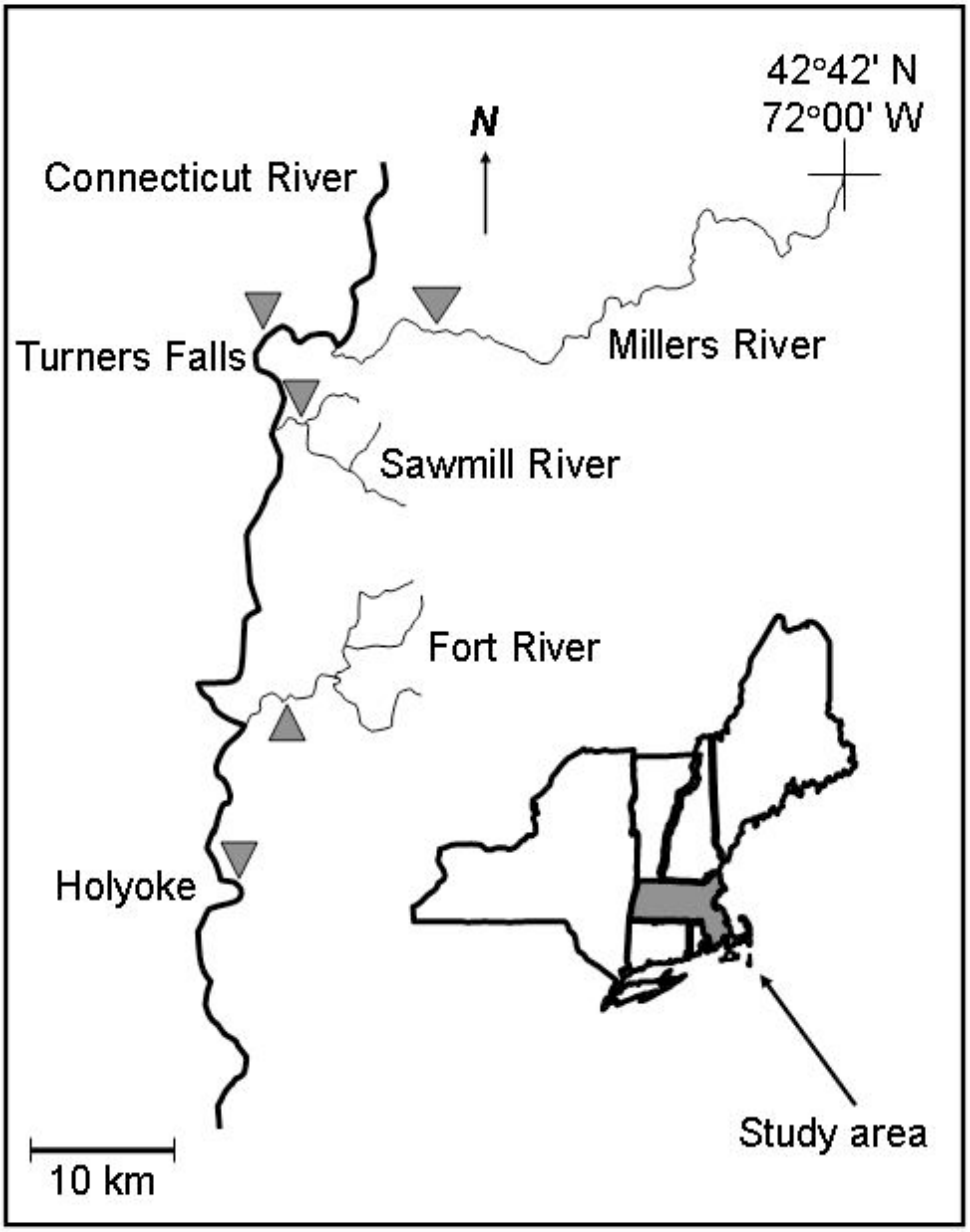


Figure 2.2. Concentrations of mercury in life stages of sea lamprey (*Petromyzon marinus*) from the Connecticut River. White and gray boxes represent ammocoetes collected in 2003 and 2004, respectively. Horizontal lines within each box represent medians. Boxes represent interquartile ranges (IQ; middle 50% of values). Vertical lines extend to lowest and highest values which are no greater than 1.5 times IQ. Circles indicate outliers. Numbers indicate number of individuals analyzed.

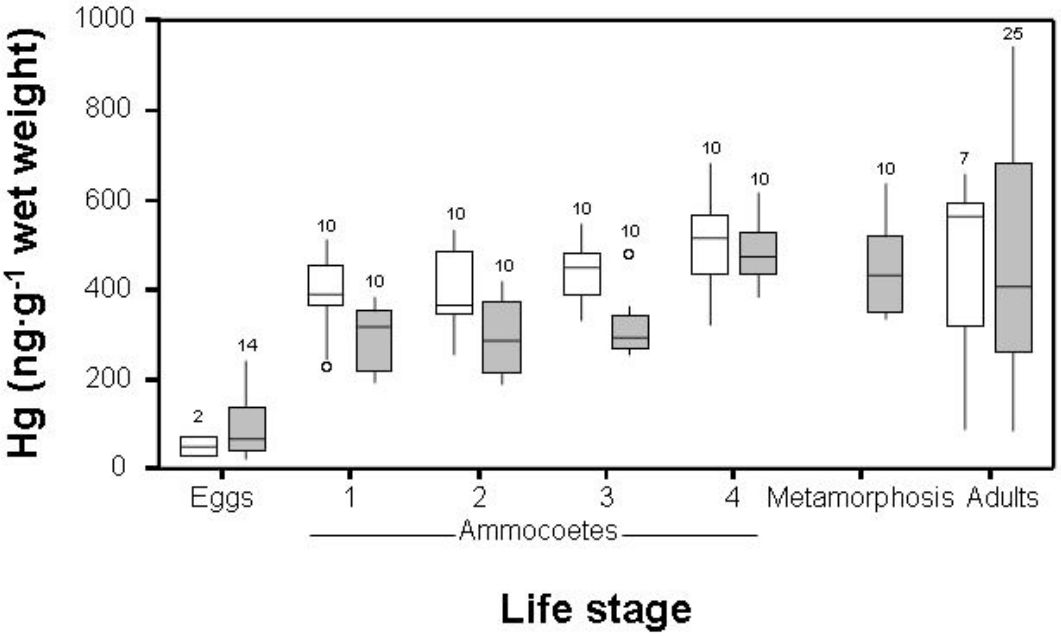


Figure 2.3. Relationship between concentrations of mercury in eggs and female sea lamprey (*Petromyzon marinus*) collected from the Connecticut River. Circles and triangles represent samples from 2003 and 2004, respectively. Regression equation: $y = 19.15e^{0.0027x}$; $r^2 = 0.935$; $p < 0.001$.

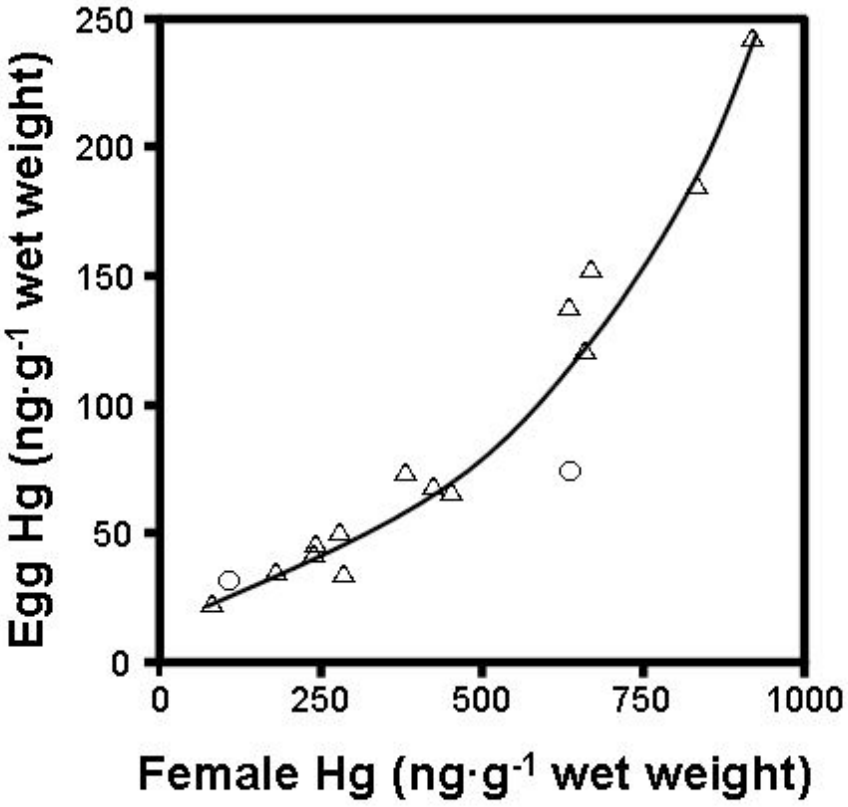


Figure 2.4. Relationships of carbon (a) and nitrogen (b) isotope compositions with mercury concentrations in adult sea lamprey (*Petromyzon marinus*) collected from the Connecticut River. Circles and triangles represent samples from 2003 and 2004, respectively. The symbol "x" represents an outlier from 2003.

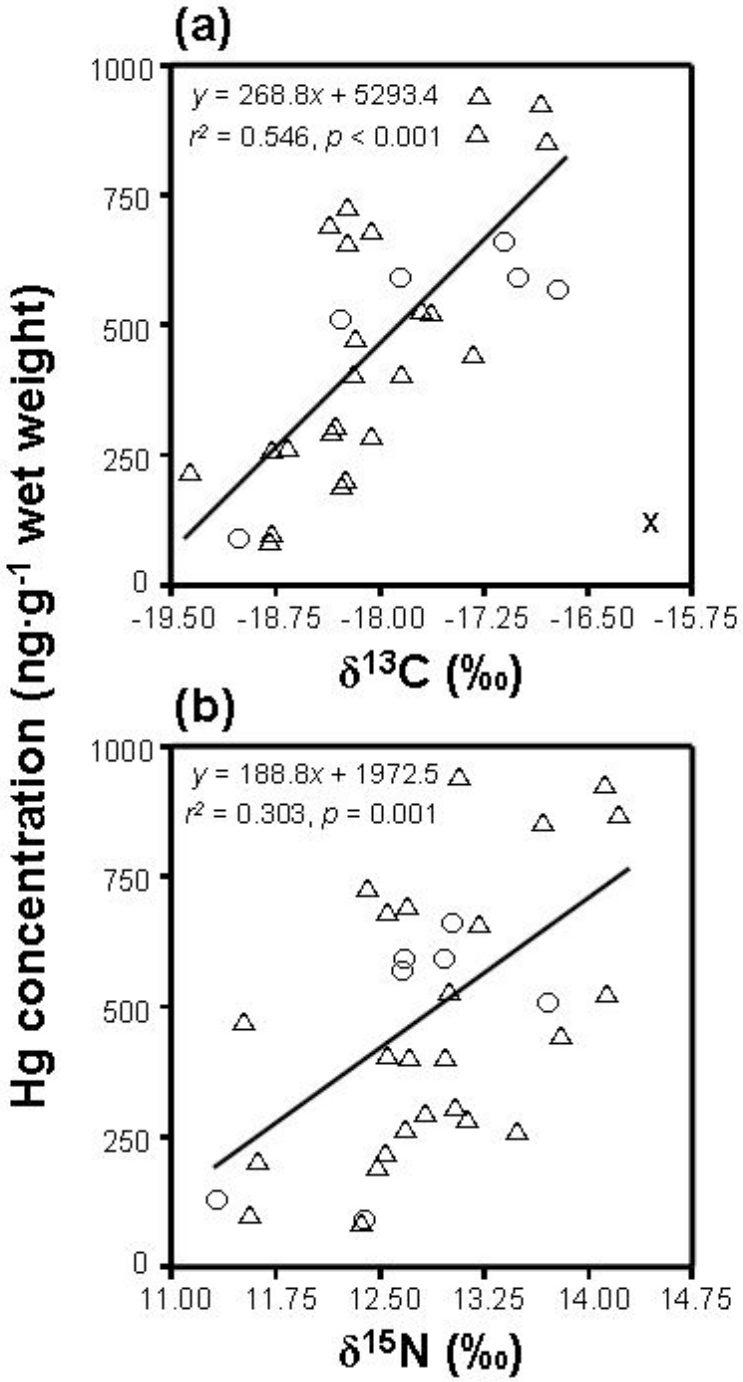
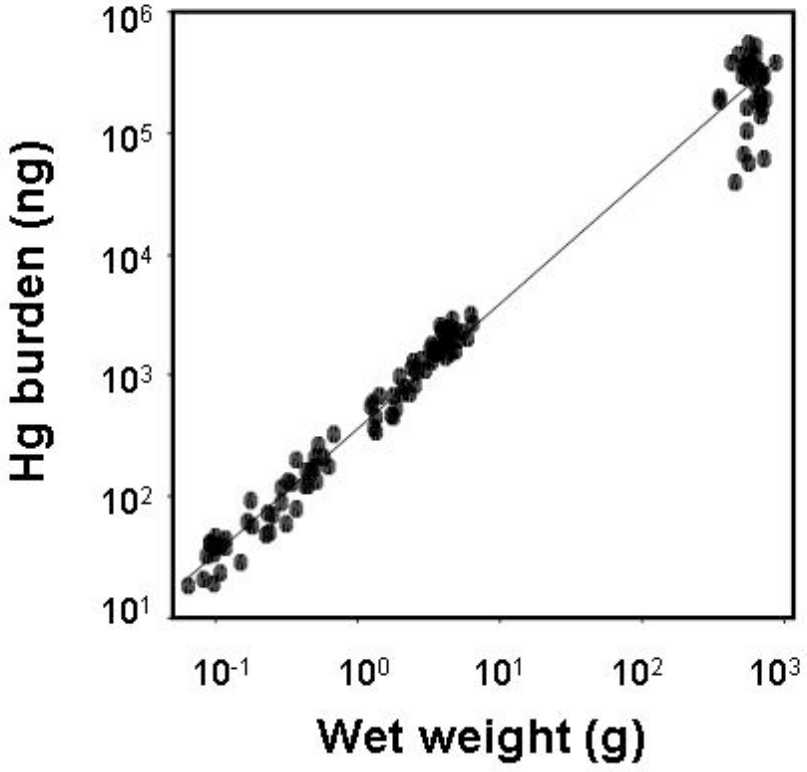


Figure 2.5. Relationship between mercury burdens and wet weights of sea lamprey (*Petromyzon marinus*) collected from the Connecticut River. Regression equation: log y = 1.02 (log x) + 2.57; r^2 = 0.982; p < 0.001.



CHAPTER 3
INCREASED OVARIAN FOLLICULAR APOPTOSIS IN FATHEAD MINNOWS (*PIMEPHALES PROMELAS*) EXPOSED TO DIETARY METHYLMERCURY[2]

3.1. INTRODUCTION

Exposure to methylmercury impairs the reproduction of fish. Recent laboratory studies have documented that concentrations of dietary methylmercury encountered by fish in some surface waters of North America result in (i) reduced and delayed spawning, (ii) small gonads and less fecundity, (iii) altered reproductive behavior, and (iv) suppressed levels of sex steroid hormones (Matta et al., 2001; Hammerschmidt et al., 2002; Drevnick and Sandheinrich, 2003; Sandheinrich and Miller, unpublished). In light of these findings, Wiener et al. (2003) called for the critical examination of the reproductive effects of methylmercury in fish.

The mechanism of reproductive toxicity of methylmercury in fish is unknown, but is likely related to the suppression of sex steroid hormones (Gross et al., 2003). Sex steroid hormones, especially testosterone (T) in males and 17β-estradiol (E2) in females, regulate the processes necessary for reproduction (Fostier et al., 1983). Our previous research demonstrated that reproductive failure in fathead minnows (*Pimephales promelas*) exposed to dietary methylmercury was related to suppressed levels of T and E2 (Drevnick and Sandheinrich, 2003).

Sex steroid hormones are synthesized by somatic cells in the testes (Leydig cells) and ovaries (follicular cells) of fish (Fostier et al., 1983). These "steroidogenic gonadal cells" are sensitive to environmental stressors. Weber and colleagues (Weber and Janz, 2001; Weber et al., 2002; Weber et al., 2004) have demonstrated that some natural and xenobiotic chemicals induce apoptosis in steroidogenic gonadal cells. Apoptosis is a process of selective cell deletion to prevent proliferation of damaged cells and subsequent tissue inflammation (Corcoran et al., 1994). Induction of apoptosis in steroidogenic gonadal cells can result in the suppression of sex steroid hormones. For example, increased ovarian follicular cell apoptosis in white sucker

[2] Note: This chapter has been published in *Aquatic Toxicology* (79:49-54) with Sandheinrich, M.B., and Oris, J.T. as coauthors.

(*Catostomus commersoni*) exposed to bleached kraft pulp mill effluent likely resulted in reduced capacity of ovaries to synthesize E2 (McMaster et al., 1995; Janz et al., 1997).

We hypothesize that methylmercury induces apoptosis in steroidogenic gonadal cells in fish, thereby interfering with the synthesis of sex steroid hormones critical for the regulation of reproduction. The effect of methylmercury on levels of sex steroid hormones in fathead minnows is consistent with the inhibition of steroidogenesis (Drevnick and Sandheinrich, 2003). Moreover, low levels of methylmercury induce apoptosis in mammals (Shenker et al., 1998). Methylmercury has not previously been shown to induce apoptosis in fish, however.

The objective of our study was to determine the effects of dietary methylmercury on apoptosis of steroidogenic gonadal cells in fathead minnows. We report significant increases in the number of apoptotic ovarian follicular cells in females fed diets contaminated with methylmercury at environmentally relevant concentrations. Moreover, increased ovarian follicular apoptosis was significantly related to suppressed levels of plasma E2 and smaller ovaries. These results suggest increased apoptosis of steroidogenic gonadal cells as a possible mechanism for the suppression of sex steroid hormones and ultimately the impairment of reproduction in fish exposed to methylmercury.

3.2. MATERIALS AND METHODS

Experimental design

The design of this experiment was reported previously in our study of the effects of dietary methylmercury on reproductive endocrinology of fathead minnows (Drevnick and Sandheinrich, 2003). Fathead minnows were exposed to methylmercury in diets containing (mean ± 1 SE of total mercury) 0.058 ± 0.004 (control), 0.87 ± 0.02 (low MeHg), or 3.93 ± 0.08 (medium MeHg) μg $Hg \cdot g^{-1}$ dry weight. Concentrations of methylmercury in these diets are representative of those found in invertivorous and piscivorous fish from surface waters of North America (Hammerschmidt et al., 2002). Each of the three treatments consisted of five replicate groups each containing 200 fathead minnows. Exposures started at 90 d post hatch and continued through sexual maturity. At sexual maturity, five breeding pairs of fathead minnows were selected at random from each treatment replicate and allowed to spawn. After spawning or after 21 d if no spawning occurred, fathead minnows were euthanized. Blood plasma, gonads,

and the carcass of each fish were collected for the determination of sex steroid hormones (T in males, E2 in females), gonad size (expressed as the gonadosomatic index), and total mercury. Methods, results, and quality assurance procedures of these analyses were reported previously (Drevnick and Sandheinrich, 2003). Ovaries from female fathead minnows were frozen (-20°C) or preserved in phosphate-buffered formalin for use in this study.

Quantification of apoptosis

We qualitatively confirmed apoptosis in ovaries of female fathead minnows with a DNA Laddering Kit (Cayman Chemical, Ann Arbor, MI, USA). DNA laddering (i.e., the appearance of fragmented chromatin DNA in agarose gels) is the hallmark of apoptosis (Sweet et al., 1999). Frozen ovaries from two female fathead minnows per treatment were thawed, homogenized in phosphate-buffered saline, and pelleted by centrifugation. Pellets were treated with lysis buffer and centrifuged. Resulting supernatants were diluted to 1% sodium dodecyl sulfate and incubated with Rnase A for 60 min at 56°C and proteinase K for 60 min at 37°C. DNA was precipitated with ammonium nitrate and ethanol, mixed with gel-loading buffer, and separated by electrophoresis on a 1.8% agarose gel. Visualization on an ultraviolet transilluminator confirmed apoptosis in ovaries from females from all treatments. This finding is consistent with our previous studies (Drevnick and Oris, unpublished) that determined apoptosis occurs in gonads during the normal physiology of fathead minnows. Because DNA laddering is qualitative and not cell specific, we used a different method (terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP nick end labeling; TUNEL) to quantify apoptosis in ovaries of female fathead minnows.

Ovaries of female fathead minnows were staged for quantification of apoptosis by TUNEL according to criteria described by Leino et al. (2005). Stage 1b (late primary growth) follicles were characterized by their small size and squamous follicular cells. Stage 2 (cortical alveolus) follicles were characterized by the presence of yolk vesicles. Stage 3 (early vitellogenic), 4 (late vitellogenic), and 5 (mature) follicles were not quantified for apoptosis because too often the vitelline envelope ruptured during sectioning or labeling, making it difficult to accurately count apoptotic follicular cells.

Apoptosis was quantified in ovaries by TUNEL with an ApoAlert^{TM} DNA Fragmentation Assay Kit (BD Biosciences Clontech, Palo Alto, CA, USA). Preserved ovaries were embedded

in paraffin, cut into 10-μm thick sections, and mounted on glass slides. Mounted sections were deparaffinized, rehydrated, and incubated with 20 $\mu g \cdot mL^{-1}$ proteinase K for 60 min at 37°C. Proteinase K-treated sections were incubated with TdT and fluorescein-dUTP in the dark for 60 min at 37°C to label 3' ends of fragmented DNA. Fluorescein labeling of fragmented DNA resulted in the strong, nuclear green fluorescence of apoptotic cells (Figure 3.1). Fluorescence microscopy at 400 x magnification was used to count the number of apoptotic follicular cells in three stage 1b and three stage 2 ovarian follicles selected at random in each section (i.e., each fish). Values presented for each female fathead minnow represent the average of the three observations for each stage within a single section.

We ensured the validity of our data by adherence to quality assurance procedures. Apoptosis was quantified without bias by assigning each female fathead minnow a unique random number for blind study. Sections were evaluated without knowledge of treatments. Every assay included negative and positive controls. Negative controls were incubated without TdT (necessary for the incorporation of fluorescein-dUTP at the 3' ends of fragmented DNA). Positive controls were incubated with 1 $U \cdot \mu L^{-1}$ DNase I (Ambion, Austin, TX, USA) for 10 min at 20°C prior to fluorescein labeling. Assays were considered valid if zero follicular cells in negative controls and >70% of follicular cells in positive controls fluoresced green. Method precision was estimated by assaying in duplicate ovaries from five randomly selected female fathead minnows. Median coefficients of variation were 16.6% for stage 1b follicles and 28.3% for stage 2 follicles.

Data analysis

One-way analysis of variance (ANOVA) was used to test our hypothesis that dietary methylmercury induced apoptosis in ovarian follicular cells in female fathead minnows. Bonferroni post hoc tests were used to determine differences between dietary treatments. Data were square root transformed to meet the assumptions of normality and homogeneity of variance. A Type I error (α) of 0.05 was used to judge the significance of statistical tests. Tests were performed with SPSS for Windows software (version 13.0, SPSS, Chicago, IL, USA).

Quantile regression was used to describe relationships between the number of apoptotic follicular cells in stage 1b and stage 2 follicles and (i) levels of plasma E2 and (ii) the gonadosomatic index. Regression quantiles are useful for modeling the effects of limiting

factors (e.g., methylmercury-induced apoptosis) on biological responses, especially for data with wedge-shaped distributions (Cade and Noon, 2003). Blossom statistical software (version 2005.05.06, Fort Collins Science Center, U.S. Geological Survey, Fort Collins, CO, USA) was used to determine for each relationship the largest quantile for which the slope was statistically different from zero ($P < 0.05$). This quantile was used as a best estimate of the maximum biological response when methylmercury-induced apoptosis is the limiting factor. Hypothesis tests for regression quantiles assume homogeneous variation across variables; apoptosis and plasma E2 data were square root and log transformed, respectively, to meet this assumption.

3.3. RESULTS

Methylmercury significantly increased the number of apoptotic follicular cells in stage 1b ($F_{2,12} = 30.6$, $P < 0.001$) and stage 2 ($F_{2,12} = 7.6$, $P = 0.007$) ovarian follicles (Figure 3.2). For stage 1b follicles, each dietary treatment was statistically different from the others. Females fed the low MeHg diet had 3-fold more apoptotic follicular cells than females fed the control diet ($P = 0.045$). Females fed the medium MeHg diet had 6-fold and 2-fold more apoptotic follicular cells, respectively, than females fed the control ($P < 0.001$) and low MeHg ($P = 0.012$) diets. For stage 2 follicles, females fed the control and low MeHg diets had equal numbers of apoptotic follicular cells ($P = 1$). Females fed the medium MeHg diet, however, had 3-fold more apoptotic follicular cells in stage 2 follicles than females fed both the control ($P < 0.001$) and low MeHg ($P < 0.001$) diets.

Plasma E2 levels and the gonadosomatic index were each inversely related to the number of apoptotic follicular cells in stage 1b and stage 2 follicles (Figure 3.3). The 99th ($b_0 = 1.316$, $b_1 = -0.379$, $P < 0.001$, $n = 63$) and 85th ($b_0 = 1.291$, $b_1 = -0.281$, $P = 0.046$, $n = 63$) regression quantiles for stage 1b and stage 2 follicles, respectively, were the best estimates of maximum levels of plasma E2 when methylmercury-induced apoptosis is the limiting factor. The maximum response of the gonadosomatic index to methylmercury-induced apoptosis was best estimated by the 99th regression quantile for both stage 1b ($b_0 = 15.394$, $b_1 = -4.148$, $P = 0.019$, $n = 75$) and stage 2 ($b_0 = 15.347$, $b_1 = -2.363$, $P < 0.001$, $n = 75$) follicles.

3.4. DISCUSSION

To our knowledge, this is the first study to report the induction of apoptosis in fish by methylmercury. Methylmercury contamination of aquatic food webs, especially fish, is a serious environmental health problem (Wiener et al., 2003). Understanding the toxicological significance of methylmercury to fish is an important area of research (Bodaly and Kidd, 2004). Mechanistic studies, such as reported here, are important for developing biomarkers that can be used to evaluate the effects of methylmercury on wild fish populations (Chan et al., 2003).

Methylmercury may induce apoptosis in ovarian follicular cells directly via oxidative stress or indirectly via alterations in follicle survival factors. Oxidative stress induces apoptosis (Holden, 2000) and is a known symptom of methylmercury toxicity (Berntssen et al., 2003). Follicle survival factors are necessary for the successful ovulation of oocytes (Nagahama et al., 1999). Reduced levels of follicle survival factors can induce apoptosis in follicular cells (Janz and Van Der Kraak, 1997; Wood and Van Der Kraak, 2002). The most potent follicle survival factors are hormones, especially pituitary gonadotropins (FSH, LH) and E2 (Wood and Van Der Kraak, 2002). We find it unlikely, however, that reduced hormone levels result in follicular apoptosis in female fish exposed to methylmercury. In another study, we observed increases in mRNA levels of the β-subunits of FSH and LH in zebrafish (*Danio rerio*) exposed to dietary methylmercury (Drevnick and Oris, unpublished). Moreover, even though methylmercury suppressed plasma levels of E2 in female fathead minnows of this study, it is doubtful that apoptosis could be caused by reduced levels of a single follicle survival factor (Janz et al., 2001). We find it more likely that suppressed levels of plasma E2 were a result of increased ovarian follicular apoptosis.

Apoptosis of ovarian follicular cells is a possible mechanism for the suppression of E2 in female fish exposed to methylmercury. Ovarian follicular cells (i.e., granulosa, theca) are responsible for the production of E2 and other sex steroid hormones. Therefore, deletion of follicular cells by methylmercury-induced apoptosis could result in the reduced capacity of ovaries to synthesize E2. We did observe significant relationships between increased ovarian follicular apoptosis in stage 1b and stage 2 follicles and suppressed levels of plasma E2. Our argument would be stronger, however, if we had also quantified apoptosis in follicular cells from vitellogenic follicles (stages 3, 4). In female asynchronous spawning fish such as fathead

minnows, all stages of ovarian follicles synthesize E2, but the vitellogenic stages are the major contributors to plasma levels (Goto-Kazeto et al., 2004). As stated previously, we were not able to quantify apoptosis in vitellogenic follicles because the vitelline envelope too often ruptured during tissue preparation. To conclusively demonstrate that ovarian follicular apoptosis results in suppressed levels of plasma E2 in female fish exposed to methylmercury, the most appropriate approach may be to specifically measure E2 synthesis of follicular cells *in vitro* subjected to graded levels of methylmercury-induced apoptosis.

Little is understood of the association between apoptosis of follicular cells and ovary size in female fish exposed to methylmercury. Ovarian follicular apoptosis is the underlying mechanism of atresia (i.e., the degeneration of ovarian follicles not selected for ovulation; Hsueh et al., 1994). Atresia inhibits growth of ovaries in female fish exposed to methylmercury (Kirubagaran and Joy, 1988). We did observe significant relationships between increased ovarian follicular apoptosis in stage 1b and stage 2 follicles and reductions in the gonadosomatic index, but we did not measure atresia. Alternatively, apoptosis of follicular cells and ovary size may simply be correlated (i.e., no causal relationship inferred) because of a common association with levels of plasma E2. As expected, there was a significant positive relationship between gonadosomatic index and levels of plasma E2 in female fathead minnows (Figure 3 in Drevnick and Sandheinrich, 2003). From the few studies that have each measured plasma E2 levels, ovary size, and atresia in female fish exposed to methylmercury (Adams et al., 1999; Arnold, 2000; Fynn-Aikins et al., 1998), the most consistent effect has been the suppression of E2. Effects on ovary size and atresia have been equivocal. Wood and Van Der Kraak (2001) recently cast doubt on the importance of apoptosis in atresia of ovarian follicles in fish. Their findings could help explain the inconsistent effect of methylmercury on ovary size and atresia in female fish.

3.5. CONCLUSION

This research demonstrates that dietary methylmercury increases ovarian follicular apoptosis in female fish. Our previous research demonstrated that dietary methylmercury impaired reproduction and suppressed levels of plasma E2 in female fish (Drevnick and Sandheinrich, 2003). The inverse relationship between ovarian follicular apoptosis and levels of plasma E2 suggests increased apoptosis as a possible mechanism for the impairment of

reproduction in female fish exposed to methylmercury. We further suggest that this mechanism may not be limited to fish nor females, as apoptosis of gonadal cells has been reported in other taxa exposed to methylmercury, including male mammals (Homma-Takeda, 2001).

3.6. ACKNOWLEDGEMENTS

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Figure 3.1. Ovarian follicles with apoptotic (green) follicular cells at 400x magnification.

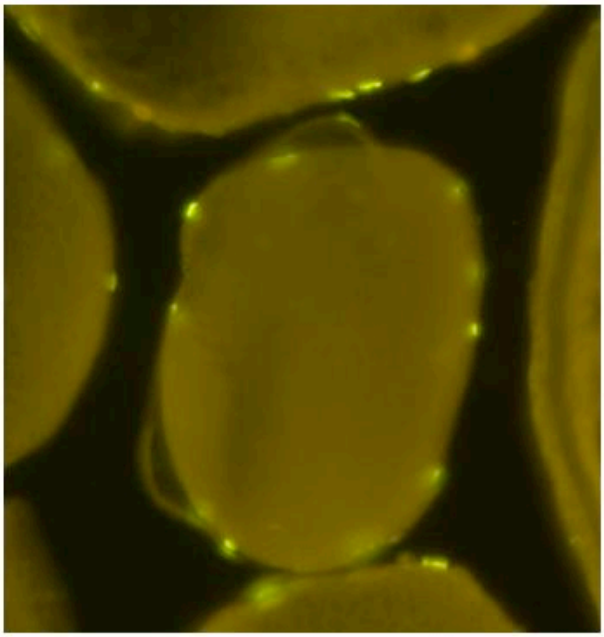


Figure 3.2. Mean number of apoptotic follicular cells in stage 1b (A) and stage 2 (B) ovarian follicles from female fathead minnows exposed to dietary methylmercury. Error bars represent 1 SE. Mean and SE based on five replicate groups per dietary treatment with five female fish sampled from each replicate. Lowercase letters denote statistically different groups ($P < 0.05$).

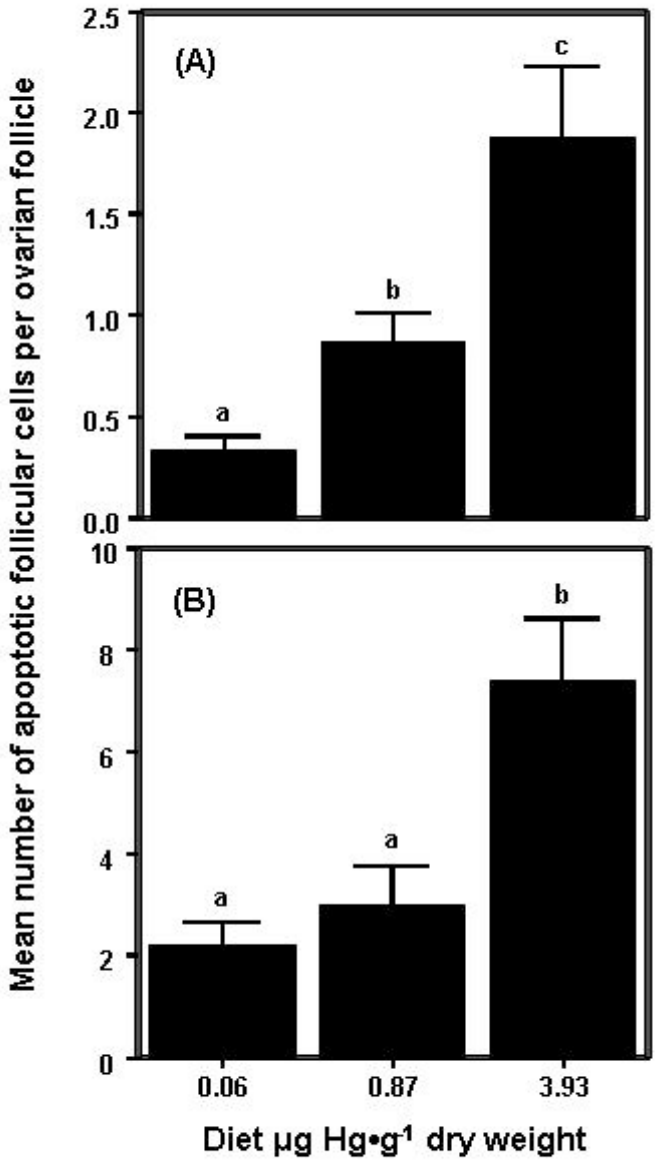
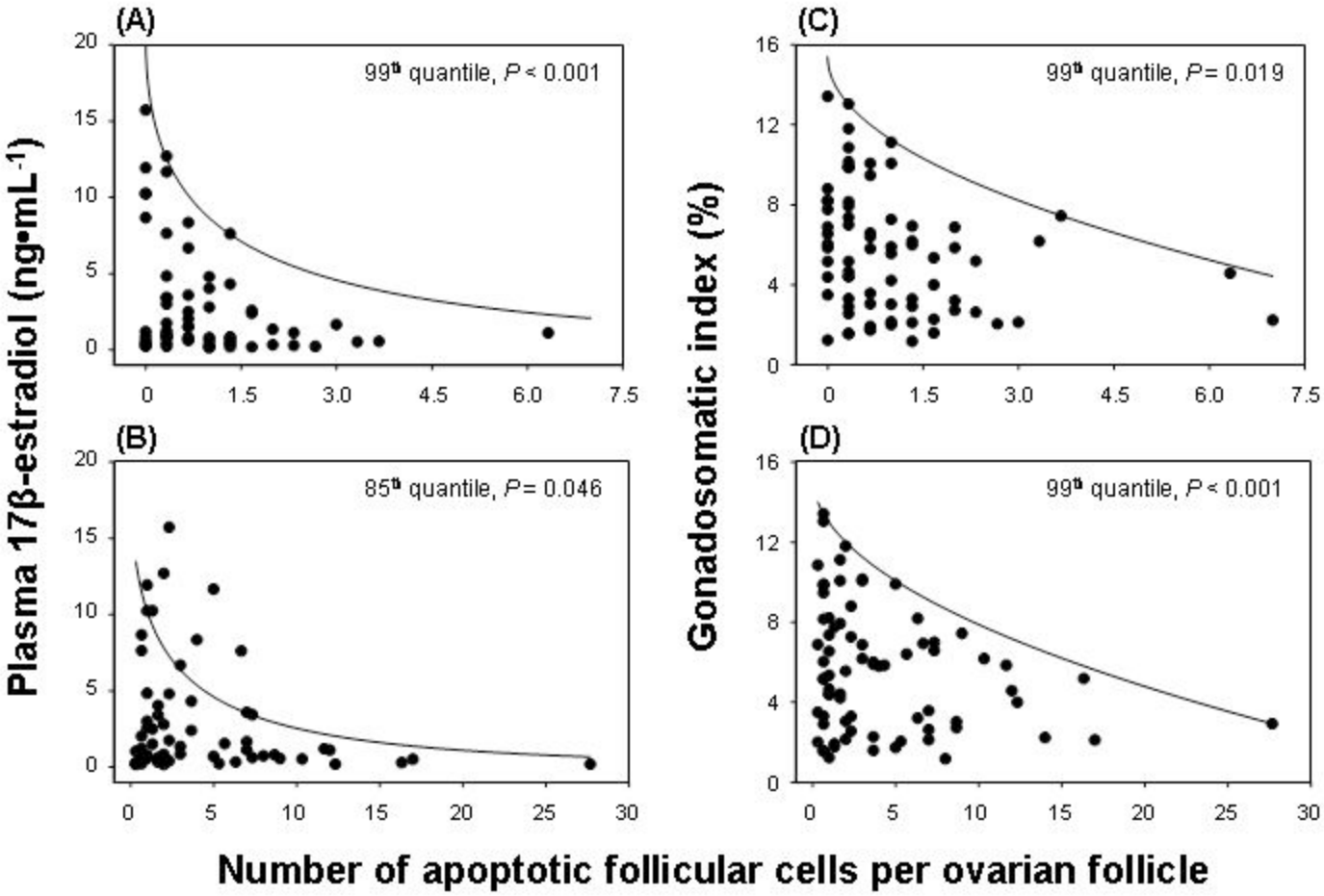


Figure 3.3. Relationships between follicular apoptosis and plasma 17β-estradiol or gonadosomatic index in stage 1b (A, C) and stage 2 (B, D) ovarian follicles from female fathead minnows exposed to dietary methylmercury.



CHAPTER 4
IMPAIRMENT OF FISH REPRODUCTION BY METHYLMERCURY MAY BE DEPENDENT UPON DIET QUALITY/QUANTITY

4.1. INTRODUCTION

Exposure to methylmercury impairs the reproduction of fish. Laboratory studies have documented that concentrations of dietary methylmercury encountered by fish in some surface waters of North America result in (1) reduced and delayed spawning, (2) small gonads and less fecundity, and (3) altered reproductive behavior (Matta et al. 2001; Hammerschmidt et al. 2002; Sandheinrich and Miller 2006). In light of these findings, Wiener et al. (2003) called for the critical examination of the reproductive effects of methylmercury in fish.

Specific mechanisms of methylmercury toxicity are beginning to be known and likely relate to disruption of the hypothalamic-pituitary-gonadal (HPG) axis, which regulates fish reproduction. Hypothalamic secretion of gonadotropin-releasing hormone (GnRH) stimulates pituitary release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which in turn act in the gonads to stimulate the synthesis of sex steroid hormones. Sex steroid hormones, such as 11-ketotestosterone (11-KT) and 17β-estradiol (E2), regulate the processes necessary for reproduction (Fostier et al. 1983). My previous research demonstrated that reproductive failure in fathead minnows (*Pimephales promelas*) exposed to dietary methylmercury was related to suppressed levels of testosterone and E2 (Drevnick and Sandheinrich, 2003). Suppressed E2 in females was due to increased numbers of apoptotic ovarian follicular cells (Drevnick et al. 2006b). Further, apoptosis-mediated E2 suppression caused a significant decline in hepatic vitellogenin (Vtg) mRNA expression (Klaper et al. 2006). Down-regulation of Vtg, an important egg yolk protein, likely resulted in small ovaries and less fecundity. With collaborators, I have thus linked methylmercury toxicity from cells (increased apoptosis) to physiology (E2 suppression) to individuals (less fecundity). Gonadotropin-releasing hormone, FSH, and LH, however, are also sensitive to toxicants (e.g., Harris et al. 2001) and are on a feedback loop with sex steroid hormones (Thomas 1990). However, very little is known about the direct or indirect effects of methylmercury on GnRH, FSH, and LH.

The initial objectives of this study were to (1) determine the effects of dietary methylmercury on the mRNA expression of GnRH, and the ß sub-units of FSH and LH in zebrafish (*Danio rerio*) and (2) relate results to known effects on sex steroid hormones, Vtg, and reproductive success. Zebrafish were used as the model fish because of the availability of genetic information for this species. Complementary DNA sequences for GNRH, FSHß, and LHß have been cloned for zebrafish and published on GenBank (National Center for Biotechnology Information 2007). Further, zebrafish have a rapid life cycle (egg to sexually mature adult in ninety days), making them an especially useful species for reproductive studies (Hoffmann and Oris 2006).

During the course of the study, a serendipitous event occurred that allowed me to also examine the combined effects of methylmercury and food quality/quantity on fish reproduction. In wild fish, food quality and quantity may influence the effects of metals on reproduction (e.g., McFarlane and Franzin 1978, Munkittrick and Dixon 1988), but this has not been shown for mercury. In my study, zebrafish were exposed in the laboratory to methylmercury in their diets, but diets were supplemented in excess with live, "clean" brine shrimp. Partway through the experiment, the apparatus used to hatch brine shrimp failed and the supplementation of diets ceased. This change in diet coincided with significant changes in reproductive success of zebrafish. Causality cannot be inferred, but results are nonetheless interesting and could be used to design further studies.

4.2 MATERIALS AND METHODS

Fish

Zebrafish were raised from embryos to sexual maturity. Embryos were collected from broodstock 24 June 2003 and placed in hatching jars with flowing water. Upon hatching, larvae were transferred to 75-L glass static aquaria and fed Larval AP100 Larval Food Supplement (< 100 microns; Ziegler Bros, Inc. Gardners, PA) and brine shrimp (*Artemia nauplii*; INVE Aquaculture Nutrition, Grantsville, UT). Twenty days after hatching, 125 larvae were transferred to each of six 75-L glass flow-through aquaria and acclimated to a diet of Sterling Silver Cup Soft-Moist Fish Feed (Starter; Nelson and Sons, Inc., Murray, UT) supplemented with brine shrimp. Beginning at 30-d post hatch (day 1), juveniles/adults were exposed to

methylmercury in these aquaria until termination of the experiment (day 134). During days 1-105, experimental diets were supplemented with ~6.5 mL (three squirts from a plastic pipette) of concentrated brine shrimp. On day 105, the apparatus used to hatch brine shrimp broke and the supplementation of diets ceased for the duration of the experiment.

Culture and handling was done in accordance with protocols approved by the Miami University Institutional Animal Care and Use Committee. Zebrafish were maintained in aquaria receiving 28.5 ± 0.2 ˚C dechlorinated tap water with a 14:10-h light:dark cycle. Water quality characteristics were measured daily (temperature, dissolved oxygen) or weekly (ph, alkalinity, hardness, ammonia) with standard methods (Clesceri et al. 1998). Wastes were siphoned daily from aquaria. Residue on aquaria was removed weekly by scraping. Separate cleaning equipment was used for each aquarium to avoid possible transmission of disease.

Methylmercury exposure

Zebrafish were fed methylmercury-contaminated food (~3% of body mass per day). Concentrations of methylmercury in diets included (mean ± sd): 0.13 (control), 0.79, and 3.59 µg/g dry wt. These concentrations approximate methlymercury in the diets of invertivorous and piscivorous fish from north temperate lakes (Hammerschmidt et al. 2002). Nearly all mercury in fish is methylmercury (Bloom 1992) and is accumulated almost entirely via dietary uptake (Hall et al. 1997). Each 75-L aquarium was randomly assigned one of the three diets to yield two aquaria per dietary treatment. Fish were fed methylmercury-contaminated food for 105 days with brine shrimp and an additional 29 days without brine shrimp.

Contaminated diets were prepared by mixing fish food with reagent alcohol (Aaper, Shelbyville, KY) containing dissolved methylmercuric chloride (Sigma Aldrich, Inc., St. Louis, MO). Control diets were prepared by mixing fish food with alcohol only. Alcohol was evaporated overnight from the fish food in acid-washed glass pans in a fume hood. Diets were prepared and frozen until use. Samples from each batch were analyzed for total mercury.

Reproduction

At day 30 of the experiment, 1-cm diameter marbles were placed in each aquarium to serve as a substrate for spawning. Eggs were collected daily from the spawning substrates, counted, measured for diameter, and incubated to determine fertilization and hatching success.

For measuring diameter of eggs, a subset of eggs was stained with rose bengal (Fisher Scientific, Pittsburgh, PA), rinsed with water, and viewed with a low-power dissecting microscope fitted with an eyepiece graticule. To determine fertilization and hatching success, a different subset of eggs was placed in a clean petri dish with culture water. Fertilization success was defined as the percentage of eggs in each clutch that reached the "eyed" stage. Hatching success was the percentage of "eyed" eggs that hatched.

Sample Collection

At day 134 of the experiment, thirty zebrafish (fifteen males, fifteen females) were selected at random from each aquarium for tissue sampling. Zebrafish were euthanized with an excess of MS-222 (Sigma Aldrich, Inc., St. Louis, MO). Total length (mm) and wet weight (g) were measured and used to calculate condition factor, with the following equation:

$$\text{Condition Factor} = (100{,}000 * \text{wet weight}) / \text{total length}^3$$

Collection of blood from the caudal vein was attempted but not successful. Livers and gonads were dissected from the fish and weighed. Gonad weights were used to calculate the gonadosomatic index (GSI), expressed as the percentage of total body contributed by the gonads. Heads were severed from the fish. Livers, gonads, heads, and carcasses were then placed in individual sample tubes, frozen in liquid nitrogen, and stored at -80 ˚C. For analysis of mercury and endocrine endpoints, samples of each tissue type were later grouped for three individuals by sex from each aquarium, resulting in ten composite samples (five from males, five from females) for each tissue type for each aquarium.

Mercury analysis

Diets, brine shrimp, and tissue from carcasses were acid digested according to US EPA Method 245.610 (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 3.9%. Mean recovery of

spiked samples was 97.0%. Mean measured concentrations of reference materials were within certified ranges.

Endocrinology

Male and female zebrafish were analyzed for mRNA expression of GnRH, FSHß, and LHß in heads and Vtg (females only) in livers. Total RNA was isolated from heads and livers with the TRI reagent protocol (Sigma Aldrich, Inc., St. Louis, MO). Total RNA concentration was determined spectrophotometrically at 260 nm. Purity was determined by calculating the ratio of UV absorbance at 260 and 280. Ratios of 1.7-2.1 were considered acceptable. Messenger RNA was then reverse transcribed to more stable cDNA with the First-Strand cDNA Synthesis Kit (Amersham Pharmacia, Uppsala, Sweden) according to manufacturers instructions. Real-time polymerase chain reactions (PCR) were then performed with cDNA with the QuantiTect SYBR-Green PCR kit (Qiagen, Valencia, CA) on a RotorGene RG-3000 thermocycler (Corbett Research, Sydney, Australia). Methods, primer design, and quality control followed Roberts (2005). The primer sequence, GenBank Accession number, and product size are provided for each primer in Table 4.1. Expression of mRNA was calculated with the equation provided by Pfaffl (2001) as modified by Hoffmann (2004). Actin was used as the internal control in calculations.

Tissue from carcasses of male and female zebrafish were analyzed for 11-KT and E2, respectively, by competitive enzyme immunoassay. Assay procedures used were specified by the manufacturer (Cayman Chemical, Ann Arbor, MI) and a previous publication (Drevnick and Sandheinrich 2003). Before analysis, total lipids were extracted from carcasses (Drevnick and Sandheinrich 2003) and quantified. Extracts were then reconstituted with petroleum ether, and polar groups (including 11-KT and E2) were separated with 70% ethanol. Ethanol was evaporated at 40 ˚C under a stream of N_2. Extracts were then reconstituted in assay buffer for analysis. Duplicate samples and spiked samples were extracted and analyzed with each batch of samples. Mean relative standard deviation for duplicate samples was 20.7% for 11-KT and 20.9% for E2. Mean recovery of spiked samples was 101.1% for 11-KT and 103.5% for E2.

Respiration

Near the end of the experiment, five zebrafish were collected from each aquarium to measure routine metabolic rate via oxygen consumption. Briefly, zebrafish were selected at random from aquaria and placed in individual isolation tanks. Twenty-two hours later, shades were pulled over isolation tanks to leave zebrafish in darkness for two hours. Zebrafish were then quickly placed in respirometers constructed according to Cech (1990). Shades were pulled over respirometers and fish were allowed to acclimate for 30 min. Measurements started with an initial measurement of dissolved oxygen and temperature in respirometer water and ended 30 min later by repeating measurements. Zebrafish were then measured for wet weight. Oxygen consumption (MO_2) was calculated with the following equation:

$$MO_2 = ((IO_2 - FO_2)_c * V) / (W * T)$$

where IO2 and FO2 are the initial and final dissolved oxygen concentrations, respectively, c is a correction for oxygen consumption in a blank respirometer, V is the volume of the respirometer, W is the wet weight of the fish, and T is the time elapsed during the measurement period.

Statistics

One-way analysis of variance (ANOVA) was used to test for effects of methylmercury on zebrafish. Bonferroni posthoc tests were used to determine differences between dietary treatments. Data were tested for normality and homogeneity of variance and, if necessary, transformed (log_{10}, square root, or arcsine square root) to meet the assumptions of ANOVA. A type I error (α) of 0.05 was used to judge the significance of statistical tests. Tests were performed with SPSS for Windows software (version 14.0, SPSS, Chicago, IL).

4.3 RESULTS

Zebrafish accumulated methylmercury in proportion to their diet (Figure 4.1). Juveniles sampled at the start of the experiment had total mercury concentrations of about 0.1 μg/g wet wt. Background contamination of zebrafish was likely due to transfer from maternal broodstock (Hammerschmidt and Sandheinrich 2005) and trace amounts of methylmercury in food

(Berntssen et al. 2004). Brine shrimp had a total mercury concentration of 0.18 µg/g dry wt. Untreated trout chow had a total mercury concentration of 0.13 µg/g dry wt or less. Zebrafish fed the control diet had total mercury concentrations that stayed near 0.1 µg/g wet wt for the duration of the experiment. Zebrafish fed the diet containing 0.79 µg Hg/g dry wt had total mercury concentrations that gradually increased to near 1 µg/g wet wt by experiment end. Zebrafish fed the diet containing 3.59 µg Hg/g dry wt had total mercury concentrations that sharply increased to more than 4 µg/g wet wt by experiment end. There was very little variability in total mercury concentrations of zebrafish within dietary treatments. There was no overlap in total mercury concentrations of zebrafish among dietary treatments.

During the first period of the experiment, with brine shrimp, zebrafish fed the control and 3.59 µg Hg/g dry wt diets spawned with the same frequency, which was greater than zebrafish fed the 0.79 µg Hg/g dry wt diet (ANOVA, $F_{2,3} = 10.307$, $P = 0.045$; Figure 4.2). Zebrafish fed the 3.59 µg Hg/g dry wt diet were the first to become sexually mature, spawning significant numbers of eggs by day 70 of the experiment. Zebrafish fed the control diet followed thereafter, spawning significant numbers of eggs by day 90 of the experiment. There were no significant effects of dietary methylmercury on the number of eggs per spawn or the diameter, fertilization success, days to hatch, and hatching success of eggs (Table 4.2).

During the second period of the experiment, without brine shrimp, zebrafish fed the control diet spawned with a greater frequency than zebrafish fed the 0.79 µg Hg/g dry wt and 3.59 µg Hg/g dry wt diets (ANOVA, $F_{2,3} = 16.050$, $P = 0.025$; Figure 4.2). Again, there were no significant effects of dietary methylmercury on the number of eggs per spawn or the diameter, fertilization success, days to hatch, and hatching success of eggs (Table 4.2).

Dietary methylmercury had little effect on the growth, body condition, and reproductive endocrinology of zebrafish (Table 4.3). Growth and body condition may have been affected by methylmercury in males, but not females. Total length of males fed the 0.79 µg Hg/g dry wt diet was significantly lower than for males fed the control and 3.59 µg Hg/g dry wt diets (ANOVA, $F_{2,3} = 16.482$, $P = 0.024$), but wet weight was unaffected (ANOVA, $F_{2,3} = 2.501$, $P = 0.230$). As a result, condition factor was higher for males fed the 0.79 µg Hg/g dry wt diet than those fed the control and 3.59 µg Hg/g dry wt diets (ANOVA, $F_{2,3} = 9.535$, $P = 0.050$). Total lipids, a separate measure of body condition, was unaffected in males, however (ANOVA, $F_{2,3} = 5.223$, $P = 0.105$). Otherwise, there were no significant effects of methylmercury on GSI, expression of

GnRH, FSHß, and LHß, and Vtg, or sex steroid hormones. There did seem to be trends in expression of GnRH, FSHß, and LHß in both males (low in those fed the 0.79 µg Hg/g dry wt diet) and females (low in those fed the control diet), but the high variability generally associated with this type of data (Roberts 2005) precluded the determination of significant relationships.

Dietary methylmercury affected the routine metabolic rate of zebrafish (Figure 4.3). In general, oxygen consumption of zebrafish decreased as concentration of methylmercury in the diet increased. The only statistically significant difference, however, was between zebrafish fed the control diet and those fed the 3.59 µg Hg/g dry wt (ANOVA, $F_{2,3} = 12.415$, $P = 0.035$).

4.4 DISCUSSION

Experiments of fish reproduction take considerable time to conduct, and the purpose of supplementing the diet was to accelerate the development of zebrafish from juveniles to adult. During the course of this study, the supplemental brine shrimp diet was terminated after 105 d due to equipment failure. Due to the change in diet, some results were unexpected. I will first discuss the results of this study in relation to determining the mechanisms for reproductive toxicity in fish exposed to methlmercury and then discuss the effect brine shrimp may have had on methylmercury toxicity.

Dietary methylmercury did not significantly affect the mRNA expression of GnRH, FSHß, and LHß, although the likelihood of detecting an effect was not high due to variability in the data. Still, this lack of effect is surprising because Joy and Kirubagaran (1989) reported methylmercury directly inhibited gonadotropic activity in the pituitary of catfish (*Clarias batrachus*), although with very high concentrations in water. Further, GnRH, FSH, and LH are tightly controlled through feedback mechanisms by sex steroid hormones, which are known to be affected by dietary methylmercury (Drevnick and Sandheinrich 2003). However, there was no significant effect of dietary methylmercury on sex steroid hormones in zebrafish in this study. In females, there was a consistent pattern of increasing values of GnRH, FSHß, LHß, E2, and Vtg with increased exposure to methylmercury. This pattern, though not statistically significant in all cases, could indicate methylmercury actually stimulated endocrine activity, possibly by mimicking calcium, as has been shown for other heavy metals (e.g., cadmium; Le Guevel et al. 2000). I question the usefulness of these data, however, because reproductive toxicity of

methylmercury appeared to have been affected by the presence/absence of brine shrimp, but zebrafish were only sampled at the end of experiment in the absence of brine shrimp.

Methylmercury did impair the reproduction of zebrafish, but this response was dependent upon dietary treatment and the presence/absence of brine shrimp. Cumulative egg production (i.e., the cumulative number of eggs produced per day) was used as the primary endpoint to measure reproductive success. Through the entirety of measuring cumulative egg production (Figure 4.2A), it appeared that the treatment order for number of eggs produced was 3.59 µg Hg/g dry wt diet ≥ control diet > 0.79 µg Hg/g dry wt diet. However, if only the period of measurement with the supplementation of brine shrimp is analyzed (days 60-105; Figure 4.2B), it is evident that zebrafish fed the control and 3.59 µg Hg/g dry wt diets spawned at an equal rate that was much greater than zebrafish fed the 0.79 µg Hg/g dry wt diet. This result was unexpected. Further, if only the period of measurement without the supplementation of brine shrimp is analyzed (days 106-134; Figure 4.2C), it is evident that zebrafish fed the control diet spawned at a much greater rate than zebrafish fed both the 0.79 µg Hg/g dry wt and 3.59 µg Hg/g dry wt diets. This result was expected; the primary effect of methylmercury on fish reproduction was reported to be reduced spawning frequency and fecundity when dietary treatments were not supplemented with brine shrimp (Hammerschmidt et al. 2002, Drevnick and Sandheinrich 2003). Similarly, there was no effect of methylmercury on fertilization and hatching success in these studies. Taken together, these results indicate that (1) the presence/absence of brine shrimp did not affect the reproduction of zebrafish fed the control diet, (2) methylmercury impaired the reproduction of zebrafish fed the low diet, regardless of brine shrimp, and (3) methylmercury impaired the reproduction of zebrafish fed the high diet only in the absence of brine shrimp.

Brine shrimp did not affect the bioaccumulation of methylmercury in zebrafish. Zebrafish in this study were fed the same diets as the control, low, and medium diets fed to fathead minnows in the studies by Hammerschmidt et al. (2002) and Drevnick and Sandheinrich (2003). Those studies did not supplement diets with brine shrimp. For each dietary treatment, total mercury concentrations in carcasses of zebrafish in this study were nearly identical to total mercury concentrations in carcasses of fathead minnows from the previous studies. Rather, the response of zebrafish reproduction to methylmercury and the presence/absence of brine shrimp can be explained with the theories of energetics and physiological stress. Mercury is known to

inhibit the metabolism of fish by interfering with enzymes necessary for glycolysis and the Kreb's cycle (Heath 1995). The routine metabolic rate of zebrafish exposed to dietary methylmercury in this study was significantly reduced (Figure 4.3). This reduction in metabolic rate could indicate that zebrafish exposed to methylmercury generated less energy via glycolysis and the Kreb's cycle and thus had less energy to meet the demands of physiological maintenance, growth, and reproduction. Fish are known to compensate for such limitations, however, with the physiological stress response (Beyers et al. 1999). An organism exposed to a threshold level of some stressor will react in a predictable, three-part response: adaptation, resistance, and exhaustion. The total mercury concentration in carcasses of zebrafish fed the 0.79 µg Hg/g dry wt diet was sufficient to impair reproduction, but apparently was not above the threshold level to prompt a stress response. The total mercury concentration in carcasses of zebrafish fed the 3.59 µg Hg/g dry wt diet, however, apparently was above the threshold level to prompt a stress response. Zebrafish fed the 3.59 µg Hg/g dry wt diet started spawning 20 d sooner than the control fish and were able to maintain a high rate of egg production until the supplementation of diets with brine shrimp stopped. The acceleration of reproduction by fish is a common and predictable response to stress (Schreck et al. 2001). The timing of reproductive events is primarily controlled by gonadotropins (Fostier et al. 1983), and the trends in increased mRNA expression of FSHß and LHß increase may be part of the stress response in female zebrafish fed the 3.59 µg Hg/g dry wt diet. The stress response of zebrafish fed the 3.59 µg Hg/g dry wt diet appears to have been dependent upon the extra food source, however, because when brine shrimp were no longer available for extra energy, spawning was immediately reduced.

The amount of brine shrimp added and its protein, fat, and caloric contents were sufficient to provide enough energy and nutrition to allow for the physiological stress response in zebrafish. At most, 2 g of trout chow (experimental diet) were added to each aquarium daily. During the period with supplementation, an additional ~3 g of brine shrimp were added to each aquarium daily (with the conservative assumption that brine shrimp constituted half of the mass of the ~6.5 mL of concentrate added daily). Thus, the mass of brine shrimp actually exceeded the mass of trout chow added to aquaria daily. Further, crude protein, crude fat, and caloric density of brine shrimp (on a dry wt basis: 56%, 12%, 4.54 kcal/g, respectively; from Garcia-Ortega et al. 1998 and Fabregas et al. 2001) were similar to that of trout chow (on a dry wt basis: 48%, 16%, 5.16 kcal/g, respectively; from manufacturer and Hammerschmidt et al. 2002).

Therefore, it is very likely that enough high-quality food was provided to meet the demands of physiological maintenance, growth, and reproduction. This is further indicated by no difference among dietary treatments in the growth of females. There was a statistically significant difference among treatments in total length of males, but I argue that this difference was not biologically significant (1 mm difference), and data for wet weight and condition factor support this argument. Further, the data for total lipids indicate zebrafish were well fed. Total lipid content of males and females in this study were ~6% and ~10%, respectively. Smolders et al. (2003) reported a total lipid content of ~2.5% for zebrafish fed a maintenance diet.

Much of this discussion is speculation. Unfortunately, the serendipitous way in which brine shrimp became an experimental treatment does not allow for legitimate comparisons of reproduction with brine shrimp to reproduction without brine shrimp. Analyses would be confounded by maturity, as, for example, the spawning frequency of fish fed the control diet increased with time. Further, because there was no experimental control for brine shrimp, it is impossible to know if reproduction of zebrafish fed the 3.59 μg Hg/g dry wt diet would have followed the same course even if the apparatus used to hatch the brine shrimp had not failed. Beyond speculation, it is also possible that the results of this experiment are an artifact of laboratory research. The stress response of zebrafish fed the 3.59 μg Hg/g dry wt diet was dependent upon the availability of extra, energy-rich food. Extra food may be common for fish in laboratory environments, but is not common for fish in natural environments (Campbell et al. 2003). Therefore, a stress response dependent upon extra food is unlikely to occur in wild fish.

In conclusion, this study does not identify the mechanisms responsible for impaired reproduction in methylmercury-contaminated fish. Research that I have conducted shows that, at least in fathead minnows, there is a specific pathway in which methylmercury impairs fish reproduction (Drevnick and Sandheinrich 2003, Drevnick et al. 2006b). Results from this study suggest that reproduction of fish exposed to dietary methylmercury may also be dependent upon energetics and the physiological stress response. This finding is highly speculative, however, because there was no experimental control for brine shrimp and conditions were unrealistic. I recommend repeating the experiment, but with a factorial design and more thought as to the quality and quantity of food added. North temperate lakes are commonly contaminated with methylmercury (Weiner et al. 2003), but many of these lakes have healthy fish populations. There must be some mechanism that allows for healthy fish populations in high methylmercury

environments. This study indicates diet quality and quantity may be part of that mechanism, but much more research is required.

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Table 4.1. GenBank Accession Number, primer sequences, and product size of all target genes used in real-time PCR.

Target	Accession #	Forward Primer	Reverse Primer	Size (bp)
Actin	AF057040	CATGGCTTCTGCTCTGTATG	GCAAAGTGGTAAACGCTTCT	143
sGnRH	AJ304429	ATGGAGGCAACATTCAGGAT	TCAGCATCCACCTCATTCAC	113
FSHβ	AY424303	TGAGCGCAGAATCAGAATG	AGGCTGTGGTGTCGATTGT	105
LHβ	AY424304	TTGGCTGGAAATGGTGTCT	TCCACCGATACCGTCTCAT	110
Vtg1	AF406784	ACACCTGCTCTCCCTGAGAA	AGCTTGGATGAAGGCAGTGT	144

Table 4.2. Effects of dietary methylmercury on reproduction of zebrafish. Values represent means (1 standard error) based on two aquaria per dietary treatment with reproduction monitored for 46 and 29 days with and without brine shrimp, respectively. F and P values are from a one-way ANOVA for each variable. Asterisks denote statistically significant effect of methylmercury, lowercase letters denote statistically significant groups; (P < 0.05).

Diet	Spawning Frequency	# Eggs per Spawn	Egg Diameter (mm)	Fertilization Success (%)	Days to Hatch	Hatching Success (%)
With brine shrimp	*					
Control	0.587 (0.109) a	62.3 (10.2)	1.09 (0.005)	91.7 (3.58)	2.31 (0.083)	32.2 (4.02)
0.79 µg Hg/g dry wt	0.402 (0,011) b	23.2 (5.19)	1.08 (0.012)	83.0 (8.51)	2.33 (0.211)	11.7 (5.69)
3.59 µg Hg/g dry wt	0.804 (0.022) a	71.8 (7.72)	1.10 (0.004)	97.1 (1.75)	2.26 (0.082)	29.4 (3.52)
$F_{2,3}$	10.3	4.07	2.00	0.498	0.337	5.44
P	0.045	0.140	0.281	0.668	0.738	0.100
Without brine shrimp	*					
Control	0.966 (0.034) a	63.4 (9.28)	1.11 (0.010)	92.7 (1.31)	2.05 (0.033)	47.7 (2.82)
0.79 µg Hg/g dry wt	0.466 (0.017) b	38.4 (8.55)	1.13 (0.014)	94.0 (2.48)	2.36 (0.203)	34.1 (8.33)
3.59 µg Hg/g dry wt	0.569 (0.086) b	31.2 (5.73)	1.14 (0.014)	93.6 (2.01)	2.47 (0.125)	40.7 (5.73)
$F_{2,3}$	16.1	2.09	1.08	0.134	2.47	2.19
P	0.025	0.270	0.443	0.879	0.233	0.260

Table 4.3. Effects of dietary methylmercury on growth, body condition, and reproductive endocrinology of zebrafish. Values represent means (1 standard error) based on two aquaria per dietary treatment with five composite samples per sex per aquarium. F and P values are from a one-way ANOVA for each variable. Asterisks denote statistically significant effect of methylmercury, lowercase letters denote statistically significant groups; (P < 0.05).

Diet	Total Length (mm)	Wet Weight (g)	Condition Factor	Total Lipids (%)	GSI (%)	GnRH	FSH	LH	Vtg	11-KT (ng/g wet wt)	E2 (ng/g wet wt)
						relative mRNA expression					
Males	*										
Control	37.9 (0.441) a	0.473 (0.015)	0.864 (0.014)	6.85 (0.941)	1.20 (0.079)	1.38 (0.340)	2.11 (0.892)	1.99 (0.692)	.	1.12 (0.117)	.
0.79 µg Hg/g dry wt	36.4 (0.363) b	0.450 (0.009)	0.943 (0.031)	5.58 (0.355)	1.05 (0.086)	1.10 (0.198)	0.342 (0.111)	0.760 (0.406)	.	1.17 (0.220)	.
3.59 µg Hg/g dry wt	37.6 (0.326) a	0.416 (0.016)	0.784 (0.024)	4.76 (0.526)	0.878 (0.092)	1.23 (0.357)	1.82 (1.00)	1.75 (0.716)	.	1.17 (0.148)	.
$F_{2,3}$	16.5	2.50	9.54	5.22	3.68	0.160	3.66	3.31	.	0.257	.
P	0.024	0.230	0.050	0.105	0.156	0.859	0.157	0.174	.	0.789	.
Females											
Control	40.0 (0.587)	0.746 (0.022)	1.18 (0.046)	10.9 (0.839)	13.2 (1.01)	0.863 (0.100)	0.924 (0.197)	0.620 (0.190)	2.43 (0.834)	.	0.402 (0.122)
0.79 µg Hg/g dry wt	39.8 (0.658)	0.770 (0.037)	1.19 (0.030)	9.37 (0.645)	13.8 (0.735)	1.06 (0.208)	5.24 (2.48)	1.95 (0.888)	2.22 (0.537)	.	0.622 (0.240)
3.59 µg Hg/g dry wt	39.9 (0.668)	0.695 (0.039)	1.12 (0.039)	10.4 (0.946)	10.7 (1.26)	1.35 (0.160)	8.08 (3.34)	3.01 (0.957)	3.17 (1.19)	.	0.937 (0.360)
$F_{2,3}$	0.013	0.427	1.66	3.65	0.612	0.994	3.06	1.41	0.006	.	0.974
P	0.987	0.687	0.328	0.157	0.598	0.467	0.189	0.370	0.994	.	0.472

Figure 4.1. Concentrations of total mercury (Hg_T) in carcasses of zebrafish fed diets containing 0.13 (control), 0.79, and 3.59 µg Hg/g dry wt. Each data point represents a composite of at least three fish.

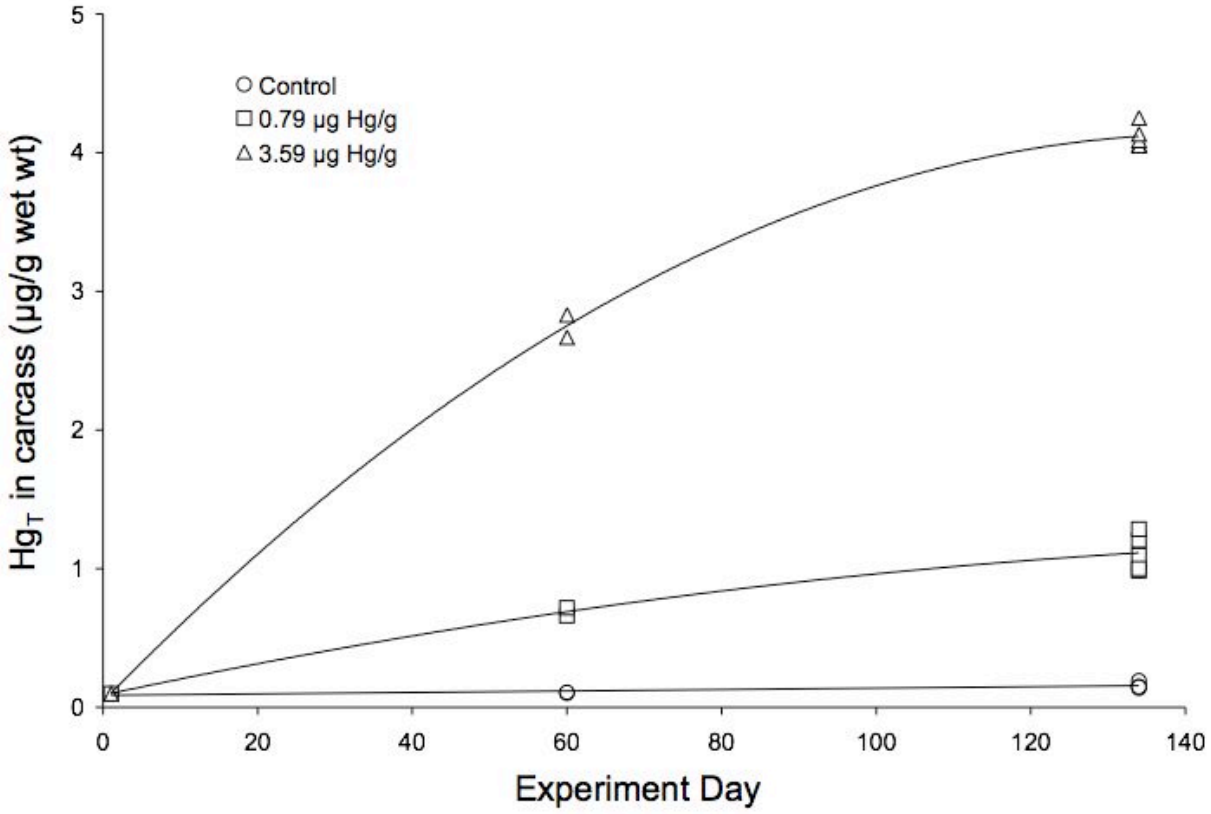


Figure 4.2. Cumulative egg production of zebrafish fed diets containing 0.13 (control), 0.79, and 3.59 µg Hg/g dry wt. Panel A represents cumulative egg production throughout the experiment; the red, dashed line separates the periods with (Days 1-105) and without (Days 106-134) the supplementation of diets with brine shrimp. Panels B and C separately represent the periods with and without brine shrimp, respectively.

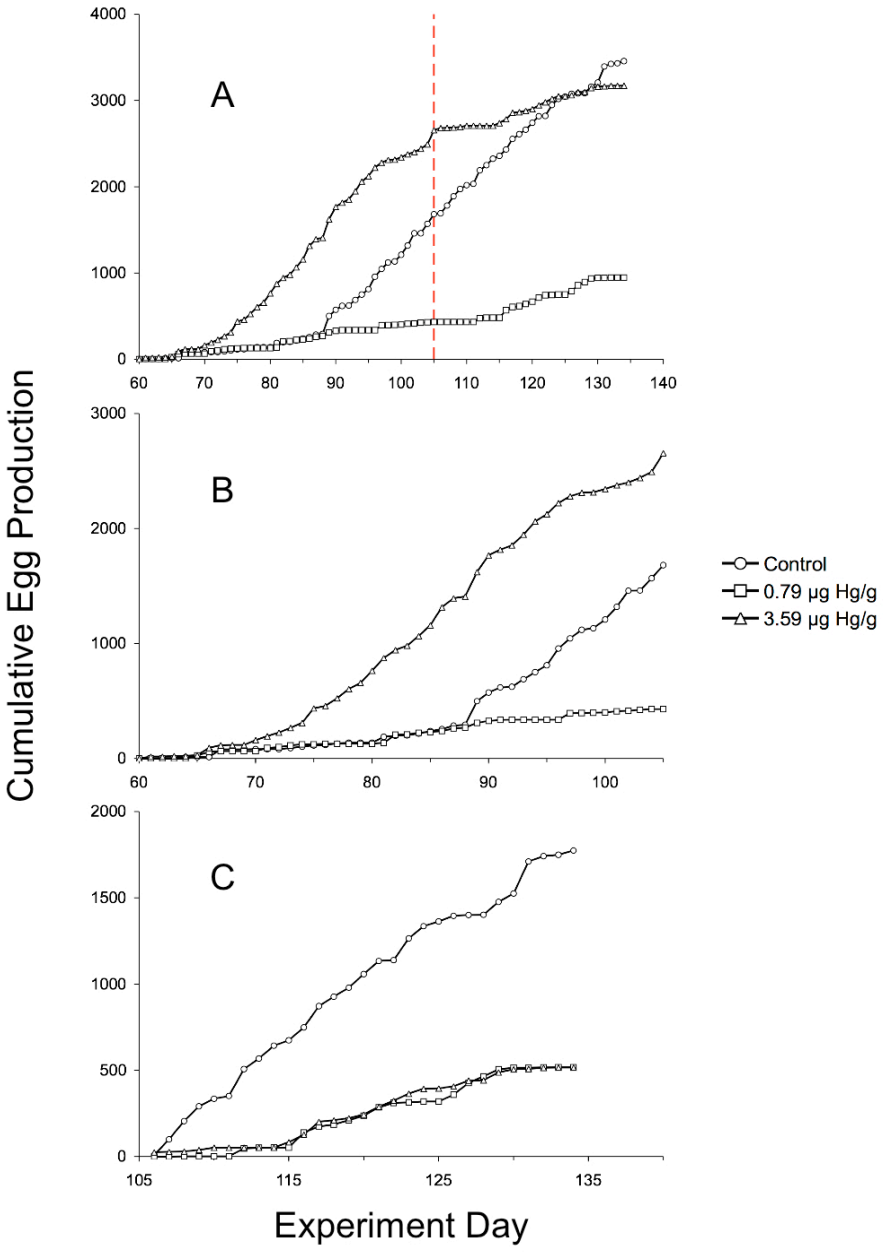
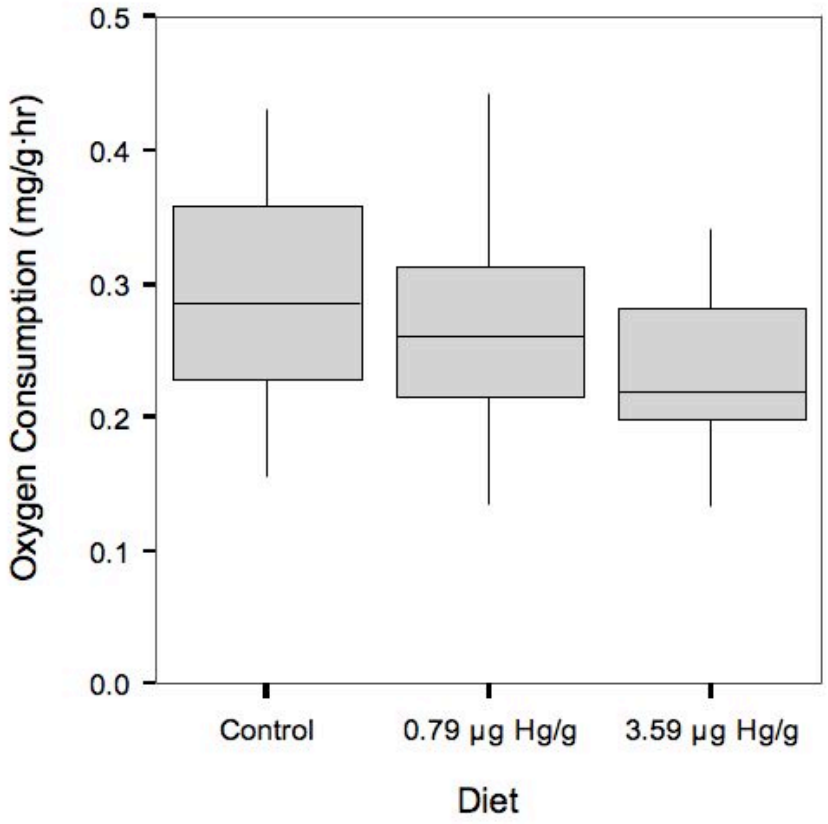


Figure 4.3. Routine metabolic rate (as oxygen consumption) in zebrafish fed diets containing 0.13 (control), 0.79, and 3.59 µg Hg/g dry wt. Horizontal lines within each box represent medians. Boxes represent middle 50% of values. Vertical lines extend to lowest and highest values.



CHAPTER 5
SULFATE DEPOSITION CONTROLS MERCURY ACCUMULATION IN FRESHWATER FISH

One-Sentence Summary

Sulfate deposition has controlled mercury accumulation in fish at Isle Royale for the past century, suggesting controls on anthropogenic sulfur emissions are necessary to reduce mercury concentrations in freshwater fish.

Abstract

Mercury contamination of fish is a global problem. Consumption of contaminated fish is the primary route of methylmercury exposure in humans and is detrimental to health. Newly-mandated reductions in anthropogenic mercury emissions aim to reduce atmospheric mercury deposition and thus mercury concentrations in fish. However, factors other than mercury deposition are important for mercury bioaccumulation in fish. In the boreal lakes of Isle Royale, USA, reduced rates of sulfate deposition since the Clear Air Act of 1970 have caused mercury concentrations in fish to decline to levels that are safe for human consumption. Therefore, a solution to the mercury problem in sulfate-limited freshwaters, including 2 million boreal lakes that contain 75% of the earth's unfrozen freshwater, must include controls on anthropogenic sulfur emissions.

Text

Anthropogenic emissions of mercury have contaminated ecosystems on a global scale (***1***). In aquatic ecosystems, microbes transform deposited inorganic mercury into methylmercury (***2***) which biomagnifies in food webs (***3***), resulting in high concentrations in fish (***4***). Consumption of contaminated fish is the major route of methylmercury exposure in humans and is detrimental to health (***5***). The Clean Air Mercury Rule, issued by the U.S. Environmental Protection Agency in 2005, and other similar initiatives aim to reduce anthropogenic mercury emissions and thus atmospheric mercury deposition. Considerable effort is now being spent to determine how mercury concentrations in fish will respond to reductions in mercury deposition, but other factors must also be important for the bioaccumulation of methylmercury in fish (***6***).

Since preindustrial times in northeastern Minnesota, USA, mercury deposition has increased by 3X while concentrations of mercury in fish have increased by 10X (***7***). In the nearby boreal lakes of Isle Royale, Michigan, USA, we discovered a recent decline in mercury concentrations in fish and point to reductions in sulfate deposition as the primary cause.

Isle Royale is an island ecosystem in Lake Superior (Fig. 5.1A) far removed from industrial and urban centers, yet it is plagued by problems of climate change (***8***) and air pollution (***9***). Mercury, perhaps the most prevalent of these pollutants, has been documented in high concentrations in fish from lakes on the island (***10-13***). Indeed, concentrations in the mid 1990s were high enough in several lakes to elicit fish consumption advisories for humans and to raise serious concerns about toxicological effects to fish and fish-eating wildlife (***11***). We returned to Isle Royale in 2004-2006 and collected 124 northern pike (*Esox lucius*) from two non-advisory, "reference" lakes and six "advisory" lakes. Northern pike are ubiquitously distributed among the island's lakes and are the predominant species in the fishery (***11***). In comparison to data from a decade ago (***11***), concentrations of total mercury in edible fillets of 55-cm northern pike (Fig. 5.1B) remained essentially the same in reference lakes (paired t-test, $t_1 = 0$, $p = 1$), but declined substantially in advisory lakes (paired t-test, $t_5 = 6.90$, $p = 0.001$) to levels considered by the U.S. Environmental Protection Agency as safe for human consumption (<0.3 μg/g wet weight) (***14***). This decline is good news for the ecosystem, and we focused on finding its cause. Three plausible hypotheses were identified: (i) atmospheric mercury deposition has declined, reducing the amount of inorganic mercury available for methylation, (ii) changes in the ecology of northern pike and/or its underlying food web have occurred, reducing the bioaccumulation and/or biomagnification of methylmercury, or (iii) environmental factors that stimulate net methylation have lessened, reducing the amount of methylmercury available for bioaccumulation. We address each of these hypotheses in turn.

The source of mercury to Isle Royale is unequivocally atmospheric (***15***), but deposition has not abated in recent years and, therefore, the amount of inorganic mercury available for methylation has not declined. Sediment records from lakes in the Park, data from the Mercury Deposition Network (linear regression, $r = 0.500$, $p = 0.069$, $n = 14$) (***16***), and literature sources (***17, 18***) all indicate a century long trend of stable or increasing rates of atmospheric mercury deposition at or near Isle Royale (Fig. 5.1C). We estimate that about 2/3 of the mercury entering the lakes at Isle Royale is mobilized from atmospheric deposition to the watershed and about 1/3

from direct deposition to the lake surface (***19***). However, this partitioning is unlikely to have changed with time, and we conclude that the deposition of mercury to the lakes has not decreased over at least the past decade, and therefore changes in mercury source cannot explain the dramatic decline in mercury concentrations we observed in fish.

We next considered whether changes in the ecology of northern pike and/or its underlying food web have occurred, influencing changes in the bioaccumulation of methylmercury. Methylmercury is primarily taken up by fish in their food (***4***). Because aquatic food webs biomagnify methylmercury (***3***), lake productivity and trophic position affect uptake (***1***). Sedimentary diatom records since 1850 indicate a slight increase in productivity in a reference lake, Lake Richie (linear regression, $r = 0.544$, $p = 0.077$, $n = 11$), but no change in productivity of an advisory lake, Sargent Lake (linear regression, $r = 0.133$, $p = 0.733$, $n = 9$) (***19***). In addition, analyses of stable carbon and nitrogen isotopes indicate no consistent, meaningful change in trophic position of northern pike in any study lake since at least 1929 (***19***). Fish growth also affects methylmercury bioaccumulation (***1***), but, as expected with little or no change in lake productivity and trophic position, growth rates of northern pike have also not changed (randomized complete block ANOVA, $F_{1,14} = 0.010$, $p = 0.936$, with linear trend test over time for males, $t_{14} = -0.540$, $p = 0.597$, and females, $t_{14} = -0.660$, $p = 0.523$) (***19***). We therefore reject the hypothesis that changes in the food web or the ecology of northern pike have influenced the biomagnification and/or bioaccumulation of methylmercury into these fish.

Finally, we examined the role of environmental factors in reducing the amount of methylmercury available for bioaccumulation. Changes in climate, landscape, and water quality could influence the net methylation of mercury in lakes (***1***). However, the environment of Isle Royale is remarkably stable. Local climate, moderated by Lake Superior, is becoming more variable with fluctuations in the North Atlantic Oscillation (***8***), but annual average temperature and precipitation have not significantly changed (***20***). Designations as a national park, a wilderness area, and an international biosphere reserve have prevented major landscape alterations for more than 50 years. In lakes on the island, water chemistry has not changed appreciably since at least 1980-1981, with the exception of sulfate (***21***). Atmospheric sulfate deposition has exhibited a downward trend at Isle Royale since monitoring began in 1985 (linear regression, $r = -0.389$, $p = 0.090$, $n = 20$; Fig. 5.1D) (***16***). Like many areas affected by high sulfate deposition (***22***), watershed outputs of sulfate to the lakes at Isle Royale have significantly

exceeded inputs to the watershed from wet deposition because of desorption from soil of sulfate deposited in past years (***20***). This excess sulfate watershed source has likely delayed recovery of lake sulfate concentrations (***22***), but as sulfate deposition has remained relatively low now for several years (***16***), watershed outputs have declined to nearly equal inputs from wet deposition (***20***). Consequently, lake sulfate concentrations have declined (randomized complete block ANOVA, $F_{2,18} = 85.2$, $p < 0.001$, with linear trend test over time, $t_{18} = -9.38$, $p < 0.001$) (***19***). Similar trends have been reported across North America and Europe and are ultimately due to decreased sulfur dioxide releases from anthropogenic sources such as coal combustion and metal smelting (***23***).

The microbial cycling of sulfur exerts a strong control on mercury methylation in lakes (***6***). Sulfate-reducing bacteria are known to methylate mercury (***1, 6***), but also of importance in freshwaters, their metabolic byproduct, sulfide, may form a neutral complex with inorganic mercury that is then readily bioavailable to a host of methylating microbes (***24***). Therefore, mercury methylation appears dependent upon the activities of sulfate-reducing bacteria. The metabolic activity of sulfate-reducing bacteria is first order with respect to sulfate concentration at low sulfate concentrations as typically found in most lakes (***25***). Thus, sulfate availability limits mercury methylation. In a direct demonstration of this relationship, additions of sulfate to Little Rock Lake, Wisconsin, USA, stimulated sulfate reduction and mercury methylation (***26***), and ultimately caused increased total mercury concentrations in fish (***27***).

We tested whether recent declines in sulfate deposition to the lakes of Isle Royale have lowered rates of sulfate reduction resulting in less methylation of inorganic mercury and thus less methylmercury in fish. We reconstructed the history of sulfate reduction by examining the concentrations of sulfur in sediment profiles (Fig. 5.2) (***28***). Before industrialization, low rates of sulfate deposition (***29***) limited sulfate reduction in lakes, as indicated deep in sediments by relatively constant and low concentrations of chromium-reducible sulfur (CRS; the ultimate end product of sulfide produced from sulfate reduction). With industrialization, rates of sulfate deposition increased steadily to a maximum of six times pre-1890 levels (***29***) in 1976. These rates have since declined in response to implementation of the Clean Air Act of 1970 (***23, 29***). This depositional history is recorded in the sediment of Sargent Lake, where the maximum CRS concentration is six times that of deep sediment and is reached at a depth (7 cm) corresponding to 1976. Thereafter, CRS concentrations decrease in accordance with declining rates of sulfate

deposition to the lake. The top few centimeters of the CRS profile could also, in part, reflect the early diagenetic addition of sulfur into organic matter through sulfate reduction. However, with low lake sulfate concentrations, most sulfate reduction and CRS formation would be expected to occur in the upper 1-3 cm of sediment (***28***) and therefore would not likely contribute to a CRS increase below this depth range.

In Lake Richie, the historic trends in CRS concentration parallel those in Sargent Lake, but the magnitude of change is much smaller (Fig. 5.2). This implies that rates of sulfate reduction in Lake Richie were consistently suppressed compared to Sargent Lake, which in turn would yield lower rates of methylmercury production and explain the lower concentrations of total mercury in northern pike from Lake Richie (Fig. 5.1B). The recent decrease in sulfate reduction rates in Sargent Lake (and presumably the other advisory lakes on Isle Royale) would also explain why fish now contain mercury below the advisory limit. Without further study, it is not immediately apparent what is causing the difference in sulfur cycling between these two lakes.

We further tested the relationship between sulfate reduction rate (as reconstructed through CRS concentration) and mercury accumulation in fish by analyzing total mercury in museum specimens of fish collected as far back as 1905 from four lakes on Isle Royale, including northern pike from Lake Richie and Sargent Lake. When concentrations of total mercury in fish are compared to time-equivalent sediment CRS concentrations, a strong positive relationship emerges (Fig. 5.3). Indeed, CRS concentration explained greater than 72% of the variability in mercury bioaccumulation during the past century (linear regression, $r^2 = 0.725$, $p < 0.001$, $n = 18$). It thus appears that sulfate deposition has controlled mercury accumulation in fish at Isle Royale for at least the past century.

Taken together, our results indicate that reductions in methylmercury contamination of fish have occurred in the absence of any change in atmospheric mercury deposition. In sulfate-limited freshwaters, such as the lakes of Isle Royale and more than 2 million other boreal lakes that contain more than 75% of the earth's unfrozen freshwater, reductions in methylmercury contamination of fish are possible solely through reductions in sulfate deposition. Existing acid rain programs and other sulfate deposition control efforts have had the unintentional additional benefit of controlling methylmercury bioaccumulation in these freshwater systems. Any significant increase in the atmospheric loading of sulfur, such as the proposed use of sulfur

dioxide to slow climate change (***30***), could reverse this positive effect. On the other hand, the eventual implementation of mercury control measures in the U.S. and globally should work synergistically with existing sulfate controls to further reduce methylmercury contamination of fish at Isle Royale and elsewhere (***31***).

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32. We thank the National Park Service for logistical support, Larry Kallemeyn and Tom Kelly for sharing data, Doug Nelson and Lilian Salling for laboratory assistance, and many volunteers for field sampling. P.E.D. received funding from Miami University, SETAC, SOAS, and the U.S. Environmental Protection Agency. This manuscript has not been subjected to the U.S. Environmental Protection Agency's required peer review policy and therefore does not necessarily reflect the views of the Agency and no official endorsement should be inferred.

Supporting Material

Additional discussion
Tables S1, S2, S3
Figure S1
Materials and methods
References

Figure Legends

Fig. 5.1. Isle Royale, mercury, and sulfate. (**A**) Location of Isle Royale (ISRO) and the nearest National Atmospheric Deposition/Mercury Deposition Network (NADP/MDN) Site. (**B**) Concentrations of total mercury (Hg_T) in edible fillets of 55-cm northern pike (*Esox lucius*) collected from eight lakes at Isle Royale during 1995-1996 (grey bars) and 2004-2006 (white bars). Lakes include Siskiwit (SIS), Richie (RIC), Intermediate (INT), Sargent (SAR), Wagejo (WAG), Angleworm (ANG), Shesheeb (SHE), and Eva (EVA). The red, dashed line represents the U.S. Environmental Protection Agency fish tissue criterion for methylmercury to protect human health. Error bars represent standard error. (**C**) Accumulation of Hg_T in sediment from four lakes at Isle Royale. Lakes include Siskiwit (dark red), Richie (blue), Sargent (orange), and Whittlesey (WHI, green). Inset graph shows wet deposition of Hg_T at the nearest NADP/MDN Site. (**D**) Wet deposition of sulfate (SO_4) at Isle Royale (open circles, dashed line) and the nearest NADP/MDN Site (filled circles, solid line).

Fig. 5.2. Concentrations of total sulfur, chromium-reducible sulfur (CRS), and non-CRS in sediment profiles from Lake Richie (RIC, blue) and Sargent Lake (SAR, orange), Isle Royale. The red, dashed lines represent 1890 (regional industrialization) and 1970 (Clean Air Act).

Fig. 5.3. History of sulfate reduction in sediment and mercury in fish at Isle Royale. (**A**) Concentrations of chromium-reducible sulfur (CRS) in sediment (lines) and total mercury (Hg_T) in edible fillets of 50-cm northern pike (N; *Esox lucius*), 30-cm walleye (W; *Sander vitreus*), and 19-cm *Coregonus* spp. (C; *Coregonus artedii*, *Coregonus clupeaformis*) from four lakes at Isle Royale. Lakes include Siskiwit (SIS, dark red), Richie (RIC, blue), Sargent (SAR, orange), and Whittlesey (WHI, green). Total lengths for standardization of Hg_T in fish (see supporting material) were chosen to overlap among years. (**B**) Scatterplot of $\log_{10}$-transformed data for CRS in sediment and Hg_T in fish.

Figure 5.1

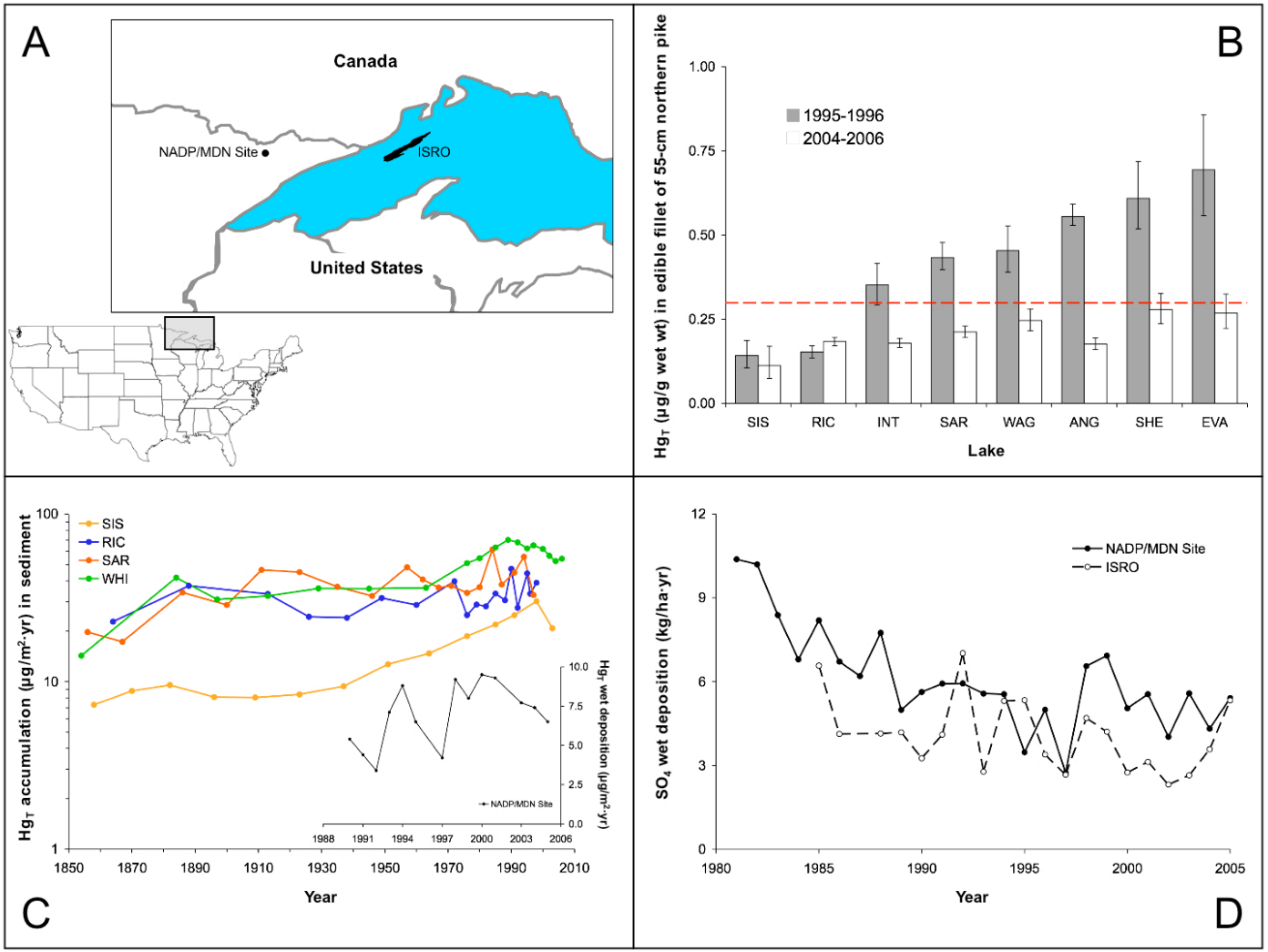

A
Canada
NADP/MDN Site
ISRO
United States
B
1995-1996
2004-2006
Hg_T (µg/g wet wt) in edible fillet of 55-cm northern pike
1.00
0.75
0.50
0.25
0.00
SIS
RIC
INT
SAR
WAG
ANG
SHE
EVA
Lake
C
SIS
RIC
SAR
WHI
Hg_T accumulation (µg/m²·yr) in sediment
100
10
1
1850
1870
1890
1910
1930
1950
1970
1990
2010
Year
Hg_T wet deposition (µg/m²·yr)
10.0
7.5
5.0
2.5
0.0
NADP/MDN Site
1988
1991
1994
1997
2000
2003
2006
D
NADP/MDN Site
ISRO
SO_4 wet deposition (kg/ha·yr)
12
9
6
3
0
1980
1985
1990
1995
2000
2005
Year

Figure 5.2

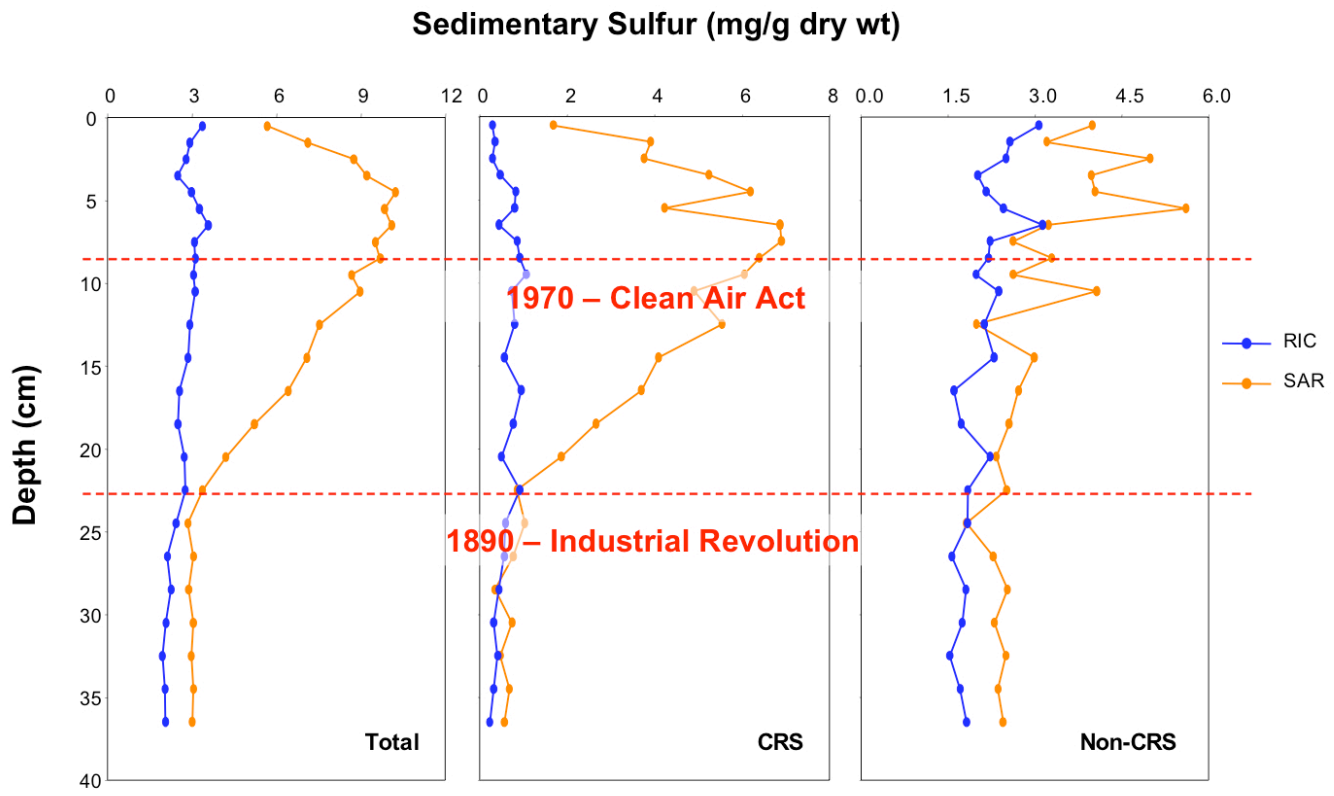
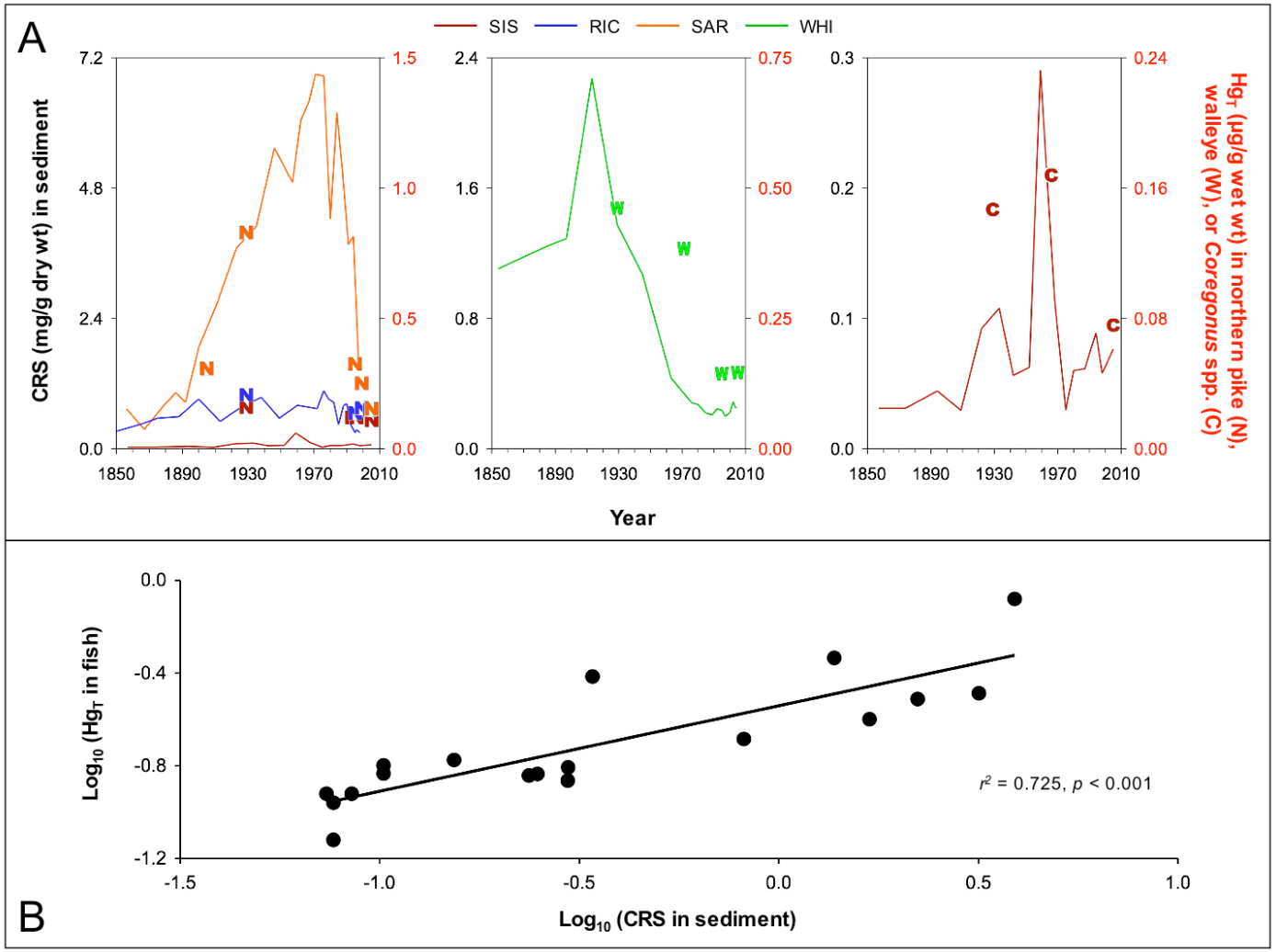

Sedimentary Sulfur (mg/g dry wt)
Depth (cm)
0
5
10
15
20
25
30
35
40
0
3
6
9
12
0
2
4
6
8
0.0
1.5
3.0
4.5
6.0
1970 – Clean Air Act
1890 – Industrial Revolution
Total
CRS
Non-CRS
RIC
SAR

Figure 5.3


A
SIS
RIC
SAR
WHI
CRS (mg/g dry wt) in sediment
Hg$_T$ (µg/g wet wt) in northern pike (N), walleye (W), or *Coregonus* spp. (C)
Year
B
Log$_{10}$ (Hg$_T$ in fish)
Log$_{10}$ (CRS in sediment)
r^2 = 0.725, p < 0.001

CHAPTER 5: SUPPORTING MATERIAL

Estimation of total mercury entering inland lakes at Isle Royale

Of the total mercury entering a given lake at Isle Royale, we estimate about 1/3 is from direct atmospheric deposition to the lake and about 2/3 is from remobilization of mercury deposited in the watershed. Our estimate is based on observed total mercury accumulation in surface sediment from four lakes and best estimates from the literature. Wet plus dry deposition (12.5 μg/m^2·yr) (***S1***) minus evasion (0.7 μg/m^2·yr) (***S2***) equals 1/3 of the flux to sediments (mean 36 μg/m^2·yr). Watershed inputs (5-43 μg/m^2·yr) (***S3***) can account for the other 2/3. Variability in watershed inputs among lakes may cause differences in total mercury accumulation in sediment.

Stable isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in northern pike

We observed statistically significant differences in $\delta^{13}C$ and $\delta^{15}N$ among years (randomized complete block ANOVA for $\delta^{13}C$, $F_{3,7} = 31.2$, $p < 0.001$, and $\delta^{15}N$, $F_{3,7} = 5.69$, $p = 0.001$), but differences are subtle and not consistent with trends in mercury concentrations in fish. Post-hoc pairwise comparisons revealed no changes in $\delta^{13}C$ ($t_7 = -0.960$, $p = 0.338$) and $\delta^{15}N$ ($t_7 = -0.380$, $p = 0.708$) during the past decade, which is the period of most interest. However, significant changes occurred between historic and recent samples (e.g., 1929 versus 2004-2006 for $\delta^{13}C$, $t_7 = 8.93$, $p < 0.001$, and $\delta^{15}N$, $t_7 = -3.88$, $p < 0.001$). For $\delta^{13}C$ and $\delta^{15}N$, recent values are on average 1.81‰ lower and 0.564‰ higher, respectively, than historic values. These are rather subtle changes, as the range for $\delta^{13}C$ in northern pike among study lakes is greater than 12‰ and Vander Zanden and Rasmussen (***S4***) reported an enrichment of 3.4‰ per trophic level for $\delta^{15}N$. Further, we cannot reasonably explain the differences in $\delta^{13}C$ and $\delta^{15}N$ among years. Differences could be an artifact from the preservation of museum specimens. Preservation with formalin and ethanol significantly affects $\delta^{13}C$ and $\delta^{15}N$ values in fish tissue, and, if possible, species-specific correction factors should be applied to adjust for these effects (***S5***). Northern pike were preserved with both formalin and ethanol (see materials and methods), but we know of no correction factors for this species. Instead, we used correction factors from the meta-analysis performed by Kelly et al. (***S5***). Otherwise, changes in $\delta^{13}C$ and $\delta^{15}N$ have

been described in lakes following the introduction of non-native species (***S6***). However, introductions of non-native species to the inland lakes of Isle Royale have not occurred (***S7***). It is also possible that source values for $\delta^{13}C$ and $\delta^{15}N$ have changed over time due to atmospheric pollution (***S8***). Regardless of possible causes, the changes in $\delta^{13}C$ and $\delta^{15}N$ are not consistent with the observed decline in mercury concentrations in northern pike from 1929 to the present. Lower $\delta^{13}C$ values indicate a shift toward food of pelagic origin. In oligotrophic lakes such as the inland lakes of Isle Royale, pelagic food webs tend to bioaccumulate contaminants to a higher degree than benthic food webs (***S9***). Higher $\delta^{15}N$ values indicate increasing trophic status. Mercury concentrations in fish increase with increasing trophic status (***S10, S11***). Therefore, as Swanson et al. (***S12***) conclude, subtle changes in trophic position may not result in changes in mercury bioaccumulation.

Tables

Table 5.S1. Mean (standard error) stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope compositions in edible fillets of northern pike in lakes of Isle Royale, 1905, 1929, 1998-1999, and 2004-2006.

	1905			1929			1998-1999			2004-2006		
Lake	*n*	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	*n*	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	*n*	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	*n*	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
ANG	.	.	.	3	-25.27 (0.10)	8.47 (0.20)	.	.	.	12	-28.20 (0.22)	9.10 (0.15)
EVA	.	.	.	3	-28.51 (0.51)	9.76 (0.30)	.	.	.	12	-29.59 (0.27)	9.73 (0.11)
INT	.	.	.	3	-24.44 (0.77)	8.84 (0.11)	.	.	.	20	-27.13 (0.12)	10.21 (0.12)
RIC	.	.	.	2	-25.99 (0.36)	9.98 (0.19)	11	-26.06 (0.34)	9.99 (0.14)	16	-26.50 (0.25)	9.70 (0.28)
SAR	1	-24.39 (na)	9.49 (na)	3	-25.97 (0.35)	9.80 (0.32)	14	-27.90 (0.17)	10.29 (0.14)	20	-26.95 (0.14)	10.58 (0.16)
SHE	.	.	.	3	-28.79 (0.16)	8.17 (0.48)	.	.	.	14	-30.92 (0.11)	9.57 (0.08)
SIS	.	.	.	1	-22.02 (na)	8.39 (na)	.	.	.	11	-23.18 (0.50)	8.48 (0.17)
WAG	.	.	.	5	-26.61 (0.29)	8.28 (0.13)	.	.	.	19	-28.63 (0.40)	8.47 (0.12)

Table 5.S2. Mean back-calculated total length (cm) of northern pike in lakes of Isle Royale, 1995-1996 and 2004-2006.

Kallemeyn (*S7*); 1995-1996

			Age (Years)											total length = a + b Ln(age)		
Lake	Sex	*n*	1	2	3	4	5	6	7	8	9	10	11	a	b	r^2
ANG	F	16	18.1	36.4	48.4	53.1	56.9	60.2	65.3	.	.	.	.	19.606	23.595	0.991
	M	11	17.5	34.6	46.9	52.4	55.4	56.9	.	.	.	.	.	18.925	22.822	0.981
EVA	F	5	20.1	31.1	43.0	50.3	52.6	.	.	.	.	.	.	18.995	21.332	0.984
	M	2	18.2	32.4	42.4	47.9	.	.	.	.	.	.	.	18.007	21.671	0.998
INT	F	24	19.9	35.9	46.4	52.2	54.8	55.3	.	.	.	.	.	21.373	20.711	0.977
	M	21	21.0	36.6	46.3	50.0	52.4	53.5	54.7	.	.	.	.	23.651	17.471	0.960
RIC	F	39	20.8	34.5	42.9	51.1	56.1	64.7	.	.	.	.	.	19.083	23.650	0.983
	M	23	19.4	32.2	42.8	50.5	52.7	.	.	.	.	.	.	18.797	21.643	0.991
SAR	F	50	20.9	38.9	49.4	53.7	56.5	58.2	59.8	.	.	.	.	23.813	20.024	0.967
	M	27	19.6	36.3	46.5	51.5	53.7	55.7	54.5	54.2	.	.	.	23.722	17.184	0.919
SHE	F	2	17.8	30.3	42.2	49.3	52.4	53.4	.	.	.	.	.	17.610	21.240	0.984
	M	3	14.2	27.2	38.4	47.1	51.5	53.7	.	.	.	.	.	13.246	23.198	0.991
SIS	F	3	22.0	41.4	53.0	65.6	73.0	74.6	79.9	82.9	93.7	95.9	98.3	19.997	32.015	0.990
	M	2	22.0	37.9	58.7	63.9	68.6	.	.	.	.	.	.	20.987	30.531	0.975
WAG	F	10	18.5	32.8	42.8	47.0	48.6	50.2	50.1	.	.	.	.	20.921	16.839	0.954
	M	7	16.3	32.1	41.5	44.6	46.5	46.3	47.2	48.2	.	.	.	20.519	14.951	0.913

This study; 2004-2006

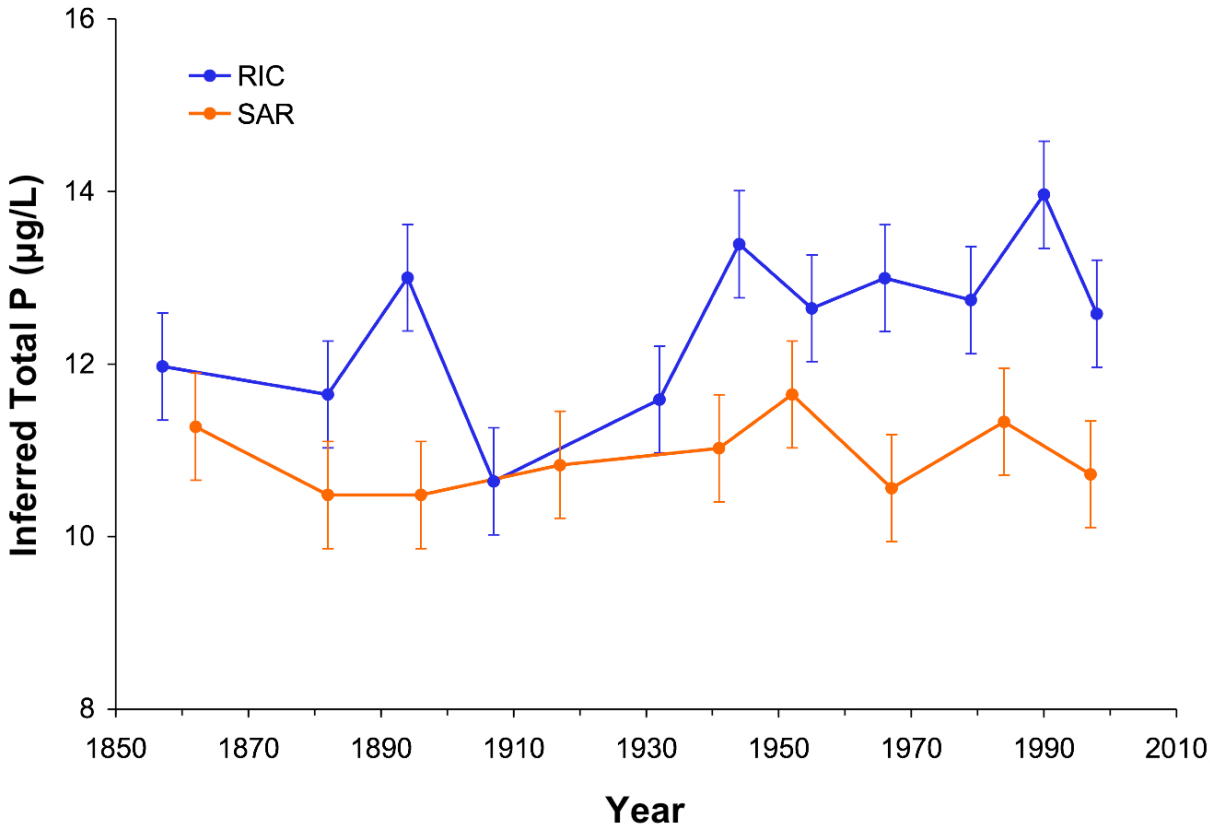
			Age (Years)											total length = a + b Ln(age)		
Lake	Sex	*n*	1	2	3	4	5	6	7	8	9	10	11	a	b	r^2
ANG	F	7	19.4	34.3	42.7	51.9	56.3	60.4	63.7	67.9	.	.	.	18.627	23.356	0.997
	M	5	18.4	32.3	41.7	51.3	57.2	59.7	61.6	62.9	.	.	.	18.052	22.685	0.988
EVA	F	8	21.5	32.3	40.4	46.5	51.5	54.3	58.7	.	.	.	.	19.142	20.302	0.995
	M	4	21.0	31.1	37.4	43.5	50.5	54.0	.	.	.	.	.	19.270	18.523	0.980
INT	F	13	20.9	35.8	43.3	49.4	52.9	55.2	57.3	59.3	62.3	.	.	22.413	18.329	0.993
	M	7	21.0	36.1	43.2	47.8	51.6	54.6	57.2	63.6	65.0	66.0	66.5	21.581	19.145	0.993
RIC	F	13	19.7	34.5	44.0	49.9	54.9	59.5	64.9	.	.	.	.	19.068	22.756	0.997
	M	3	19.1	34.1	42.2	47.6	.	.	.	.	.	.	.	19.364	20.615	0.999
SAR	F	13	22.4	38.2	45.4	49.3	53.3	56.5	59.1	61.3	63.5	.	.	24.105	18.118	0.994
	M	6	21.0	34.7	43.6	46.9	52.0	53.7	55.8	.	.	.	.	21.988	18.031	0.992
SHE	F	10	19.2	32.5	42.3	48.7	53.3	55.8	.	.	.	.	.	18.880	21.040	0.998
	M	4	16.6	30.3	41.5	46.4	50.5	52.9	.	.	.	.	.	16.801	20.991	0.994
SIS	F	9	23.0	40.1	52.8	63.7	69.4	74.2	80.7	83.4	.	.	.	21.371	29.824	0.996
	M	2	23.2	38.3	48.2	56.3	.	.	.	.	.	.	.	22.712	23.664	0.997
WAG	F	11	19.5	33.5	43.3	49.3	53.4	58.3	59.5	.	.	.	.	19.505	21.140	0.998
	M	5	19.6	31.4	40.1	46.6	51.5	54.6	59.0	.	.	.	.	18.528	20.311	0.997

Table 5.S3. Sulfate (SO_4) in epilimnetic lake water, Isle Royale, 1980-1981, 1995-1996, and 2006.

	SO_4 (mg/L)		
Lake	Stottlemyer et al. (***S13***); 1980-1981	Kallemeyn (***S7***); 1995-1996	This study; 2006
AHM	.	2.01	.
AMY	5.00	2.52	.
ANG	6.15	3.11	.
BEA	.	3.22	.
BEN	.	2.19	.
CHI	4.71	2.94	.
DES	4.32	2.55	.
DUS	4.61	2.99	.
EPI	4.32	1.48	.
EVA	.	2.10	.
FEL	.	3.18	.
FOR	4.37	1.44	.
GEO	3.70	2.29	.
HAL	4.66	1.76	.
HAR	.	2.72	.
HAT	4.37	2.29	.
INT	6.34	2.95	1.19
JOH	5.96	1.81	.
LES	.	2.84	.
LIN	.	2.68	.
LIV	4.23	3.00	.
MAS	.	3.18	.
MCD	.	3.33	.
NEW	3.22	.	.
OTT	.	2.83	.
PAT	.	1.19	.
RIC	5.62	3.24	1.67
SAR	.	3.26	.
SCH	4.95	2.69	.
SHE	.	1.74	.
SIS	.	4.49	3.35
THE	2.59	.	.
WAG	.	3.34	.
WHI	4.61	2.66	2.31

Figures

Figure 5.S1. Historical reconstruction of total phosphorus (P) in lake water of Richie (RIC, blue) and Sargent (SAR, orange), Isle Royale, from analysis of diatom records in sediment. Error bars represent standard error.



Materials and methods

Live fish were collected by gill net or hook-and-line and sacrificed according to protocols approved by the Miami University Institutional Animal Care and Use Committee. Preserved fish were obtained from the University of Michigan Museum of Zoology. Since 1920, the museum's protocol for preserving fish has involved fixation in 10% formalin for up to 1 wk, washing in water for 1 d, and storage in 70% ethanol. It is possible a different method was used to preserve the fish from 1905. Live and preserved fish were sexed, measured for total length, and sampled for scales and skin-on edible fillets. At least two scales from each fish were examined to determine age (***S14***) and back-calculated total length at age (***S15***). Skin-on edible fillets were analyzed for carbon and nitrogen stable isotopes and total mercury. For carbon and nitrogen stable isotopes, fillet subsamples were dried, ground, packaged into tin capsules, and sent to the University of California-Davis Stable Isotope Facility for analysis (***S16***). Laboratory standards for carbon (Pee Dee Belemnite) and nitrogen (atmospheric N_2) were analyzed before and after every twelve samples and were within 0.1‰ of known values. Ten percent of samples were analyzed in duplicate. Mean relative standard deviation for duplicates was 0.3% for carbon and 2.3% for nitrogen. To adjust for effects of museum preservation on $\delta^{13}C$ and $\delta^{15}N$ values of preserved fish, correction factors were applied according to the meta-analysis performed by Kelly et al. (***S5***). For total mercury, fillet subsamples were acid digested according to US EPA Method 245.6 (***S17***) and analyzed by cold-vapor atomic absorption spectroscopy. 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 6.0%. Mean recovery of spiked samples was 94.2%. Mean measured concentrations of reference materials were within (TORT-2) or 4.0% below (DORM-2) the certified ranges. It is unlikely museum preservation affected total mercury concentrations of preserved fish, as total mercury was not detectable in preservation fluids before or after use (***S18***). Concentrations of total mercury were standardized to a chosen total length for northern pike, walleye, and coregonids for each lake from regressions of $\log_{10}$-transformed data (***S19***).

Sediment cores were obtained with piston or gravity corers from depositional basins within lakes. Cores were sectioned and freeze dried. Analysis of ^{210}Pb was performed on each core to determine age and sedimentation rates (***S20***). Except for Siskiwit Lake, sedimentation rates were multiplied by a correction factor (calculated as the atmospheric ^{210}Pb flux divided by

the core-specific ^{210}Pb flux) to adjust for focusing. Analysis of total mercury was performed on each core according to previously published methods (***S21-S23***). As for fish, duplicate samples, spiked samples, and certified reference material (PACS-2) were digested and analyzed with each batch of samples. Mean relative standard deviation for duplicate samples was 6.0%. Mean recovery of spiked samples was 96.4%. Mean measured concentration of PACS-2 was within the certified range. Values for total mercury accumulation were calculated by multiplying focusing-corrected sedimentation rates by total mercury concentrations. Analysis of diatoms (***S24, S25***) was performed on cores from Lake Richie and Sargent Lake to reconstruct lake productivity (as total phosphorus). Total phosphorus was inferred from diatom assemblages by comparison with a calibration data set from 64 lakes in northern Wisconsin (***S26***). Analysis of total sulfur (TS) and CRS was performed on each core according to Aspila et al. (***S27***) and Canfield et al. (***S28***), respectively. Ten percent of samples were analyzed in duplicate. Mean relative standard deviation for duplicates was 10.5% for TS and 13.1% for CRS. Values for non-CRS were derived by subtracting CRS from TS.

Epilimnetic water was collected by hand into polyethylene cubitainers, filtered through 0.45 μm membranes, and analyzed for sulfate according to standard methods (***S29***). One of four samples was analyzed in duplicate with a relative standard deviation of 1.7%.

Data were analyzed with paired t-tests, least-squares regression, and analysis of variance (ANOVA) with SAS or SPSS. Assumptions of statistical tests were validated before analysis. Because of inherent variability in monitoring data (***S30***), a type I error (α) of 0.1 was used to judge the significance of statistical tests.

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CHAPTER 5: APPENDIX A
STABLE ISOTOPIC COMPOSITION OF SEDIMENTARY SULFUR IN LAKE RICHE AND SARGENT LAKE

The stable isotopic composition of sulfur ($\delta^{34}S$, the ratio of $^{34}S/^{32}S$ relative to Cañon Diablo Troilite) in lake sediments records historical trends in sulfate reduction (***A1***). Historical trends are recorded because the dissimilatory reduction of sulfate results in the production of ^{34}S-depleted sulfides (***A2***), which are subsequently stored in sediments. The magnitude of depletion (i.e., fractionation) is directly proportional to the concentration of sulfate (***A3, A4***). Thus, increases in sulfate concentration stimulate sulfate reduction and ultimately lower $\delta^{34}S$ values in lake sediments. This phenomenon has been used in relatively few studies (e.g., ***A5-A7***), however, to reconstruct past sulfate reduction.

We used $\delta^{34}S$ values in sediments to gain further insight into the history of sulfate reduction in the lakes of Isle Royale. Lake Richie and Sargent Lake sediments were analyzed for $\delta^{34}S$ in TS, CRS, and non-CRS fractions. Briefly, fractions were isolated according to Canfield et al. (***A8***), packaged into tin capsules, and sent to Iso-Analytical (Sandbach, UK) for analysis. Details of analytical methods (***A8***) and derivation of $\delta^{34}S$ values (***A2***) have been reported elsewhere. Laboratory standards (IAEA-S-1, IA-R025, IA-R036) were analyzed during each run sequence and were within 0.07‰ of known values.

Isotope results (Figure 5.A1) below 10-cm sediment depth agree with our use of CRS concentrations to reconstruct past sulfate reduction in Lake Richie and Sargent Lake. As for CRS concentrations, relatively constant $\delta^{34}S$ values in deep sediment indicate low rates of sulfate deposition (***A9***) limited sulfate reduction in both lakes before industrialization. With industrialization, up-core decreases in $\delta^{34}S$ values indicate increased sulfate deposition (***A9***) stimulated sulfate reduction in both lakes. This interpretation relies on the fair assumption that $\delta^{34}S$ values of source sulfate have remained constant over time (***A10***). A test of this assumption is now possible with the analysis of $\delta^{34}S$ in annual tree rings (***A11***) and could be done in the future.

Isotope results for the top 10 cm of sediment may reflect contemporary rather than historic processes. With decreased lake sulfate concentrations since implementation of the Clean Air Act (***A12***), we expected $\delta^{34}S$ in recent sediments to return to values found deeper in the

cores. This response occurred in Lake Richie, as $\delta^{34}S$ in all sulfur fractions returned to values typical before industrialization. However, we cannot state with confidence that these changes are due to decreased lake sulfate concentrations. Detailed studies by Fry et al. (***A3***) indicate increased $\delta^{34}S$ values in the top few centimeters of sediment may alternatively be due to sulfide oxidation. Sulfide oxidation is a poorly understood process, but is known to occur in well-mixed sediments (***A13***) and to involve phototrophic sulfur bacteria (***A2***). Lake Richie has well-mixed sediments (Figure 5.A2) and contains an abundant population of phototrophic sulfur bacteria (***A14***). Thus, the observed increase in $\delta^{34}S$ values may very well be due to sulfide oxidation, which could account for the lower CRS concentrations in Lake Richie than in Sargent Lake (***A3***). In contrast, values of $\delta^{34}S$ in Sargent Lake decrease to the sediment surface. This result is at odds with evidence from CRS concentrations that sulfate reduction rates have declined. Unfortunately, we have little additional information from Sargent Lake to understand this disparity. It is possible, but highly speculative, that water in Sargent Lake has been depleted in ^{34}S due to sulfur exchange with the sediment (***A15***). For future investigations, we recommend determining the $\delta^{34}S$ value of water as well as sediments.

In sum, the stable isotopic composition of sulfur in sediments contributed little to our understanding of sulfate reduction in the lakes of Isle Royale due to both active processes in recent sediment and limitations of our study. Future research can improve upon our study by analyzing $\delta^{34}S$ in more lakes, in multiple cores from each lake, and in source sulfate through time. Values of $\delta^{34}S$ in lake sediments from the Great Lake region may not provide reliable records of sulfate reduction (***A7***), however, because of sulfide oxidation, isotope exchange, or other factors.

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Figure 5.A1. Stable isotopic composition of total sulfur, chromium-reducible sulfur (CRS), and non-CRS in sediment profiles from Lake Richie (RIC, blue) and Sargent Lake (SAR, orange), Isle Royale.

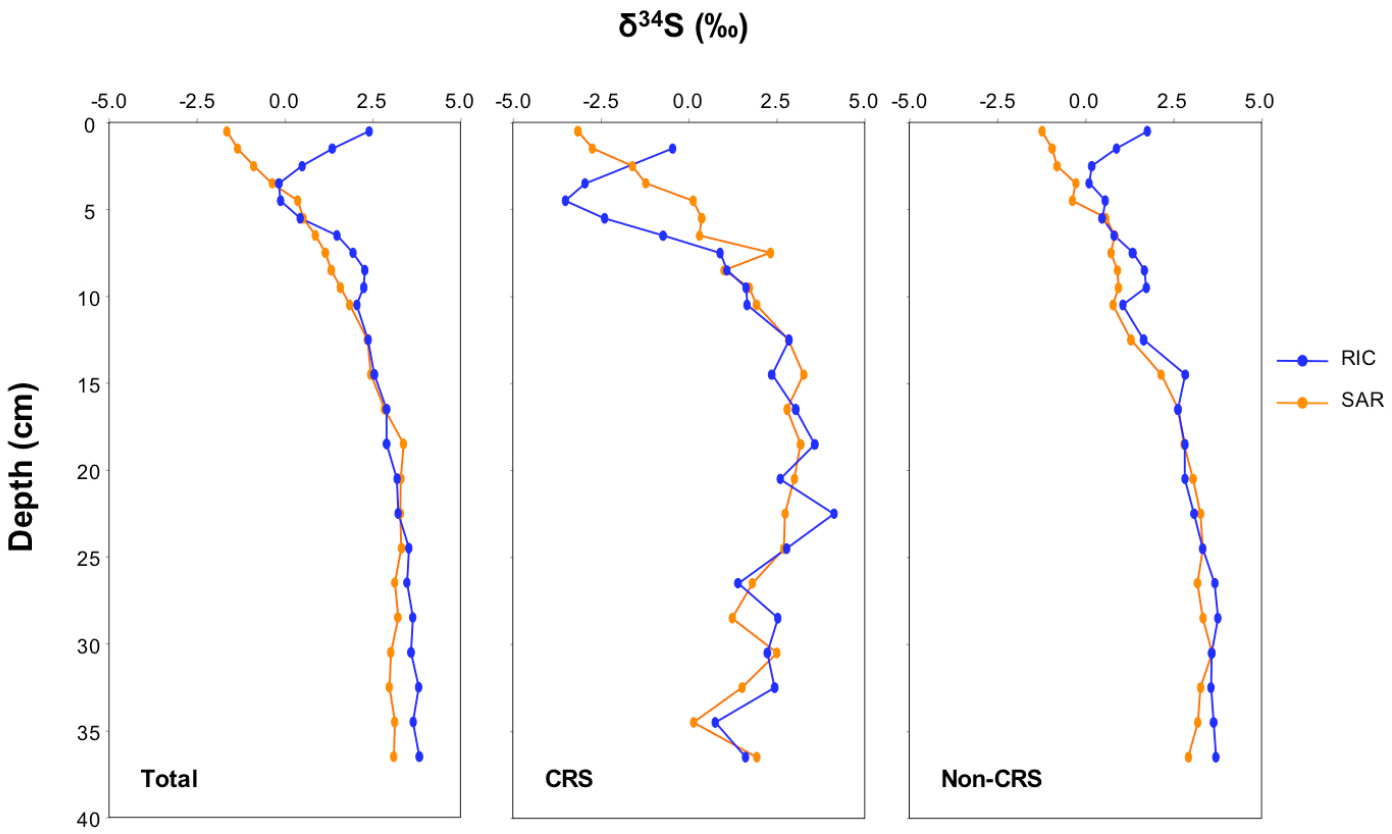
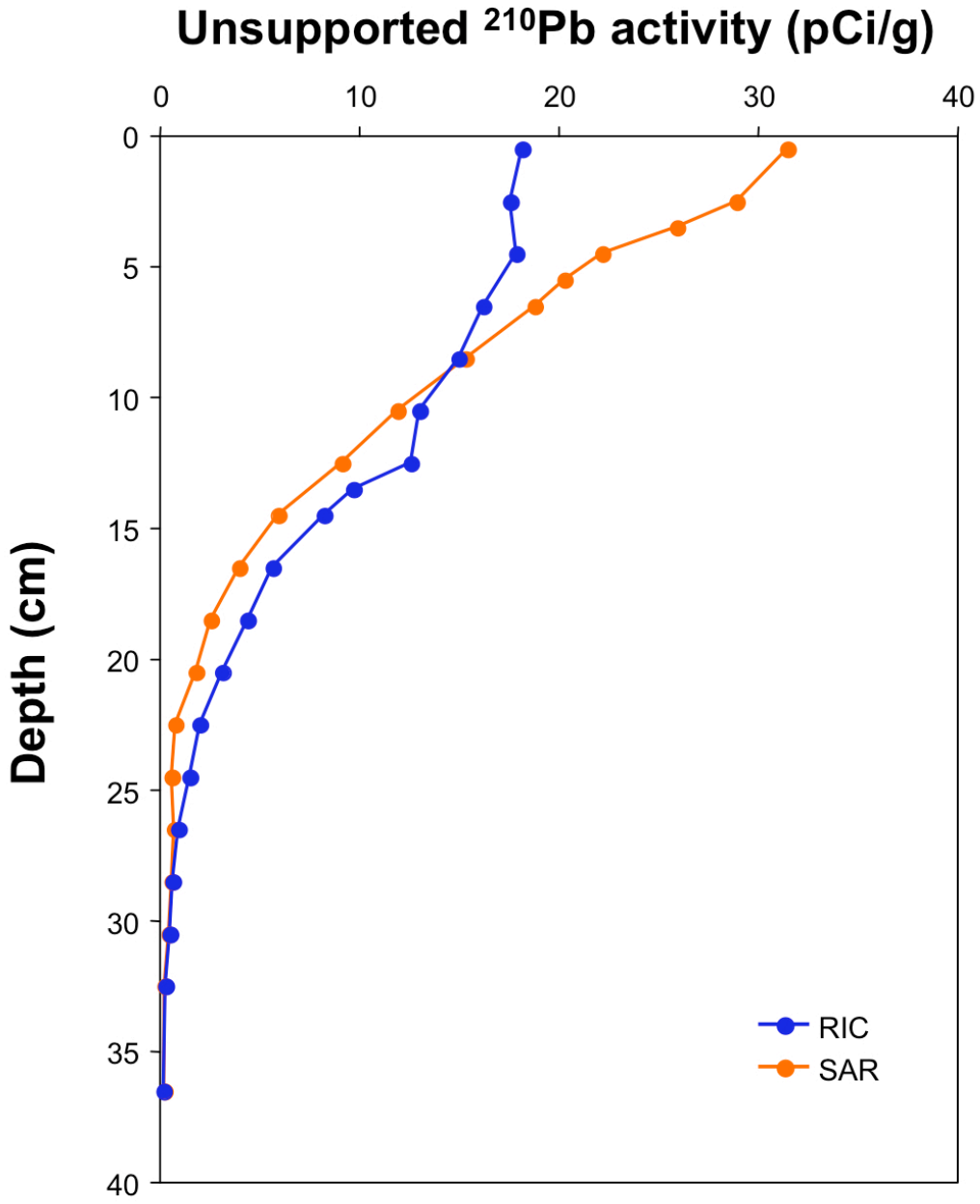


Figure 5.A2. Indication of mixing, from unsupported ^{210}Pb activity, in sediment profiles from Lake Richie (RIC, blue) and Sargent Lake (SAR, orange), Isle Royale. Total ^{210}Pb activity is the sum of supported ^{210}Pb, which is continuously produced as uranium in the sediment decays over time, and unsupported ^{210}Pb, which was deposited atmospherically. Inputs of unsupported ^{210}Pb cease once the sediment is buried and shielded from further deposition. Relatively constant values in the top few centimeters of Lake Richie indicate mixing of sediment.



CHAPTER 5: APPENDIX B
NO SEASONAL TREND IN MERCURY CONCENTRATION OF GREAT LAKES REGION NORTHERN PIKE

In dimictic lakes at Isle Royale and elsewhere, environmental factors that influence microbial cycling of sulfur and mercury exhibit considerable variation with season (***B1***). Most pronounced, temperature and dissolved oxygen alternate through predictable seasonal phases: summer stratification, spring turnover, winter stratification, and fall turnover (***B2***). Warm temperatures and hypolimnetic oxygen deficit during summer stratification result in net methylation of mercury (***B3***). Cold temperatures and/or oxygenated waters during winter stratification and turnover result in net demethylation of mercury (***B3***). Thus, variation in environmental factors with season likely influences the amount of methylmercury available for bioaccumulation by fish. However, it is not well known if mercury concentrations in fish also exhibit seasonal variation. Fish in our study were collected during at least three seasons (spring, summer, fall). To fairly represent seasonal variation in our analysis, we conducted a parallel study to investigate seasonal trends in mercury concentrations of fish.

We tested the hypothesis that mercury concentrations in northern pike vary with season. Isle Royale was not used as the study site because access is restricted during November-April. Rather, the well-studied and dimictic Douglas Lake, Michigan, was used (Figure 5.B1). Douglas Lake is located near Lake Huron and Lake Michigan and is the site of the University of Michigan Biological Station (UMBS). This setting provides a climate similar to Isle Royale, but with year-round access and support through UMBS. Twenty northern pike were sampled September 2005 and January, May, and July 2006 with non-lethal methods (***B4***) approved by the Miami University Institutional Animal Care and Use Committee. Briefly, fish were captured by angling, sexed, measured for total length, sampled for skin-on axial muscle tissue with a 6-mm diameter biopsy punch, treated with tissue adhesive to seal the biopsy wound, and released to the water. Muscle tissue was acid digested according to US EPA Method 245.6 (***B5***) and analyzed for total mercury by cold-vapor atomic absorption spectroscopy. 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 8.4%. Mean

recovery of spiked samples was 94.9%. Mean measured concentrations of reference materials were within (TORT-2) or 0.3% below (DORM-2) the certified ranges.

Data for northern pike are presented in Table 5.B1. Rather than analyze data by sampling period, we chose to utilize temperature and dissolved oxygen profiles from UMBS (***B6***) to analyze data by seasonal phase. Analyzing by seasonal phase maximizes our studies relevance to the biogeochemistry of mercury in the lake (***B3***). Samples from September/July, January, and May were collected during summer stratification, winter stratification, and spring turnover, respectively (Figure 5.B2). Contrary to expected for fish (***B1***), total mercury concentration was not significantly related to total length (linear regression with $\log_{10}$-transformed data; $r = -0.078$, $p = 0.744$, $n = 20$), making comparisons across seasonal phases difficult. Significant relationships allow for the standardization of total mercury by total length (***B7***), which facilitates spatial and temporal comparisons. The lack of a positive relationship may be due to an apparent separation of northern pike in the lake into two groups (Figure 5.B3). Group 1 is comprised of larger individuals with relatively low total mercury concentrations. Group 2 is comprised of smaller individuals with high total mercury concentrations. There is some overlap in size between the groups. With the data separated into these groups, significant relationships emerge between total length and total mercury (linear regression with $\log_{10}$-transformed data; Group 1: $r = 0.612$, $p = 0.026$, $n = 13$; Group 2: $r = 0.766$, $p = 0.044$, $n = 7$). We therefore standardized total mercury concentrations for group and seasonal phase to the minimum total length that northern pike may be caught and kept by anglers (61 cm). Standardized total mercury concentrations were then compared across seasonal phase by random block (on group) ANOVA.

Test results indicate there is no change in total mercury concentration of northern pike across seasonal phase (ANOVA, $F_{2,2} = 0.701$, $p = 0.588$; Figure 5.B4). Another recent study reported no significant effect of season on total mercury concentrations of smallmouth bass (*Micropterus dolomieu*) (***B8***). This lack of seasonal effect in fish is likely due to the relatively large reservoir of accumulated methylmercury in skeletal muscle (***B9***) and the long elimination time of methylmercury (***B10***). These results further indicate other factors not accounted for in our study, such as seasonal mercury flux from the atmosphere or watershed, also do not affect total mercury concentrations in fish. Mercury deposition is measured at UMBS by the University of Michigan Air Quality Laboratory, but data was not made available to us for this study. We conclude that there is no seasonal variation in total mercury concentrations of Great

Lakes region northern pike. Thus, collecting fish during three seasons at Isle Royale did not bias our results.

As a side note, it is interesting that in relation to mercury there are two distinct groups of northern pike in Douglas Lake. This pattern has not been reported for populations elsewhere. Zebra mussels (*Dreissena polymorpha*) have recently invaded Douglas Lake (personal communication, Bob Vande Kopple, UMBS), and we speculate they may be causing elevated total mercury concentrations in Group 2 northern pike. Mechanisms may include (i) a diet shift of small northern pike to contaminated zebra mussels, (ii) a lengthening of the lake's food web, and (iii) a shunting of mercury to benthic food resources. For Group 2, the standardized total mercury concentration of a 61-cm northern pike (1.286 μg/g wet wt) exceeds by more than 4 fold the U.S. Environmental Protection Agency (***B11***) fish tissue criterion for mercury. Northern pike are a popular sport fish in Douglas Lake (***B12***). To protect public health, it is important to investigate mercury in Douglas Lake northern pike further.

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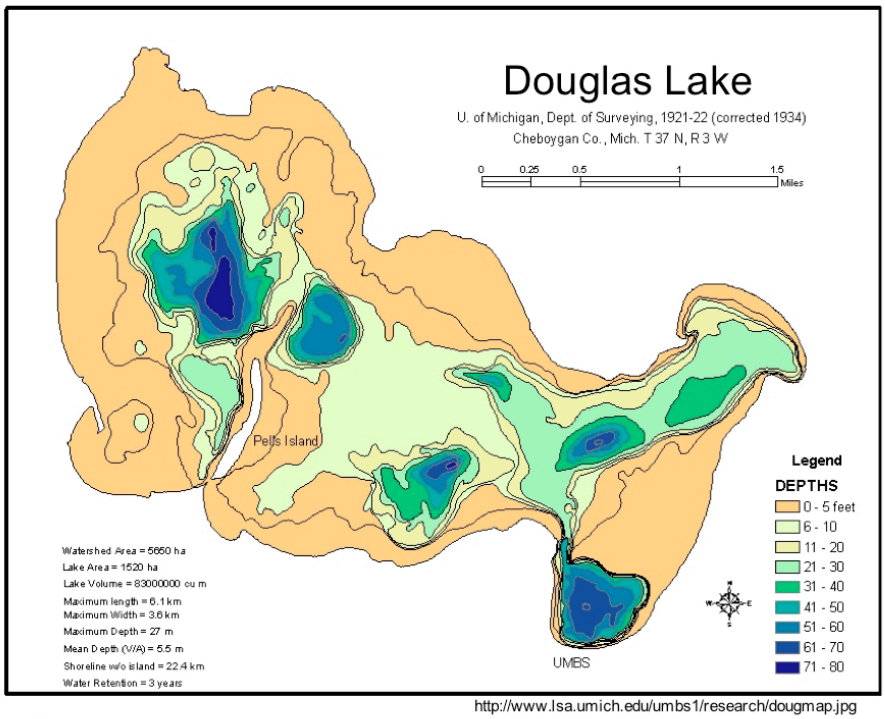
Table 5.B1. Data for collection date and season, age, total length, and total mercury concentration (Hg_T) of northern pike collected from Douglas Lake during 2005-2006.

Fish ID	Year	Month	Age (Years)	Total length (cm)	Hg_T (µg/g wet wt)	Seasonal phase	Group
1	2005	September	3	54.3	0.346	summer stratification	1
2	2005	September	6	68.0	0.603	summer stratification	1
3	2005	September	4	61.0	1.355	summer stratification	2
4	2005	September	2	46.5	0.627	summer stratification	2
5	2005	September	4	61.5	0.516	summer stratification	1
6	2005	September	4	58.0	0.447	summer stratification	1
7	2005	September	5	64.0	0.437	summer stratification	1
8	2005	September	5	64.0	0.338	summer stratification	1
9	2006	January	4	58.4	0.543	winter stratification	1
10	2006	January	6	70.7	0.442	winter stratification	1
11	2006	January	4	59.0	1.005	winter stratification	2
12	2006	January	6	71.0	0.692	winter stratification	1
13	2006	January	5	61.9	1.047	winter stratification	2
14	2006	January	3	55.7	1.663	winter stratification	2
15	2006	May	4	58.6	0.520	spring turnover	1
16	2006	May	6	73.3	0.685	spring turnover	1
17	2006	May	3	54.1	0.343	spring turnover	1
18	2006	May	3	53.9	1.013	spring turnover	2
19	2006	May	2	47.2	0.597	spring turnover	2
20	2006	July	7	75.1	0.516	summer stratification	1

Figure 5.B1. Location (star) and map of Douglas Lake, Michigan.



http://www.lsa.umich.edu/umbs1/research/dougmap.jpg

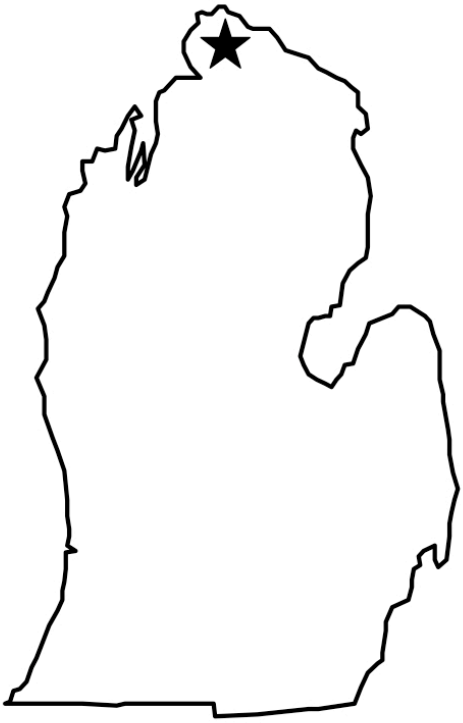


Figure 5.B2. Seasonal phases of temperature (closed circles, solid lines) and dissolved oxygen (open circles, dashed lines) in Douglas Lake. Data is from the University of Michigan Biological Station <http://www.lsa.umich.edu/umbs/research/data/>.

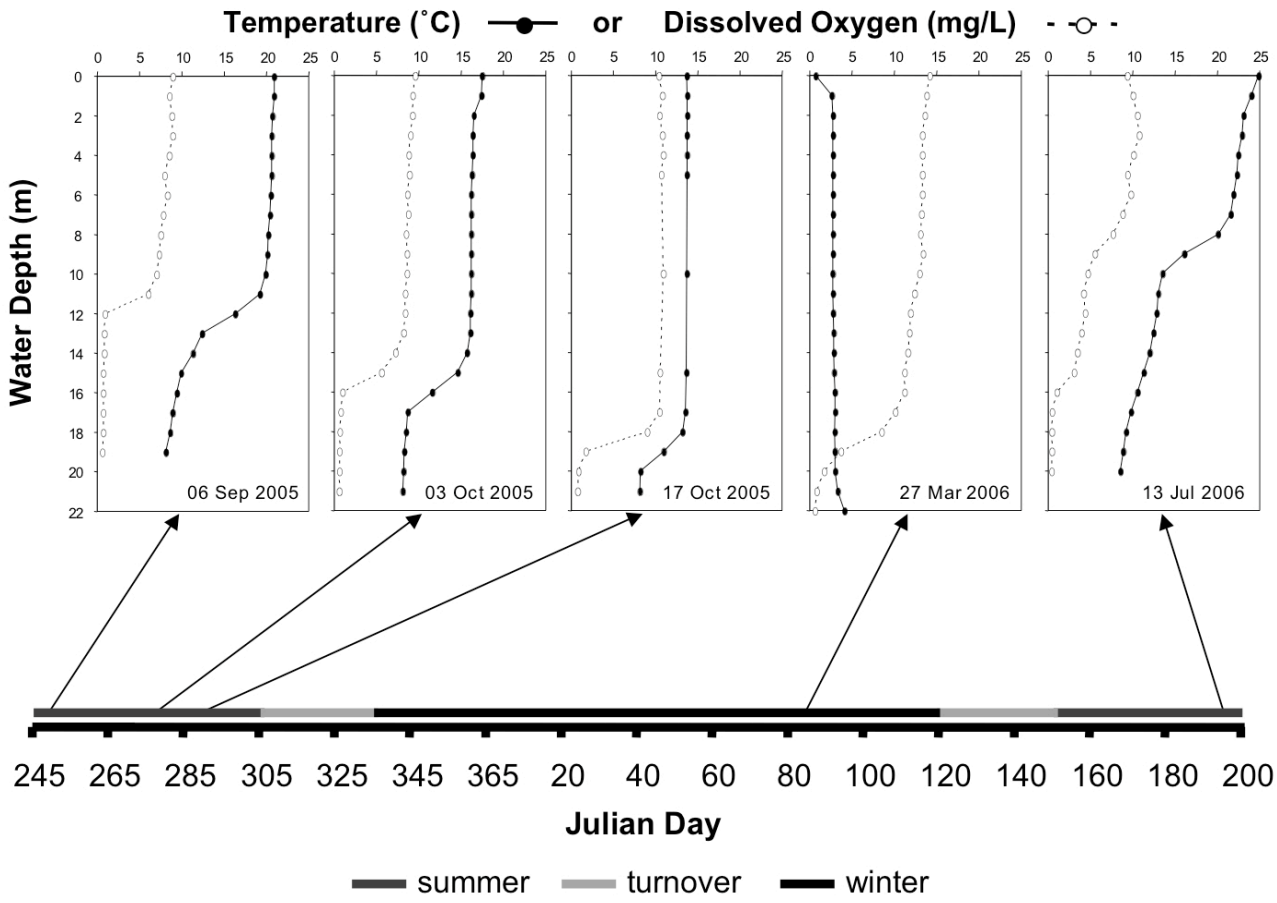


Figure 5.B3. Total length and total mercury concentrations of Group 1 (open circles) and Group 2 (closed circles) northern pike from Douglas Lake.

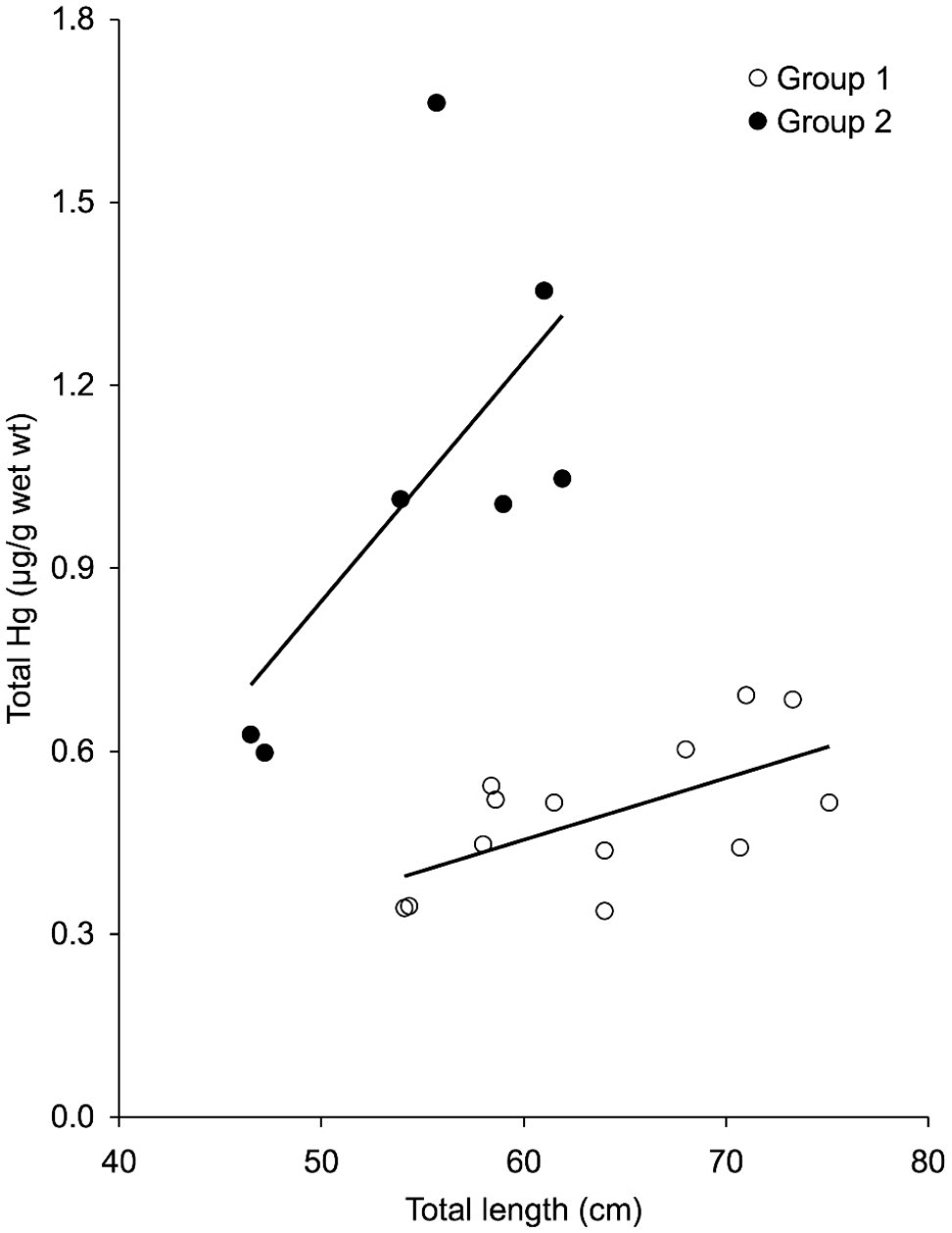
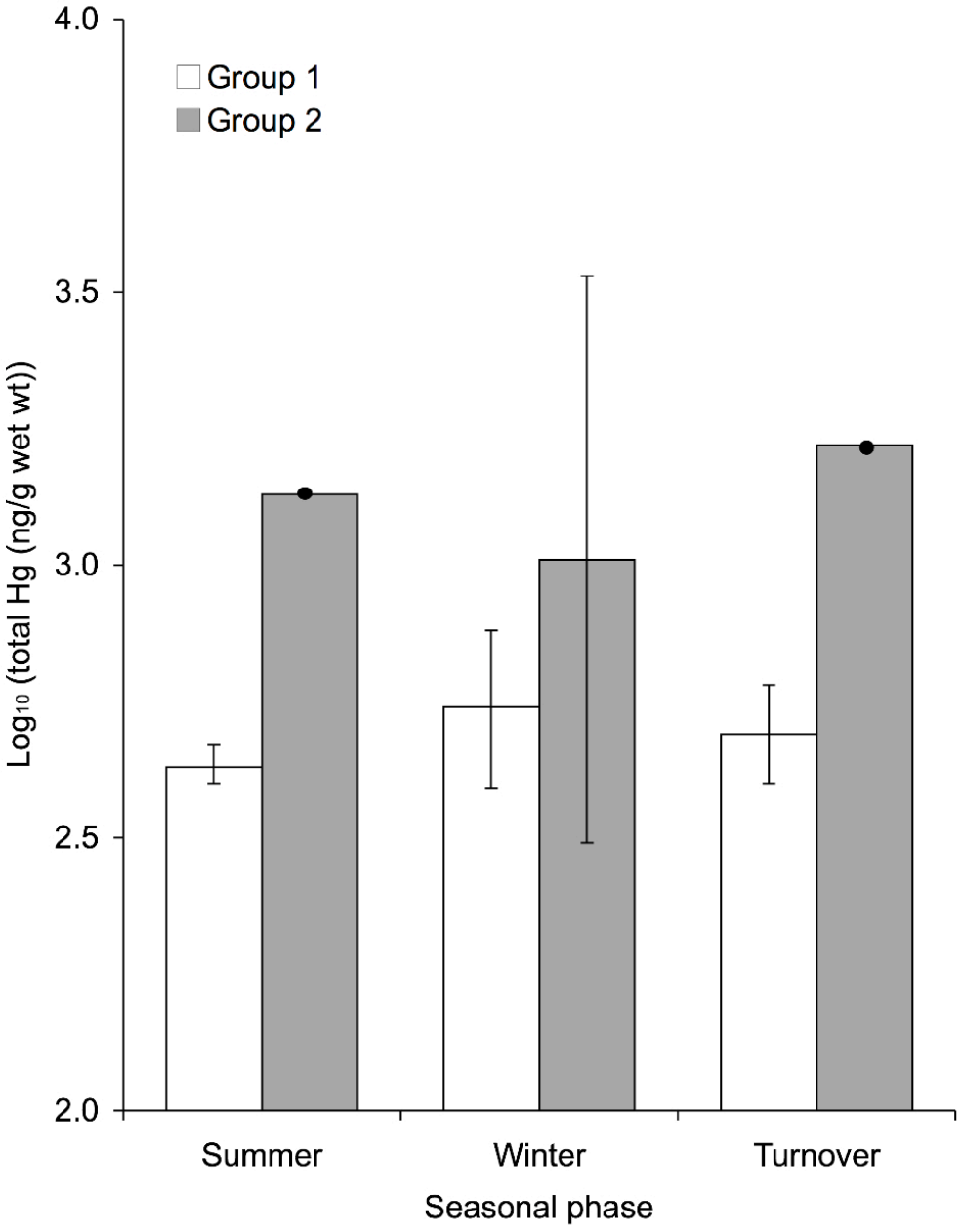


Figure 5.B4. Calculated concentrations of total mercury (log_{10} transformed) in axial muscle of 61-cm Group 1 (white bars) and Group 2 (grey bars) northern pike collected during summer stratification, winter stratification, and spring turnover. Error bars, calculated from log_{10}-log_{10} regressions of total length versus total mercury, represent standard error. Bars displayed with dots indicate that standard error could not be calculated due to sample size restrictions.



CHAPTER 6
CONCLUSION

As stated throughout this dissertation, mercury contamination of fish is a serious environmental problem. I conducted research to contribute new knowledge to help better understand and hopefully solve this problem. My research has been guided by two questions that are integral to ongoing consideration of policy options for a solution. In this conclusion, I will address how my research has played a role towards answering these questions. I will also suggest research, management, and regulations that could further my work.

Question 1: What is the evidence that humans, fish, wildlife, and other biota are being adversely affected by exposure to methylmercury (Scheuhammer et al. 2007)?

This topic (excluding effects on wildlife and humans) has been the primary focus of my research, and I will discuss the exposure of fish to methylmercury and its effects on reproduction. The bioaccumulation of mercury in teleost fish is well characterized (Wiener et al. 2003). Comparatively, little is known about the bioaccumulation of mercury in non-teleost fishes, such as lampreys and sharks (de Pinho et al. 2002). My research on the ontogenetic dynamics of mercury accumulation in northwest Atlantic sea lamprey (Chapter 2) thus fills a gap in knowledge. I discovered there are fundamental differences in mercury accumulation between sea lamprey and teleosts. Further, all life stages of sea lamprey are exposed to relatively high concentrations of mercury, especially adults. More research should be directed towards understanding mercury contamination of diverse groups of fishes and its toxicological effects. I also filled a gap in knowledge in the toxicological effects of methylmercury on reproduction. Many recent studies have shown altered reproduction in fish exposed in the laboratory to dietary methylmercuy (e.g., Matta et al. 2001; Hammerschmidt et al. 2002; Sandheinrich and Miller 2006). My previous research showed that reduced fecundity in fathead minnows exposed to dietary methylmercury was due to the suppression of sex steroid hormones (Drevnick and Sandheinrich 2003). Research for this disseration shows the suppression of sex steroid hormones in these same fathead minnows was caused by increased ovarian follicular apoptosis (Chapter 3). With collaborators, I have thus linked methylmercury toxicity from cells (increased apoptosis) to physiology (suppression of sex steroid hormones) to individuals (reduced fecundity). These

studies provide biomarkers to study the effects of methylmercury on reproduction in wild fish. A study involving zebrafish, however, suggests that reproduction of fish exposed to dietary methylmercury may also be dependent upon diet quality and quantity (Chapter 4). Aquatic ecosystems are commonly contaminated with methylmercury (Wiener et al. 2003), but many of these ecosystems have healthy fish populations. There must be some mechanism that allows for healthy fish populations in high methylmercury environments. This study indicates diet quality and quantity may be part of that mechanism, but much more research is required. In sum, my research has provided knowledge of (1) methylmercury exposure in fish, (2) a possible mechanism for reproductive toxicity, (3) biomarkers to study wild fish populations, and (4) a hypothesis to explain how fish populations can remain healthy in high methylmercury environments. I provide no evidence, however, that wild fish are being adversely affected by exposure to methylmercury.

To determine whether wild fish are adversely affected by methylmercury, I recommend the framework presented in Figure 6.1. This framework builds on my research and includes both direct (toxicological) effects and indirect (food web) effects of methylmercury. I defer to the figure legend for a complete explanation.

Within this framework, I suggest the use of (1) wild sea lamprey to investigate direct effects and (2) laboratory experiments to initially investigate indirect effects of methylmercury. Sea lamprey are an ideal species to study the direct effects of methylmercury on wild fish because they exhibit natural gradients of mercury contamination within populations (Chapter 2), are easy to observe and collect (Boyd Kynard, personal communication), and much is known of their genetics and endocrinology (Sower 2003). Further, although sea lamprey do bioaccumulate organic contaminants (MacEachen et al. 2000), most of these contaminants have little effect on sea lamprey because their aryl hydrocarbon receptor has little binding affinity for organics (Hahn et al. 1997) and does not induce CYP1A (Hahn et al. 1998). Therefore, organic contaminants may not confound studies of methylmercury toxicity, as has been reported in teleost fish (Friedmann et al. 2002, Webb et al. 2006). Lastly for sea lamprey, there are huge, unwanted populations in the Great Lakes that are highly contaminated with methylmercury (MacEachen et al. 2000) and easily sampled. To study indirect effects of methylmercury on fish reproduction, an experiment such as that in Chapter 4 could be conducted, but with a factorial design with dietary concentration and food quality/quantity as factors. If the impairment of reproduction by

methylmercury is again dependent on food quality/quantity, but with an experimental control to infer causality, then field studies will also be warranted to investigate the indirect effects of methylmercury on wild fish reproduction.

Question 2: How would methylmercury concentrations in fish respond to reduced anthropogenic emissions of mercury (Munthe et al. 2007)?

Reductions in methylmercury concentrations in fish are of utmost importance for ameliorating methylmercury toxicity in humans, fish, wildlife, and other biota, but my research has determined this result is possible without reductions in anthropogenic emissions of mercury. Plans to reduce mercury emissions (such as those outlined by the U.S. Environmental Protection in the Clean Air Mercury Rule) are noble indeed, but may not matter in the face of greatly increasing emissions in Asia. Mercury emissions from Asia are transported across the Pacific Ocean (Steding and Flegal 2002, Jaffe et al. 2005) and may contribute as much as 36% of the total mercury deposited in the U.S (Seigneur et al. 2004). Factors other than mercury deposition that are important for the bioaccumulation of methylmercury in fish must be considered for the management of mercury in the U.S.

I have discovered that in sulfate-limited freshwaters, reductions in methylmercury concentrations in fish are possible solely through reductions in sulfate deposition. Reduced sulfate deposition to the lakes of Isle Royale, for example, has recently caused methlymercury concentrations in fish to decline to levels safe for human consumption. Any significant increases in the loading of sulfate to freshwaters would thus impede this effect.

Therefore, to solve the mercury problem in sulfate-limited freshwaters, I suggest policy and management of sulfur at local, regional, and global scales. At the local scale, sulfate should not be added to freshwaters unnecessarily. It is common practice for lake managers to add copper sulfate to lakes to control invasive and/or unwanted species (Flemming and Trevors 1989). For example, more than 1,9000,000 kg of copper sulfate was added to lakes in Michigan during 1970-2000 (Michigan Deparment of Environmental Quality, written communication). At the regional level, existing acid rain programs should continue. Acid rain programs have been shown to be very successful in reducing regional sulfate deposition (Stoddard et al. 1999). My research shows these successful programs are indirectly reducing methylmercury concentrations in fish. At the global scale, the proposed use of sulfur dioxide to slow climate change (Crutzen

2006, Wigley 2006) should be carefully considered for its potential impact on methylmercury accumulation in fish, wildlife, and humans. Wigley (2006) suggested the addition of 5 Tg of sulfur dioxide to the stratosphere every year would lower global temperatures by 1-2 ˚C. This amount of sulfur represents 7% of current sulfur dioxide emissions from the burning of fossil fuels (Wigley 2006). Through stratospheric processes, sulfur dioxide would be converted to sulfate and deposit to the earth's surface (Crutzen 2006), quite eventually increasing methylmercury concentrations in freshwater fish. This result would be a detriment to ecosystems and humans globally.

Parting words

Many questions, including the two I have highlighted in this dissertation, remain to be answered about mercury pollution. However, there is a substantial body of evidence, including this dissertation, that shows mercury pollution has serious toxicological, social, and economic consequences. This evidence provides ample reason to create better policy on mercury pollution. The future of mercury science should be focused on providing policymakers with the knowledge to quickly and cost-effectively control mercury pollution.

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Figure 6.1. Framework to investigate direct (toxicological) and indirect (food web) effects of methylmercury on wild fish. Relations among methylmercury-induced effects on fish include: (1) indirect effects mediated through the food web; (2) direct toxicological effects (i.e., interaction of methylmercury with target molecule); (3) food web effects dependent upon ecological factors (e.g., system productivity); (4) nutritional deficiencies due to poor food quality/quantity; result of mercury effects on food base; (5) energetic costs associated with detoxication; (6) toxicological effects on reproduction (i.e., alterations in steroidogenesis, vitellogenesis, gonadal development, gametogenesis, and behavior); (7) reduced energy for somatic growth; (8) reduced energy for reproduction; (9) fish optimize fitness by balancing somatic growth and reproduction (i.e., energy trade-off); size of fish determines fecundity; (10) altered growth affects competitive interactions and delays seasonal reproductive activity and age to sexual maturation, resulting in negative effects on year-class strengths of fish; (11) impaired reproduction may cause detrimental, long-term effects to populations; compensatory mechanisms may prevent alterations in recruitment.