Molecular, histological, and behavioral differences in largemouth bass (*Micropterus salmoides*) and topsmelt (*Atherinops affinis*) exposed to methylmercury

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By

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Abstract

Molecular, histological, and behavioral differences in largemouth bass (*Micropterus salmoides*) and topsmelt (*Atherinops affinis*) exposed to methylmercury

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Methylmercury (MeHg) pollution in aquatic environments is recognized as a serous threat, yet the sublethal effects of MeHg exposure on fish are not well understood. Assessing the sublethal effects of MeHg is vital in order to understand how this exposure may affect reproductive fitness and long-term survival, important regulators of population growth and survival. To evaluate the sublethal effects of MeHg on a wild fish population, I examined the relationship between environmental MeHg exposure and two biomarkers of effect (i.e., hepatic metallothionein gene expression, histopathological assessment in liver, spleen, and kidney tissues) in largemouth bass (*Micropterus salmoides*) collected from the Sacramento-San Joaquin Delta, CA, USA. The relationship between MeHg exposure and histopathological parameters (i.e., immune responses, parasite density) was compared between juvenile and adult largemouth bass. The total mercury (Hg) concentration range for muscle tissue from largemouth bass was 0.117-0.975 ppm (wet weight). Metallothionein gene expression was lower, yet not statistically significant ($p=0.081$), in high Hg exposed adults (0.63-0.97 ppm), suggesting an inhibition of the metallothionein response mechanism with increased MeHg exposure. Histopathological results indicated that juvenile largemouth bass could be more susceptible than adults to MeHg exposure, as high-exposed juveniles showed indication

of immunosuppression, with significantly lower macrophage density in kidney and liver tissues ($p=0.018$, and 0.020, respectively), higher trematode density in liver tissue ($p=0.014$), and a greater number of trematodes that survived to maturation. To evaluate the behavioral effects of environmentally realistic MeHg exposure in fish, a dosing study was performed on topsmelt (*Atherinops affinis*) and change in school area was examined after control, low, and high MeHg exposure treatments. Mean total Hg concentrations in muscle tissue and standard error for treatment groups were: 0.059 (0.002), 0.323 (0.012), and 0.584 (0.028) ppm (wet weight), respectively. While school area was highly variable, results suggested an increased school area with MeHg exposure, but these findings were not statistically significant. Examining sublethal markers of effect representing multiple physiological processes can help to elucidate the impacts of MeHg exposure to fish, as significant and suggestive evidence of MeHg toxicity were observed with both molecular and histopathological markers. These results suggested that chronic, low level MeHg exposure could cause adverse effects on largemouth bass before reaching reproductive maturity, as high MeHg exposed juveniles exhibited markers of immunosuppression.

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INTRODUCTION

Mercury is a well-known environmental contaminant (National Research Council 2000), and coal-burning power plants and mining activity are the primary sources of anthropogenic mercury pollution (http://www.epa.gov/mercury/control_emissions/emissions.htm). Mercury pollution is particularly problematic in wetlands (Hurley et al. 1995, St. Louis et al. 1994), as wetland sediments are rich in sulfate-reducing bacteria that metabolize inorganic mercury into methylmercury (MeHg; Compeau and Bartha 1985). MeHg is a lipophilic compound that is easily absorbed (Boudou et al. 1991), sequestered and bioaccumulated within an individual (Mason et al. 1995, Watras and Bloom 1992), and biomagnified in aquatic food webs (Schwarzbach and Adelsbach 2002, Davis et al. 2002a). Accumulating high concentrations of MeHg causes a wide range of toxic effects (e.g., reproductive impairment, neurological damage) in fish (Friedmann et al. 1996, Hammerschmidt et al. 2002, Webber and Haines 2004), birds (Heinz 1979, Spalding et al. 2000, Barr 1986), and humans (Lebel et al. 1998, Grandjean et al. 1997, Takaoka, et al. 2004, Harada 1995). The documented high mercury concentrations in aquatic biota, has resulted in fish and shellfish consumption advisories designated by the U.S. Environmental Protection Agency and Food and Drug Administration (2004).

California state advisories warn that women of childbearing age and children should not eat more than one meal per month containing sportfish (e.g., largemouth bass, striped bass) from the San Francisco Bay and Sacramento-San Joaquin Delta (Bay-Delta), in central California, USA, as these fish are known to contain high tissue concentrations of mercury (http://www.oehha.ca.gov/fish/general/sfbaydelta.html). The Bay-Delta region is

heavily polluted by mercury due to gold and mercury mining activities in the 1800s as mercury was utilized to amalgamate gold particles in the gold mines along the foothills of the Sierra Nevada Mountains. Tailings containing an estimated $4.5x10^6$ kilograms of mercury (Churchill 2000) were deposited into various tributaries connected to the Delta (Alpers and Hunerlach 2005), and contaminate present-day Bay-Delta biota (Davis et al. 2002 a, b).

Even though there has been extensive research on mercury toxicity in humans that consume fish from polluted areas (Lebel et al. 1998, Grandjean et al. 1997, Harada et al. 1995), a clear understanding of mercury toxicity on wild fish populations has yet to be determined. The majority of the reported effects of MeHg on fish are derived from laboratory studies (Berntssen et al. 2004, Gonzalez et al. 2005) that administered dietary MeHg at concentrations higher than that found in potential prey fish tissues collected from polluted environments [5 ppm (dry weight); 13.5 ppm (dry weight); 1 ppm (dry weight; Davis et al. 2002a), respectively]. Therefore, studies that examine effects from environmentally relevant MeHg exposures to fish are needed. Furthermore, combined examinations of biochemical, histological, neurological, behavioral, and reproductive markers would provide valuable information for assessing fish health (Fossi 1998, Huggett et al. 1992, McCarthy and Shugart 1990), as changes in biological processes may indirectly lead to decreased fitness and survival.

Biological markers (biomarkers) integrate multiple factors (e.g. individual susceptibility, chemical bioavailability) and stressors (e.g. parasite exposure, predation pressure) that influence responses to contaminant exposure and thus are valuable tools in

determining the effects of contaminant exposure in wild populations. Many biomarkers have been used to determine potential effects of mercury exposure in wild species (Finkelstein et al. 2007, Adams et al. 1995, Barr et al. 1986).

One molecular biomarker that has been used to assess contaminant exposure effects is the production of metallothionein (MT), a detoxification protein that binds to and sequesters many metals, including inorganic mercury, copper, cadmium, and zinc, causing upregulation of the protein (for review, see Smirnov et al. 2005, Hamilton and Mehrle 1986). Previous studies have found a positive relationship between MeHg exposure and MT protein or mRNA concentrations in fish under laboratory (Berntssen et al. 2004, Gonzalez et al. 2005) and field conditions (Schleck et al. 1995). However, MT's binding affinity for MeHg has not been determined in fish, and might differ between inorganic mercury and MeHg because of the covalent methyl bond in MeHg, and more studies are needed to assess this relationship, especially with environmentally relevant exposure levels.

Histopathology examines tissue damage, which is known to occur after contaminant exposure to fishes (Teh et al. 1997, Hinton et al. 1992), and is a good marker of MeHg pollution (Wester and Canton 1991, Wester and Canton 1992). Two sunfish species exhibited increased frequency of liver and spleen lesions after environmental exposure (Adams et al. 1999), and arctic charr (*Salmo salar*) had depleted glycogen reserves and liver necrosis in a laboratory experiment (Oliveira Ribeiro et al. 2000). Further investigations of histopathological parameters and MeHg exposure would help to elucidate the impacts of MeHg exposure on the individual health of wild fish species.

Elevated MeHg exposure is neurotoxic to vertebrates (Klaassen 2001), and contributes to sensory impairment in humans (Lebel et al. 1998). Neurotoxicity in fishes, observed by behavioral changes, has been recorded (Samson et al. 2001, Zhou et al. 2001) and may cause adverse effects under environmental conditions. Golden shiners (*Notemigonus crysoleucas*) fed environmentally realistic concentrations of MeHg displayed altered shoaling ability after a fright response (Webber and Haines 2003) which could enhance the probability of predation. Further investigations of low level MeHg exposure and neurotoxicity are needed as behavioral changes in wild fish populations could have measurable consequences for fitness and survival.

The aim of this study was to investigate MeHg toxicity to fish by examining the relationships between several biological markers and environmental or low-level laboratory-controlled MeHg exposure. The objectives of this study were to 1) determine the relationship between mercury tissue concentration and hepatic metallothionein gene expression in adult largemouth bass, 2) compare the relationship between histopathological markers and mercury exposure in juvenile and adult largemouth bass, and 3) determine the relationship between a range of MeHg exposure treatments and a behavioral indicator of sensory impairment (school area) in topsmelt (*Atherinops affinis*). Largemouth bass (*Micropterus salmoides*) were collected from the Sacramento-San Joaquin Delta (Delta); to our knowledge, the effects of environmental MeHg exposure to fish have not previously been examined in this ecosystem, although the Delta supports several sensitive species (e.g. delta smelt). A comparison between juvenile and adult largemouth bass was conducted to determine whether juveniles may be more sensitive to

MeHg exposure, a finding that has been observed in mammals (National Research Council 2000). The results of this study contribute to the evaluation of the use of biomarkers for MeHg exposure in fish species.

METHODS

Field study

Site selection and sample collection

The sampling efforts for this study (Figure 1, Table 1) were combined with that of the Fish Mercury Project, a collaborative project funded by California Bay-Delta Authority, with data sharing permitted by San Francisco Estuary Institute. The aim of the Fish Mercury project is to determine mercury concentrations in fishes collected from the Sacramento and San Joaquin watersheds and provide information required to update fish consumption advisories. Sample collection for this project took place in the Sacramento-San Joaquin Delta, which, combined with the San Francisco Bay, forms the largest estuary (approximately 40,000 hectares) on the west coast of the United States, and provides essential habitat for many wildlife species. Largemouth bass, a popular freshwater sport fish, were selected for this study because of their high trophic level, with juveniles as small as 50 mm feeding on smaller fishes (Moyle 2002), allowing for biomagnification of MeHg. Largemouth bass are abundant and widely distributed throughout the Delta (Davis et al. 2002a), and were collected during the summer of 2005 and 2006 using boat-electroshocking methods. Length and weight measurements of adults and lengths of juveniles were obtained upon collection. In 2005, 24 juveniles (ages

2-3) and 26 adults (ages 4-6) were collected, and in 2006, 43 adults (ages 3-8+) were collected. Ages were determined based on fork length measurements, using growth estimates determined by Schaffter (1998).

Given the Fish Mercury Project sampling regime, sites were sampled in 2005 if adult largemouth bass (305-450 mm TL) were expected to average mercury concentrations of less than 0.3 ppm or greater than 0.9 ppm, based on previous sampling efforts of Davis et al. (2002a). The Fish Mercury Project sampled fewer sites in 2006, thus all sites were sampled for this study.

Metallothionein Expression Analysis

Hepatic MT gene expression for thirteen adult largemouth bass collected in 2006 (Table 1, Figure 1) was analyzed using real-time polymerase chain reaction (PCR). Liver tissue was collected immediately after sacrifice, stored in RNAlater®, and subsequently stored at -20°C. RNA was extracted from tissue (0.015g +/- 0.001) using TRIZOL (Life Technologies), and sample integrity was determined by assessing the quality of the two ribosomal RNA bands present on a 1.2% formaldehyde agarose gel (Addendum A). When these ribosomal RNA bands were bright and discrete, RNA concentrations were determined using a Nanodrop spectrophotometer ND-1000, and RNA [mean = 1556 ng, standard error (SE) = 94.9 ng] was DNased (Fisherbrand) to minimize amplification of genomic DNA in the Real-Time PCR reaction. Reverse transcription (Reverse-iT First Strand Synthesis Kit, AB gene) was performed using random hexamer primers to synthesize cDNA from equal volumes of RNA. MT expression was standardized against a reference gene to normalize for sample integrity and technical error. Three commonly

used genes (β-actin, elongation factor 1α, and 18S) were evaluated for use as a reference gene by examining crossing threshold stability between samples and treatments.

Largemouth bass MT was isolated using a degenerate forward primer designed for conserved regions of Percoideian MT sequences using Primer 3 software (Rozen and Skaletsky 2000), and a 3'-RACE T_{17} adapter (Hastings et al. 1999) primer (Table 2). The resulting PCR products were cloned using TOPO TA Cloning Kit for Sequencing (Invitrogen) and sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) so that largemouth bass-specific primers could be designed (Table 2). Primers for the β-actin and elongation factor 1α were designed based on highly conserved regions in several Perciform and Percoideian sequences (Table 2). A universal primer (Table 2) was used to amplify 18S (Hillis and Dixon 1991, Halanych et al. 1995). The conditions for the Real-Time PCR reactions were optimized to eliminate primer-dimer, and were as follows: MT, β-actin, elongation factor 1α : 1x Sybr Green Master Mix with Fluoroscein (AB Gene), 0.5 μM primers, 30 ng cDNA; 18S: 1x Sybr Green Master Mix with Fluoroscein (AB Gene), 1.0 μM primers, 30 ng cDNA. Cycling parameters for all genes were as follows: initial 95°denaturing step (15 min), followed by 40 cycles of 95° (30 sec), 55° (30 sec), 72° (45 sec). Thirty ng of cDNA was determined appropriate for this study, as a dilution series (2, 10, 30, 60, or 100 ng) of starting cDNA suggested that gene expression is not measured in a linear fashion (Addendum A), with delayed crossing thresholds of PCR at 60 and 100 ng of template cDNA.

Assessment of genomic DNA contamination in cDNA was examined using 5 ng of DNased RNA as template, and negative controls (no template) were performed for each

gene. PCR products were visualized on a 1.2% gel to confirm single expected PCR product and limited or no primer-dimer (Addendum A).

Histopathological analyses

Liver, spleen, and kidney tissues were evaluated because they experience high blood flow, and are thus exposed to MeHg after dietary intake (Keating et al. 1997). Juvenile and adult largemouth bass liver, kidney, and spleen tissues were fixed in 10% buffered formalin immediately after sacrifice, and histological slides were prepared and stained with hematoxylin and eosin using standardized techniques (Histo-Tec in Hayward, CA, USA, and the Community Hospital of Monterey Peninsula in Monterey, CA, USA). One section was prepared for each sample tissue from each fish, which were analyzed under blind conditions to ensure unbiased results. Tissue damage was quantified using multiple histopathological parameters (Table 3) that were chosen based on ease of identification, presence in our samples, and findings from Adams et al. (1999). Parasites observed in these tissues included trematodes and protozoans, and immune parameters included melanomacrophage centers (referred to as macrophages), inflammatory cells (eosinophilic granulocytes and lymphocytes), and granulomas.

Laboratory study

Species collection and dosing regime

Juvenile topsmelt (*Atherinops affinis,* 5-10 cm in length) were collected from Elkhorn Slough, CA, USA using a beach seine, and acclimated to laboratory conditions for at least six weeks before beginning the experiment. Ten fish were maintained in a 280-liter flow-through tank for each treatment. Fish were fed 5% body weight of dried coarse

flakes (AquaDine), six days per week. One of three diets were administered to each exposure treatment, which was repeated in triplicate: control (0.012 μg MeHg wet weight/fish/day), low (0.235 μg MeHg wet weight/fish/day), and high (0.434 μg MeHg wet weight /fish/day) for 20 days. MeHgCl (Alfa Aesar) was diluted in ethanol and added to flakes to distribute the MeHg evenly throughout the food, and flakes were dried overnight at 70° C to evaporate residual ethanol. An attempt was made to quantify histopathological parameters in liver after 20 days of MeHg exposure, but tissue integrity was compromised due to severe lipidosis (fatty liver) in all treatment groups suggesting problems with the food quality or quantity.

Behavioral analysis

Methods for analysis of schooling behavior were adapted from Webber and Haines (2004). School area was measured daily by placing a grid marked with 2.5 cm increments at the bottom of each tank, and recording the school's movements for five minutes per observation with a digital video camera that was placed approximately 2 m away from the school. Recording began two minutes after approaching the tank to allow the fish to return to a normal resting state. Five-minute recordings were made at the same time of day, six days per week, beginning three days before the experiment for baseline data collection. Video footage quantification of school area was a limiting factor as only the surface area of the school, not the total volume, was measured. Nonetheless, surface area of the school was measured every thirty seconds using ImageJ software (http://rsb.info.nih.gov/ij/), and divided by the total number of fish in the tank (adjusted school area), as some topsmelt jumped out of their tanks and died.

Mercury Analysis

Total mercury (tHg) concentrations were determined in topsmelt and adult largemouth bass muscle tissue by Marine Pollutions Studies at Moss Landing Marine Labs as tHg is known to be indicative of MeHg exposure in fishes, with greater than 95% of tHg found in fish is MeHg (Bloom 1992). All concentrations reported are wet weight concentrations. U.S. Environmental Protection Agency Method 7437 (www.epa.gov/ epaoswer/ hazwaste/test/pdfs/7473.pdf) was used with a Milestone DMA-80 Automatic Mercury Analyzer. For each treatment replicate, in the laboratory experiment, three subsamples, each composed of three homogenized individuals, were analyzed for tHg concentration, and the mean tHg concentration was calculated. Juvenile largemouth bass samples were not analyzed for tHg, but instead were categorized by low and high MeHg exposure sites based on the mean adult tHg concentration for each site analyzed as part of the Fish Mercury Project in 2005 (number of adults examined per site = 4-8). The extrapolation of juvenile exposure level was possible because mercury pollution from mine tailings has point-sources in the tributaries, causing the Northern, Southern, and Eastern Delta largemouth bass to contain higher tHg concentrations than Central Delta largemouth bass (Davis et al. 2002a). Furthermore, largemouth bass exhibit strong site fidelity (Hassler and Wisby 1958, Fish and Savitz 1983), suggesting that largemouth bass collected from low exposure regions have likely experienced low mercury exposure throughout most of their life. Low exposure sites were located in the Central Delta (Figure 1, Table 1) and were assigned when mean adult tHg concentrations were less than 0.400 ppm. High exposure sites were found in the Northern and Eastern Delta (Figure 1,

Table 1) and were designated when mean adult tHg concentrations above 0.400 ppm. Overall mean adult tHg concentrations were 0.249 ppm (SE=0.013) in low exposure sites, and 0.510 ppm (SE=0.055) in high exposure sites.

Data Analysis

General physiological assessment

The body condition [100x (body weight/ body length^3)] for each adult largemouth bass was compared to tHg concentration in muscle tissue ($\alpha = 0.05$) using linear regression. Body condition and hepatosomatic index [100x (liver weight/total body weight)] of topsmelt were determined and compared to mercury exposure group using an ANOVA ($\alpha = 0.05$). All analyses were performed in SYSTAT (Systat Software Inc., 11th ed. 2004, San Jose, CA, USA).

Metallothionein expression analysis

Thirteen adult largemouth bass were analyzed for gene expression, and were selected as they had the lowest (0.27-0.37 ppm; n=7) and highest (0.63-0.97 ppm; n=6) tHg concentrations in the dataset, and had good RNA integrity. To validate that there was no treatment effect on β-actin, elongation factor 1α, and 18S gene expression, crossing thresholds (Ct) for each gene were examined by treatment using a two sample t-test (α=0.05; p=0.376, 0.625, 0.438, respectively). Gene expression stability was determined using BestKeeper (Pfaffl et al. 2004), which considers a gene to be unstable for use as a reference gene when the standard deviation exceeds 1.00. β-actin [standard deviation (SD)=1.37] and 18S (SD=1.10) were excluded as reference genes, and elongation factor 1α (SD= 0.92) was used exclusively to standardize MT expression which was determined

using the $2^{-\Delta Ct}$ method (Livak and Schmittgen 2001). Data were transformed [log (standardize MT expression +1)] to meet assumption of equal variances for parametric statistics, and the relationship between standardized MT expression and tHg concentration in muscle tissue was examined using two sample t-test (α=0.05) in SYSTAT.

Histopathological analyses

All statistical tests (α = 0.05) were performed using SYSTAT. Each histopathological parameter in adults was statistically compared to tHg concentration using linear regression analyses, and data were transformed when necessary to meet parametric assumptions. Data collected in 2005 and 2006 were combined, as preliminary analysis found no difference in the distribution and trends between years (data not shown). Juveniles were analyzed using a two-sample t-test or Mann-Whitney U (when data did not meet parametric assumptions) to compare the mean of each histopathological parameter in low and high exposure groups.

Individuals were excluded from the appropriate analysis when a parameter (i.e. trematodes, granulomas) was absent in 33% or more of the samples because a high proportion of zeros heavily influences statistical relationships. These data were not examined using a logistic regression because the numerical values are accurate and the distributions of zeros do not appear to be explained by tHg concentration. Juvenile spleen tissues were not statistically tested for trematode and granuloma densities due to the low sample size of spleens collected and the rarity of trematodes and granulomas present.

Although up to fourteen statistical analyses were performed to examine the relationship between tHg concentrations or MeHg exposure and histopathology, alpha was not adjusted for multiple comparisons, as this procedure can inappropriately invalidate relationships that are important and warrant further research (Rothman 1990, Gotelli and Ellison 2004). As a result, recent studies that examined relationships between contaminant exposures and biological effects in wildlife did not perform multiple comparison adjustments (Verreault et al. 2004, Bustnes et al. 2003, Finkelstein et al. 2007).

Behavioral analysis

The geometric mean for adjusted school area was determined each day, and the geometric mean for baseline (three days before the start of the experiment) and final school area (the last three days of the experiment) were calculated to obtain the percent change in adjusted school area for each treatment: (Average school area $_{final}$ / average school area $_{baseline}$) x 100. Geometric mean was used for this study as it is commonly used in determining the average of percentages. Treatment effect was statistically analyzed ($\alpha = 0.05$) using a one-way ANOVA in SYSTAT.

RESULTS

Field Study

tHg concentrations and general physiological assessment

Adult largemouth bass tHg concentrations ranged from 0.117-0.652 ppm in 2005, and 0.270-0.975 ppm in 2006, with 72 % of the samples collected exceeding the EPA

screening value of 0.3 ppm (Figure 2). Overall, more females (n=34) were collected than males (n=27), and body condition did not appear to be affected by tHg concentration ($p=0.555$, $r^2=0.005$, linear regression).

Metallothionein expression

A weak negative relationship was found between standardized MT expression and high tHg concentrations (0.63-0.97 ppm) in adult largemouth bass (Figure 3b, two sample t-test, $p=0.081$). The expression of standardized MT was highly variable [variance = 0.326 (low exposure), 0.035 (high exposure)] and was not equal between exposure groups (Figure 3a, F-test, $F= 9.314$; $F_{0.05(2), 6,5}$: 6.98). No trend was apparent between any of the genes examined and age [as determined by Schaffter (1998)], and sample sizes were not sufficient to detect site effects or gender differences.

Histological analyses

Fourteen histopathological parameters with respect to age class and tissue type were evaluated in largemouth bass tissue samples (Table 4). Mean macrophage densities in kidney tissues were lower in juveniles (0.58, SE=0.06) compared to adults (0.41, SE=0.08). Mean larval trematode densities in kidney and mean adult trematode densities in liver were greater in juveniles (0.40, SE=0.16; 0.23, SE=0.13, respectively) than in adults (0.21, SE=0.05; 0.08, SE=0.02, respectively). Furthermore, mean protozoan parasites densities were greater in juveniles (17.2, SE=2.9) than in adults (8.5, SE=0.98).

In general, the relationship between histopathological parameters and MeHg exposure was highly variable (Table 6; representative scatterplots are shown in Figure 4 a, b). Suggestive differences between genders were observed for some parameters measured

(Figure 5 a, b); however, detecting significant effects was not always possible due to the decrease in samples size, and therefore power, by separating analysis by gender. Upon preliminary examination, body condition and geographical region of sample collection did not greatly contribute to the variability observed in the histopathological parameters evaluated.

The relationships between histopathological parameters and mercury exposure for juveniles and adults differed, with more parameters trending towards significance ($p \leq 0.10$) in juveniles than adults (Table 5). Macrophage densities appeared to be related to MeHg exposure, as noted by significant relationships in juvenile kidney and liver tissues ($p=0.018$, 0.02, respectively; two sample t-test) and in adult kidney tissue ($p=0.020$, $r^2=0.084$, linear regression), and this relationship trended towards significance in juvenile spleen tissue ($p=0.087$, two sample t-test). Inflammation significantly decreased with mercury exposure in adult kidney ($p=0.041$, $r^2=0.06$, linear regression), and this relationship trended towards significance in juvenile kidney tissue ($p=0.066$, two sample t-test). The occurrence of granulomas may be weakly, but were not significantly, related to MeHg exposure, as observed by adult liver data (Figure 6), adult spleen data (Figure 6), and juvenile spleen data, where granulomas were present in 1 in 4 low exposure individuals, and 3 in 5 high exposure individuals.

High exposure juveniles appeared to have more adult trematodes than low exposure juveniles in all three tissue types [liver: $p=0.014$, two sample t-test; spleen: 25% ($n=4$) of low exposed and 60% ($n=5$) of high exposed juveniles had trematodes present; kidney: 18% ($n=11$) of low exposed and 63% ($n= 8$) of high exposed juveniles had trematodes

present]; however, no relationship was found between adult trematode density and tHg concentration in all three tissue types of adult largemouth bass. Protozoan parasite density decreased only in adults with respect to tHg concentration, and was marginally significant ($p=0.055$, $r^2=0.055$, linear regression). Larval trematode density was not significantly related to mercury exposure level; however, high exposure juveniles with larval trematodes present has adult trematodes present more frequently than that of low exposure juveniles. Of the low exposure juveniles with larval trematodes present (55%; n=11), 17% had adult trematodes in kidney tissue, and 17% had adult trematodes in liver tissue. However, of the high exposure juveniles with larval trematodes present (50%; n=8), 100% of the individuals that had had adult trematodes in the kidney, and 50% had adult trematodes present in the liver. Furthermore, as mentioned above, adult trematode densities also appear increased in the high exposure group. The number of spleen tissue samples collected was too small to evaluate for trematode presence trends.

Laboratory Study

tHg concentrations and general physiological assessment

The mean tHg concentration for each treatment was: control = 0.059 ppm (SE = 0.002), low = 0.323 ppm (SE = 0.012), high = 0.584 ppm (SE = 0.028). Condition and hepatosomatic index was not affected by treatment ($p=0.792$, $r^2=0.075$; 0.903, $r^2=0.034$, respectively, linear regression).

Behavior analysis

Although adjusted school area appeared to be larger with increased MeHg exposure (Figure 6), a significant treatment effect was not observed after 20 days of MeHg

exposure (p=0.542; n=3; ANOVA). The geometricmean percent change in school area was most pronounced between the control and high MeHg exposure treatment (control = 107%, SE 12.7%, high = 133%, SE 16.0%; p=0.276).

DISCUSSION

Field Study:

In summary, the field study suggested 1) adult largemouth bass MT gene expression slightly decreased with increased tHg concentration, and 2) juveniles may be more sensitive to MeHg pollution, as increased exposure was associated with decreased liver and kidney macrophage densities, increased adult trematode density in liver tissue, and increased susceptibility to trematode maturation. Post-hoc examination of the results of this study suggests that not adjusting for multiple comparisons was appropriate as the relationships that were significant or trended towards significance were consistent between tissue types (e.g. juvenile macrophage density), lifestage (e.g. kidney macrophage and inflammation densities), or year (e.g. protozoan parasite densities; data not shown). Although only weakly related to increased tHg concentrations, granuloma density trends were supported by previous observations in fish (Wester and Canton 1991).

Metallothionein expression

Median standardized MT expression and variability was greater in individuals with low tHg concentrations than in individuals with high tHg concentrations (Figure 3). The observed MT expression patterns may suggest 1) a compromise of the MT response

mechanism due to MeHg exposure or 2) confounding water quality factors that induce MT expression (e.g. trace metals) have an inverse spatial distribution to MeHg concentrations. Nonetheless, these results support the findings that MT expression is highly variable under environmental conditions, even when quantified metal concentrations were low (Laurie 2004). The lower MT expression and variability observed in high MeHg exposed adults suggests that these individuals are more likely to experience toxic effects from the excess MeHg being bioavailable to replace other metals in metalloenzymes, a phenomenon that has been observed after fish have been exposed to inorganic mercury (Brown and Parsons 1978).

The lower MT expression observed in high MeHg exposed adults is unexpected, as previous laboratory studies have found a positive relationship between tHg concentrations and MT expression or protein concentration (Berntssen et al. 2004, Gonzalez et al. 2005). The diminished MT expression observed in high MeHg exposed largemouth bass could be a result of testing the wrong MT gene, as fishes in other orders (Cypriniformes and Salmoniformes) are known to have two MT genes (Gonzalez et al. 2005, Bonham et al. 1987). Therefore, a MeHg responsive MT gene, if one exists, may not have been discovered in largemouth bass despite cloning a degenerate PCR reaction. Also, the previous lab studies (Berntssen et al. 2004, Gonzalez et al. 2005) administered high MeHg concentrations that exceed fish tissue concentrations collected from polluted environments (Davis et al. 2002a, Adams et al. 1999, Schlenk et al. 1995, Kamman et al. 2005), suggesting that MT induction may be observed following extremely elevated and

rapid administration of MeHg as an immediate stress response, rather than under low level, chronic exposure conditions.

Although not significant, a potential positive relationship might exist between standardized MT expression and body condition ($p=0.476$, $r^2=0.047$, linear regression). However, an increased sample size is required to detect these differences, as both factors are highly variable. Furthermore, the concentration of trace metals were not determined in this field study despite evidence that metals including copper, cadmium, and zinc bind to the MT protein and induce upregulation (Hamilton and Mehrle 1986), and likely influenced MT expression in these individuals. As this study does not support findings from previous studies (Berntssen et al. 2004, Gonzalez et al. 2005, Schlenk et al. 1995), further examination between the relationship of MT expression, MeHg exposure, and body condition in wild populations is needed.

Histopathology

Due to the large variability observed in each histopathological parameter evaluated (Table 5), follow up studies with an increased sample size would be beneficial to examine the effects of age, body condition, gender, and site. As an overall geographical separation exists between MeHg exposure and sites (Figure 2), the effects of other contaminants on the histopathological parameters should be noted. While laboratory studies could help control for exposure to other contaminants, replicating additional stressors that occur in the environment and contribute to tissue damage, including parasite and disease exposure, is difficult. Also, the high variability of the histopathological markers measured in largemouth bass suggests a larger sample sizes are

needed to obtain 80% power. For example, sample size would have to increase from 68 to 91 samples to obtain 80% power in the statistically significant relationship between adult kidney macrophage density and tHg concentration ($p=0.016$). Nonetheless, this study provides important information regarding the use of biological markers in fish environmentally exposed to MeHg.

Juvenile largemouth bass appeared more sensitive than adults to MeHg exposure, as juveniles appeared to have stronger associations between the histopathological parameters evaluated and MeHg exposure. Also, high exposure juveniles showed indications of immunosuppression, with decreased macrophage densities, increased adult trematode density, and increased trematode maturation (Table 5). For the trematode maturation, equal densities of larval parasites were observed between exposure groups; however, a greater number of high exposed juveniles had trematodes present and also had higher densities than low-exposed juveniles. The increase in trematode maturation suggests that high exposed juveniles do not fend off the trematodes as well as low exposed juveniles.

Immunosuppression from MeHg exposure has been documented in other fish (Roales and Perlmutter 1977) and bird (Spalding et al. 2000, Henny et al. 2002) species; however, studies that have examined the relationship between MeHg exposure and trematode density are less conclusive. Cross et al. (2001) found a decrease in digenean trematode cercariae (*Cryptocotyle lingua*) longevity in a laboratory setting after heavy metal exposure, yet a field study observed a higher abundance of adult digenean trematodes (*Posthodiplostomum minimum*) in spottail shiners (*Notropis hudsonius*) collected from

sites polluted with unidentified industrial and urban effluents compared to non-polluted sites (Thilakaratne et al. 2007). Further work is needed to understand the effects of pollution on parasite susceptibility, as many factors, including parasite age, host age, and pollution type can influence the overall parasitic response.

Significant negative relationships observed in adult largemouth bass between tHg tisssue concentrations and kidney macrophage density and inflammation may indicate that adults are also susceptible to mercury-associated immunosuppression. However, contrary to the trends observed in juvenile largemouth bass, adult trematode density did not vary with MeHg exposure in adult largemouth bass. In general, larval and adult trematode densities were greater in juveniles than in adults, suggesting adult resistance to parasites (e.g., compensatory immune mechanism, differences in feeding habits) or mercury toxicity to the trematodes themselves in adult largemouth bass, who have higher mercury concentrations (2-3 times) than juveniles in MeHg polluted environments (Davis et al. 2002a). Also, the decrease in protozoan parasites density with increased tHg concentration (Figure 3) supports the suggestion that adult largemouth bass trematodes are absent due to mortality from mercury toxicity.

Laboratory study

Behavioral Analysis

The concentration of MeHg administered in the laboratory study was environmentally realistic for piscivorous fishes; however, as topsmelt are planktivorous, the tHg concentration assimilated was three times that found in a related species, jacksmelt (*Atherinopsis californiensis*) collected from the Oakland Harbor in the San Francisco Bay

(Davis et al. 2002b). The inherent skittish behavior of topsmelt imposed limits on the behavioral tests that could be performed, and examining resting school area was minimally invasive. MeHg-associated neurotoxicity, as observed by topsmelt schooling behavior, was not conclusively determined, and a different neurological marker may be more sensitive to assessing the relationship between MeHg and altered behavior. For example, a change in fish behavior, such as altered predator avoidance (Webber and Haines 2003), decreased prey capture ability (Zhou et al. 2001, Samson et al. 2001) and decreased swimming activity (Samson et al. 2001) was reported in other fish species after elevated MeHg exposure. In addition, the time-course data suggested that school area in MeHg exposed fish slowly increased over time (data not shown). Therefore, a longer duration experiment or an increased number of replicates (n=9) may aid in understanding the relationship between behavior and MeHg exposure.

A preliminary swim performance experiment was also performed to examine behavior under increasing water velocities (Addendum B). Results indicated a slight decrease in swim strength (as measured by critical velocity and proportion of schools observed jockeying in position) and an increased frequency of startled responses (e.g. twitching, jumping) in the high MeHg exposure group (Addendum B). If either of these behaviors were to occur after environmental exposure, such as in the San Francisco Bay, the probability of schooling fish being successfully preyed upon would increase.

Conclusion

Examining multiple physiological parameters is useful tool to aid interpretation of MeHg toxicity, as sublethal differences with increased MeHg exposure were suggested by molecular and histological biomarkers. MeHg pollution in the Bay-Delta could cause adverse effects on largemouth bass before reaching reproductive maturity, as high MeHg exposed juveniles exhibited markers of immunosuppression. The high exposed juveniles of this study may have decreased resistance to disease and infection, as immune cells are responsible for antigen identification and removal. Furthermore, these findings demonstrate the importance of examining multiple age classes for toxic effects as previous field studies examine only the adult age class (Adams et al. 1999), and high MeHg exposed juveniles who survive to adulthood may represent hardy individuals that best tolerate polluted environments. Ultimately, the results from this study reflect the importance of field-based studies, as many stressors (e.g., parasites) are present, and can be important indicators of fish health. Caution should be taken with assigning lowest observable exposure effect levels because our understanding of MeHg toxicity to fish is limited by the parameters we investigate, and it is difficult to determine if a biomarker represents an adverse physiological change if the pathway of MeHg toxicity is unknown. Therefore, further investigation is required to understand the mechanism of MeHg toxicity and the relationship of MeHg exposure and parameters that likely impact wild fish populations.

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Table 1. Juvenile and adult largemouth bass sample sizes for metallothionein (MT) expression and histopathological analysis are listed by site. Adult sample sizes and adult mean tHg concentration used to assign exposure level of juvenile largemouth bass are also listed.

Site number	**# adults (MT expression)**	**# adults (histopathology)**	**# juveniles (histopathology)**	**Mean adult tHg concentration (SE); n**
1	0	1	2	0.673 (0.306); 4
2	2	4	0	NA
3	0	2	0	NA
4	5	12	0	NA
5	0	2	3	0.818 (0.215); 5
6	3	3	0	NA
7	0	4	0	NA
8	0	5	0	NA
9	0	2	0	NA
10	0	2	5	0.758 (0.096); 7
11	1	6	0	NA
12	1	4	0	NA
13	0	2	0	NA
14	1	4	0	NA
15	0	4	0	NA
16	0	1	2	0.373 (0.029); 7
17	0	4	4	0.187 (0.021); 6
18	0	2	4	0.244 (0.038); 7
19	0	2	0	NA
20	0	2	1	0.312 (0.030); 8
21	0	3	3	0.186 (0.027); 4

Table 2. Primer sequences used for standardized metallothionein (MT) gene expression. Forward and reverse primers indicated by (F) and (R), respectively. *Hillis & Dixon 1991; **Halanych et al. 1995

	Sequence (5' to 3')	**Product size**
Metallothionein		
Percoideian (F)	ACCWCSTGCAAGAAGAGCTG	242 bp
T_{17} adapter (R)	GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT	
Largemouth bass (F)	CTGCTCATGCTGCCCATC	158 bp
Largemouth bass (R)	TGCAGTTAGTCATTAGTTGTTCACAC	
β-Actin		
"Perciform" (F)	TTCCTCGGTATGGAATCCTG	166 bp
"Perciform" (R)	CCAAGGCTGTGATCTCCT	
Elongation Factor 1α		
"Perciform" (F)	CATYGAYATCGCTCTGTGG	240 bp
"Perciform" (R)	ACTCCAACGATGATCTGCTT	
18S		
Universal* (F)	CTGGTTGATCCTGCCAGT	~650 bp
Universal** (R)	GAATTACCGCGGCTGCTGGCACC	

Table 3. Histopathological parameters examined in juvenile and adult largemouth bass kidney, liver, and spleen tissues. X: parameter was examined in designated tissue type. NA: parameter was not found in designated tissue type.

	kidney	liver	spleen
Mean # macrophages in 10 fields of view (200X)	X	X	X
# of granulomas (adjusted to # of 200x fields)	X	X	X
# of adult trematodes (adjusted to # of 200x fields)	X	X	X
# of inflammatory events (adjusted to # of 200x fields)	X	X	NA
# of larval trematodes (adjusted to # of 200x fields)	X	NA	NA
Mean # protozoan parasites (Eimeriidae-like) in 10 fields of view (400x)	X	NA	NA
Mean percent coverage of eosinophilic droplets in 10 fields of view (400x)	X	NA	NA

Table 4. Mean density for each histopathological parameter in juvenile and adult largemouth bass kidney, liver, and spleen tissues. % zeros = percent of the dataset that had a zero as a data point. Mean was calculated by removing zero values when percent of zeros in the data set exceeded 33%. When percent of zeros in dataset exceeded 33% sample size was adjusted for each parameter by subtracting the number of individuals that had zeros as data points from the total number of samples collected. Juvenile sample sizes are listed for each treatment: [low exposure (adult mean tHg concentration <0.400 ppm), high exposure (adult mean tHg concentration > 0.400 ppm)]. The same number individuals were not analyzed for all tissue types. Blank cells indicate that the parameter listed was not found in the tissue type.

	macrophages	**granulomas**	**adult trematodes**	**inflammatory events**	**larval trematodes**	**protozoan parasites**	**% eosinophilic droplets**
	Mean (SE) % zero; n	**Mean (SE) % zero; n**	**Mean (SE) % zero; n**	**Mean (SE) % zero; n**	**Mean (SE) % zero; n**	**Mean (SE) % zero; n**	**Mean (SE) % zero; n**
Kidney							
Juveniles	0.41 (0.08); 26%; 11,8	0.11 (0.02); 74%; 3,2	0.18 (0.03); 63%; 2,5	0.55 (0.12); 16%; 11,8	0.40 (0.16); 47%; 6,4	17.2 (2.9); 0%; 11,8	2.0 (1.0); 63%; 4,2
Adults	0.58 (0.06); 12%; 68	0.16 (0.03); 65%; 24	0.21 (0.05); 46%; 37	0.47 (0.04); 6%; 68	0.21 (0.05); 60%; 27	8.5 (0.98); 7%; 68	2.9 (0.55); 38%; 42
Liver							
Juveniles	0.44 (0.17); 67%; 5,3	0.15 (0.02); 71%; 4,3	0.23 (0.13); 63%; 4,5	0.12 (0.03); 29%; 14,10			
Adults	0.59 (0.10); 14%; 70	0.10 (0.02); 54%; 24	0.08 (0.02); 61%; 32	0.23 (0.07); 37%; 44			
Spleen							
Juveniles	3.7 (1.7); 22%; 4,5	0.32 (0.04); 56%; 1,3	0.48 (0.22); 56%; 1,3				
Adults	2.8 (0.41); 0%; 60	0.50 (0.10); 22%; 60	0.29 (0.07); 58%; 25				

Table 5. Summary of histopathological relationships with mercury exposure in juvenile and adult largemouth bass kidney, liver, and spleen tissues. (-)/(+) indicate direction of the relationship with increased MeHg exposure. Some parameters in juvenile tissues were not statistically analyzed as fewer than three individuals in an exposure level contained the parameter indicated.

	Macrophages	**Granulomas**	**Adult trematodes**	**Inflammatory events**	**Larval trematodes**	**Protozoan parasites**	**% Eosinophilic droplets**
Juveniles	**p value** *Low exposure*: **Mean (SE)** *High exposure*: **Mean (SE)**	**p value** *Low exposure*: **Mean (SE)** *High exposure*: **Mean (SE)**	**p value** *Low exposure*: **Mean (SE)** *High exposure*: **Mean (SE)**	**p value** *Low exposure*: **Mean (SE)** *High exposure*: **Mean (SE)**	**p value** *Low exposure*: **Mean (SE)** *High exposure*: **Mean (SE)**	**p value** *Low exposure*: **Mean (SE)** *High exposure*: **Mean (SE)**	**p value** *Low exposure*: **Mean (SE)** *High exposure*: **Mean (SE)**
Adults	**p value; r^2**	**p value; r^2**	**p value; r^2**	**p value; r^2**	**p value; r^2**	**p value; r^2**	**p value; r^2**
Kidney							
Juveniles	0.018 (-); 0.56 (0.099); 0.20 (0.067)	Data not analyzed	Data not analyzed	0.066 (-); 0.76 (0.17); 0.30 (0.13)	0.748; 0.27 (0.16); 0.13 (0.077)	0.241; 20.1 (3.48); 13.2 (5.07)	Data not analyzed
Adults	0.016 (-); r^2=0.084	0.422; r^2=0.029	0.479; r^2=0.014	0.041 (-); r^2=0.062	0.463; r^2=0.022	0.055 (-); r^2=0.055	0.192; r^2=0.042
Liver							
Juveniles	0.020 (-); 0.23 (0.12); 0.10 (0.00)	0.480 0.359 (0.207); 0.066 (0.034)	0.014 (+); 0.097 (0.007); 0.19 (0.028)	0.808; 0.13 (0.045); 0.11 (0.052)			
Adults	0.772; r^2=0.001	0.100 (+); r^2=0.109	0.689; r^2=0.005	0.540; r^2=0.009			
Spleen							
Juveniles	0.087 (-); 0.76 (0.250); 0.19 (0.106)	Data not analyzed	Data not analyzed				
Adults	0.401; r^2=0.012	0.141; r^2=0.037	0.726; r^2=0.005				

Figure 1. Sample locations for largemouth bass sample collection sites in 2005, depicted with triangles (▲) and 2006, depicted with the letter x (x). Sites are labeled 1-21, in order from north to south, with low exposure juveniles collected from sites 16-21, and high exposure juveniles collected from sites 1, 5, and 10.

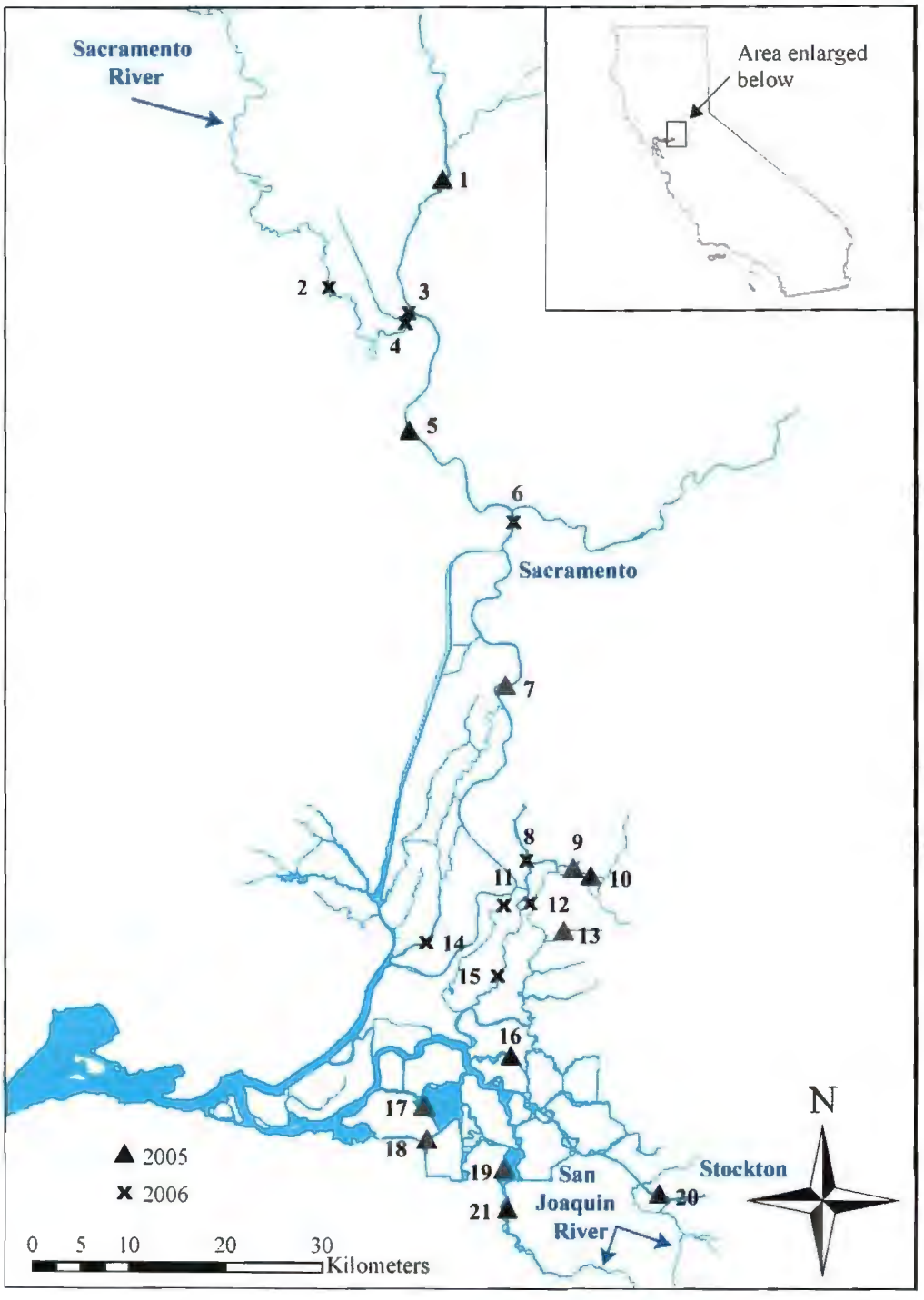


Figure 2. Mean total mercury concentrations in adult largemouth bass muscle tissue by site.

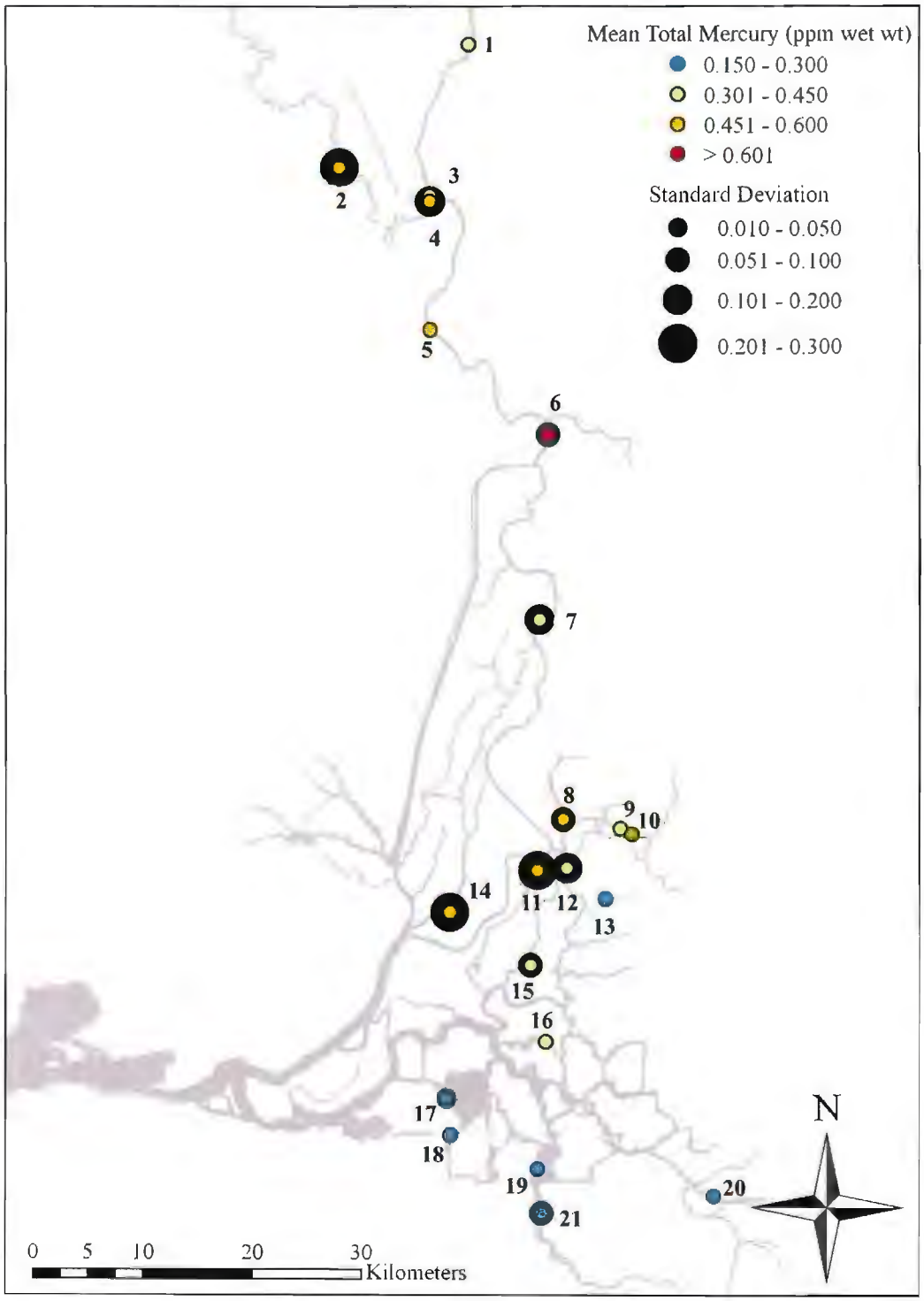
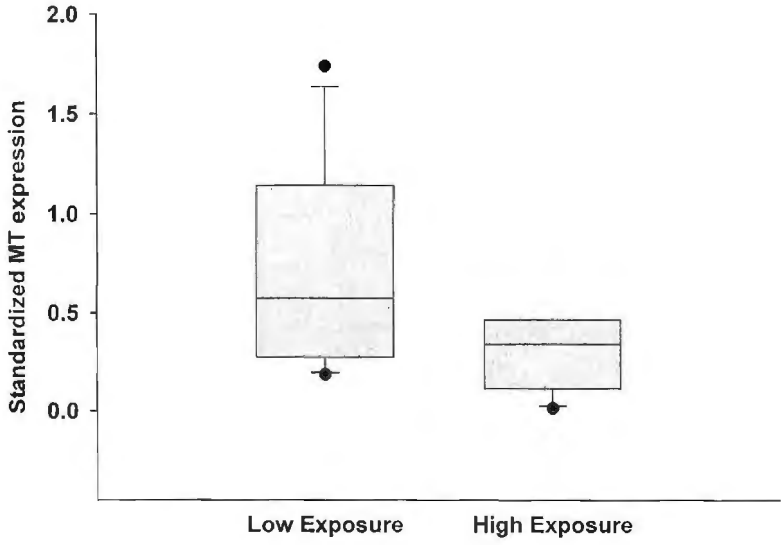


Figure 3A) Box plot displaying standardized metallothionein (MT) expression ($2^{-\Delta Ct}$) for low (0.27-0.37 ppm) and high (0.63-0.97 ppm) adult total mercury concentrations. Median (horizontal line within the box), 25th and 75th percentiles (lower and upper margins of the box), 10th and 90th percentiles (lower and upper hash marks), and outliers (circles) are depicted on the graph.
Figure 3B) Box plot displaying transformed standardized metallothionein (MT) expression [log (standardized MT expression +1 $2^{-\Delta Ct}$) for low (0.27-0.37 ppm) and high (0.63-0.97 ppm) adult total mercury concentrations.

(A)



(B)

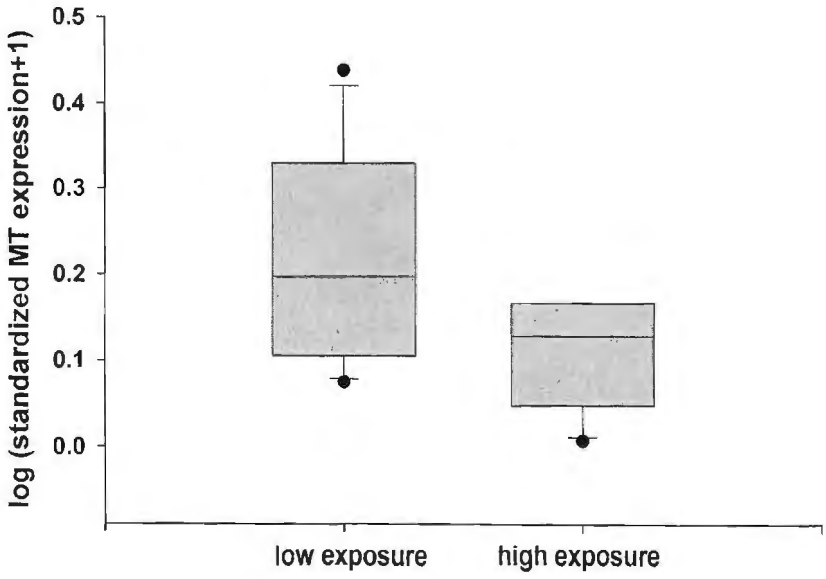
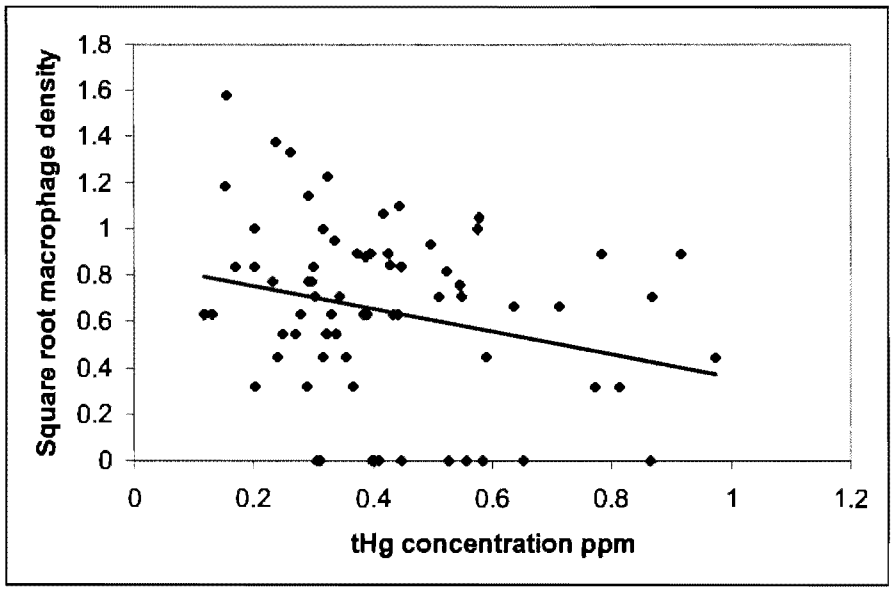


Figure 4. Representative scatterplots of histopathological parameters vs. total mercury (tHg) concentration in adult largemouth bass. Due to unequal variances, parameters were transformed using a square root transformation of the (A) average kidney macrophage density (Y= -0.5236X + 0.8969; r^2 = 0.084), and (B) average kidney protozoan parasite density (Y= -1.7438X + 3.2597; r^2 = 0.0546).

(A)



(B)

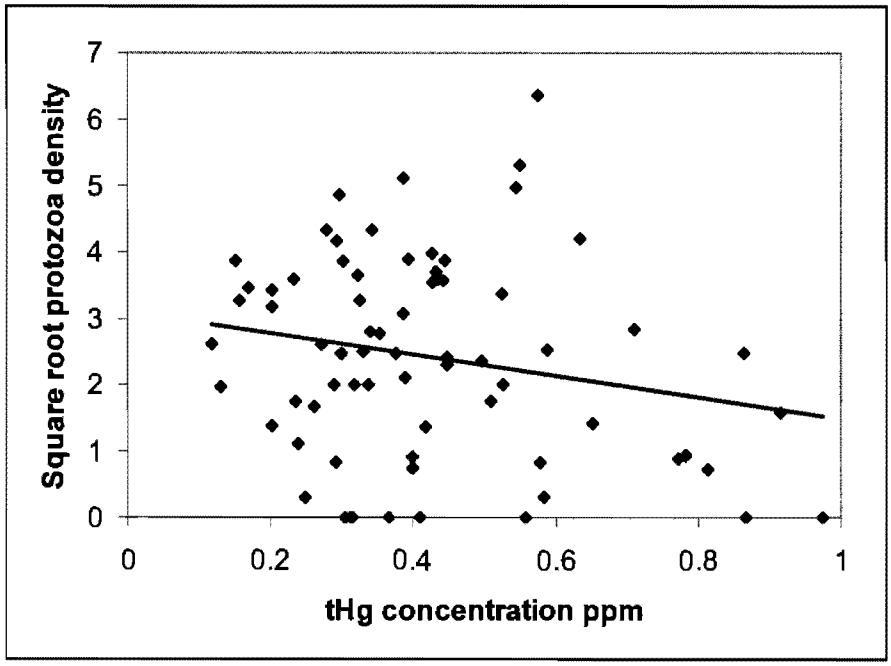
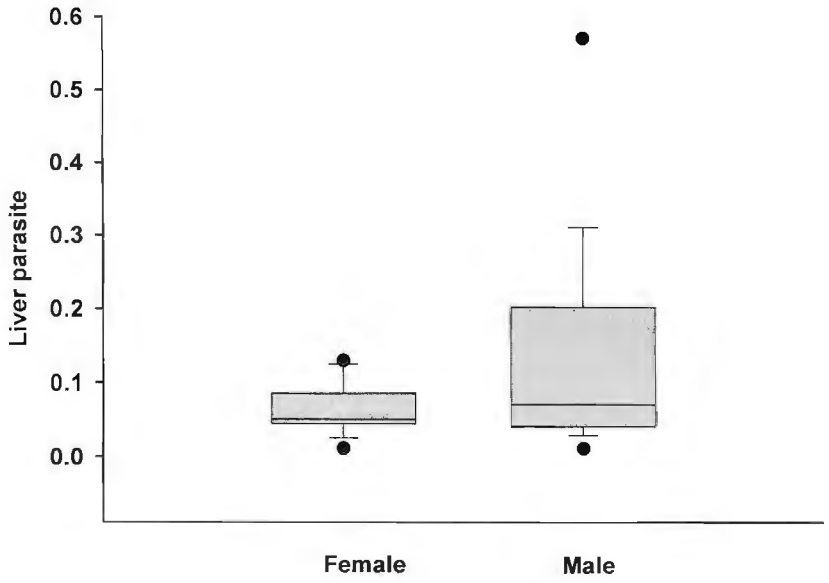


Figure 5. Box plot displaying the distribution of adult trematode density in liver tissue (A), and average eosinophilic protein droplet density (B), from male (n=15; 12, respectively) and female (n=12; 24, respectively) adult largemouth bass, with median (horizontal line within the box), 25th and 75th quartiles (lower and upper margins of the box), 10th and 90th percentiles (lower and upper hash marks), and outliers (circles).

(A)



(B)

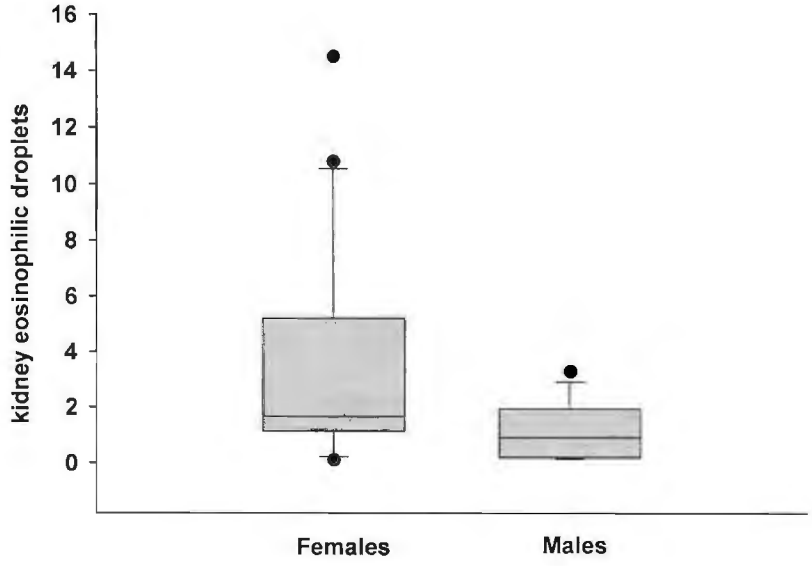
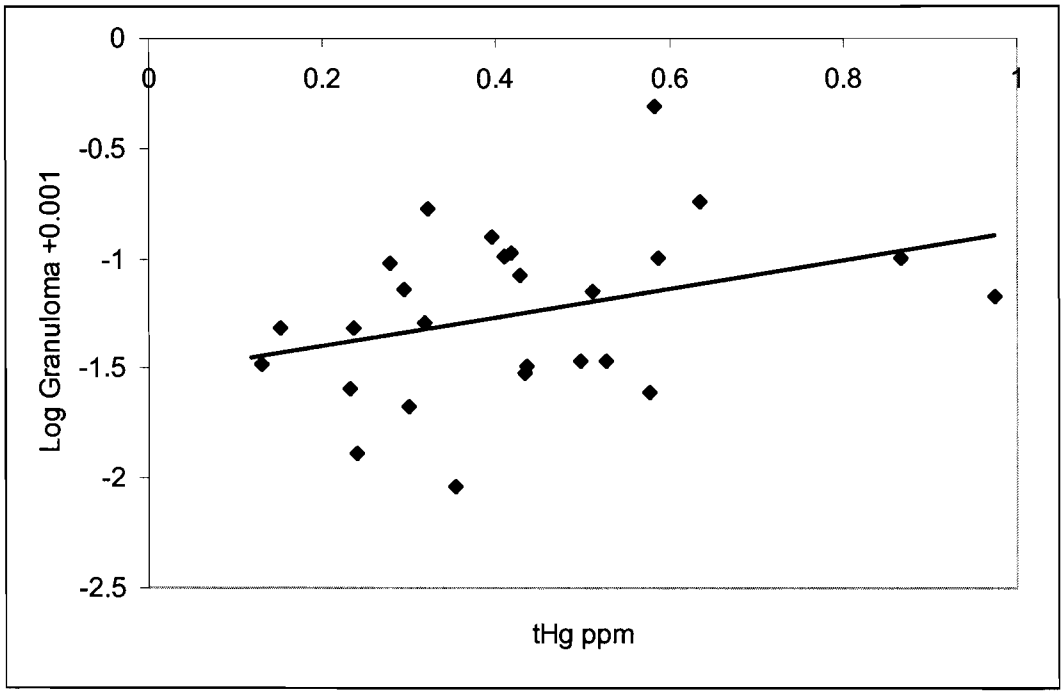


Figure 6. Relationship between total mercury concentrations (tHg) and granuloma density in (A) liver ($y=0.6522x-1.5235$, $r^2 = 0.1169$, $p=0.100$, linear regression) and (B) spleen ($y=1.1561x-1.5164$, $r^2 =0.0371$, $p=0.141$, linear regression) tissues. Data were transformed (log (granuloma density+0.001) to meet assumptions of parametric statistics.

(A)



(B)

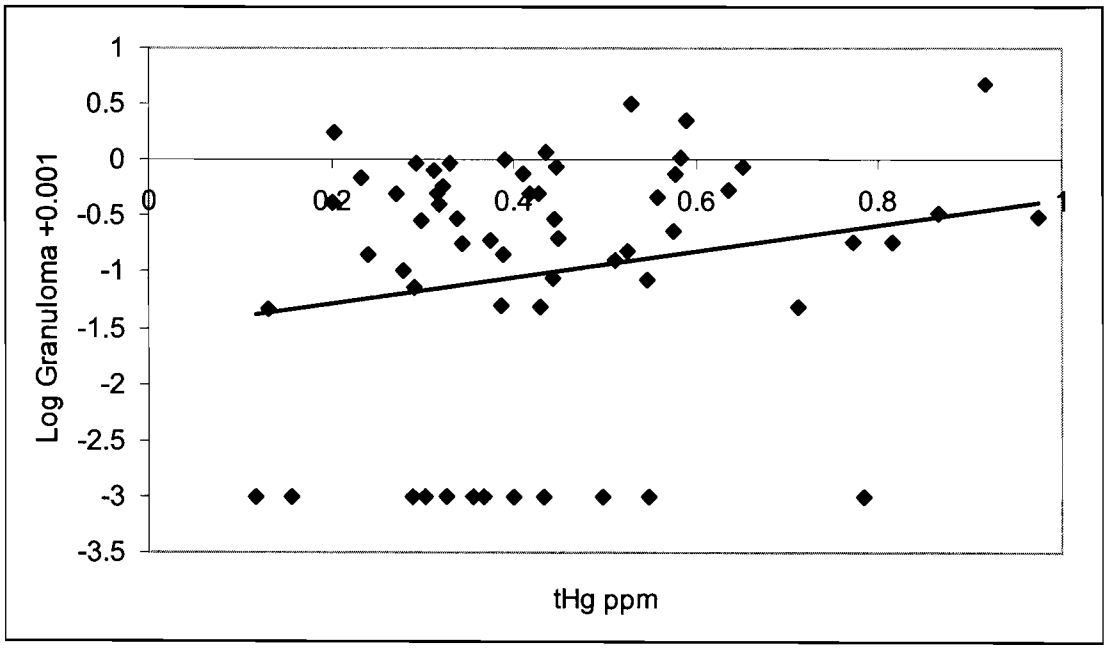
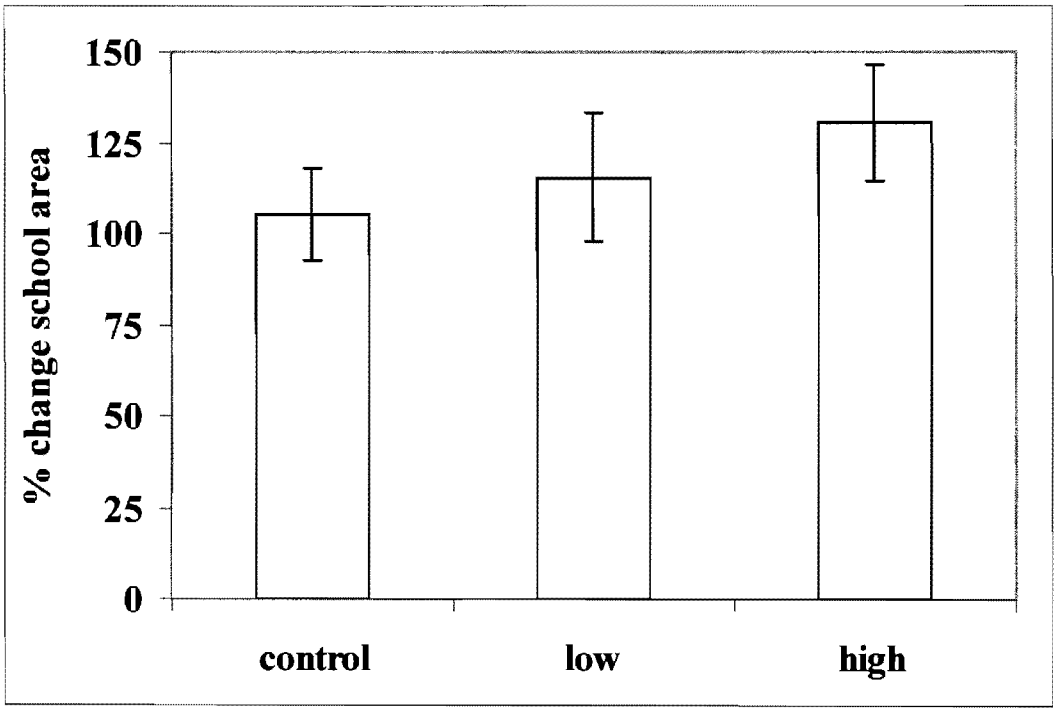


Figure 7. Geometric mean of percent change of adjusted school area of topsmelt (cm^2 per individual), separated by treatment, shown with standard error bars.



Addendum A:

Figure A. Non-linear detection of β-actin (squares), elongation factor 1α (triangles), and 18S (open diamonds) crossing threshold (Ct) in one representative largemouth bass sample with increased cDNA loading in Real-Time PCR.

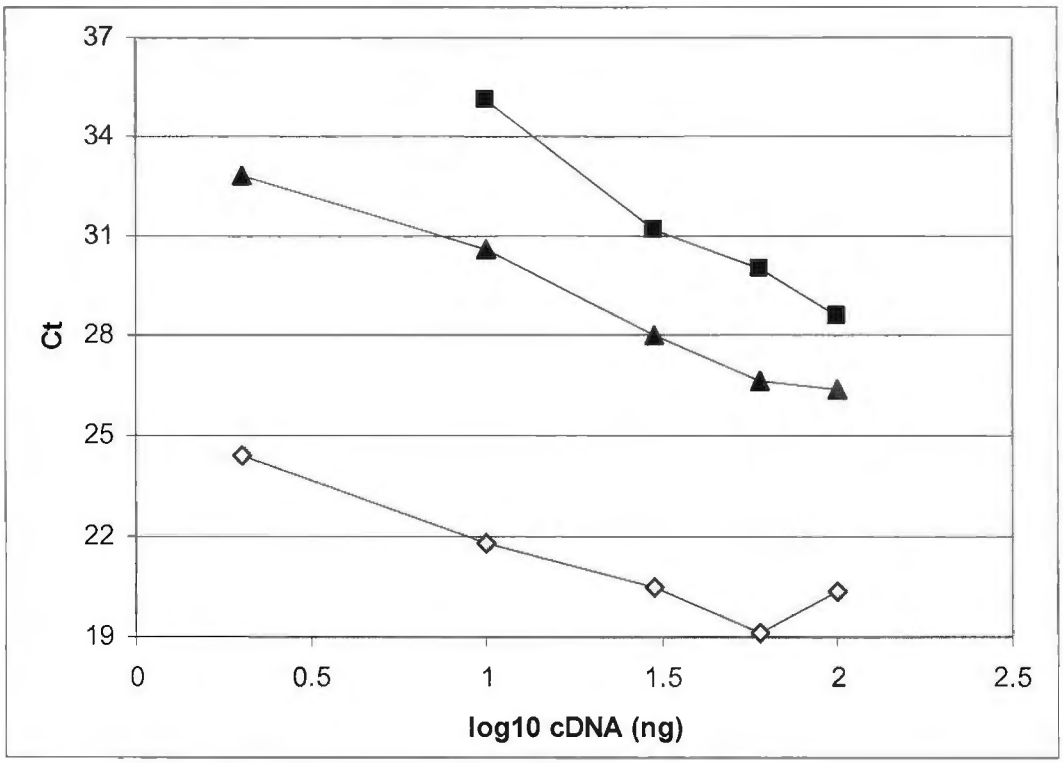


Figure B. RNA for each sample on formaline-1.2% agarose denaturing gel. No sample was loaded in lane 4. Samples in lanes 1, 2, and 5 were not used in this study, due to the low amount of rRNA visualized on the gel.

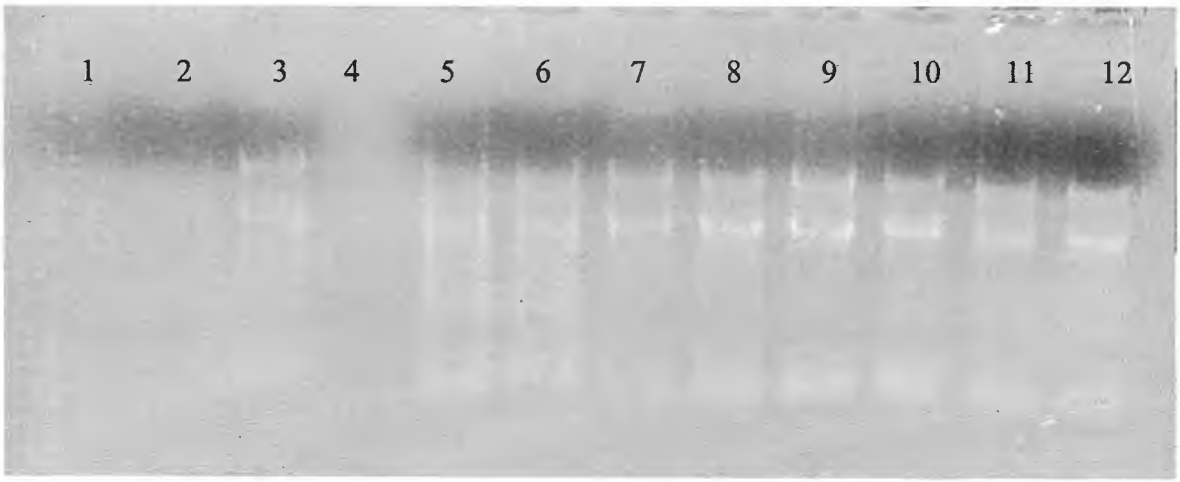
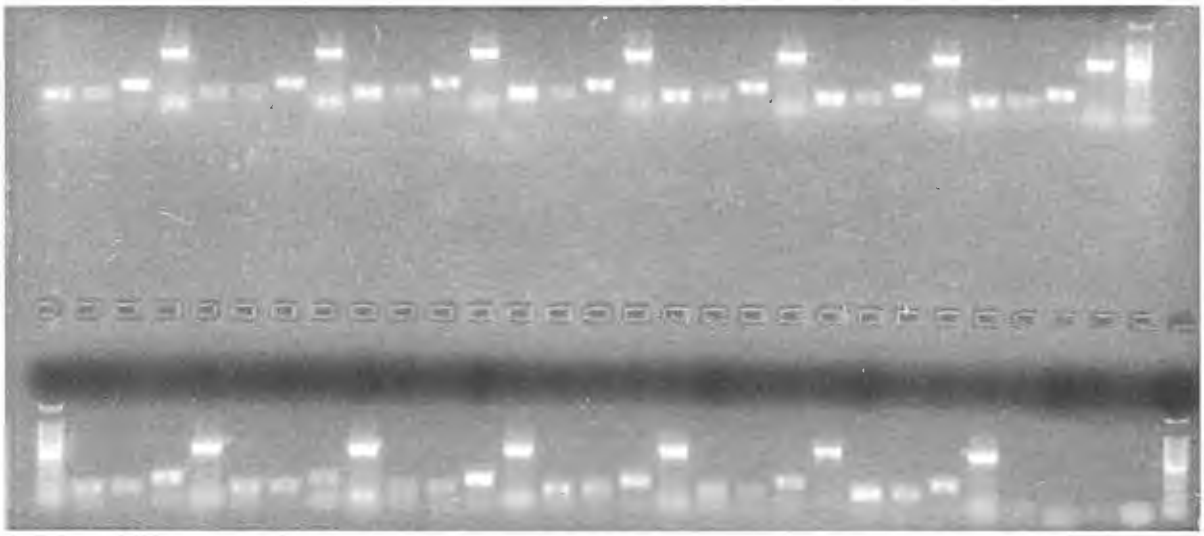


Figure C. Results from Real-Time PCR on 1.2% agarose gel. Samples are arranged by individual, and amplified genes are in the following order: metallothionein, β-actin, elongation factor 1α, and 18S. The last four samples are the negative control (no cDNA template), with primers in the same order as indicated above.



Addendum B. Flume summary

Methods

After 20 days of MeHg exposure to topsmelt, each replicate treatment was placed into the flume, or swim tunnel, for 8 hours to acclimate. Water velocities were increased by approximately one-half body lengths per second (2.5 cm/sec). Each speed was maintained for three minutes and observations were made until two fish no longer swum and were caught on the flume screen. Five swimming behavior traits were consistently observed between replicates and were easily observable without video analysis (Table A)

Results

Differences in jockeying behavior, when individuals continually change position within the school to obtain the least resistant hydrodynamic position, was observed between the control (1/3 schools) and high exposure groups (3/3 schools). The velocity at which this behavior was first observed does not appear different between treatments (Table A). Few individuals in each school were not able to maintain position (as indicated by critical velocity, when individuals exhibit bursts in swimming behavior) or to continue swimming with increased water velocities (pinned against the flume screen). The critical velocities were recorded, however, the number of bursting events was only recorded in 8/9 replicates, as the ninth replicate had too many events to observe without image analysis. The critical velocities and speed at which individuals were not able to continue swimming differed between the control and high exposure groups (Table A). General school shape was observed throughout the swim test, and the velocity was recorded when the integrity of the school diminished, and individuals were not near other

members of the school, or the school broke into two schools. Although this behavior was observed in all treatments, the dissociation of the school occurred at a lower velocity in the high exposure treatment (Table A). Lastly, increased frequency of skittish behavior (i.e., twitching, darting) was also observed in the low and high exposure groups.

Discussion

A qualitative examination of the patterns in the data suggested that the high exposure groups experienced more rapid exhaustion, as indicated by the speed at which critical velocity was observed and by the speed at which two individuals no longer swam. Furthermore, individuals became tired more quickly in the high exposure group than the control, as they jockeyed for better positions within the school more frequently, and school structure dissociated at a lower water velocity (Table A). If these alterations were exhibited in the environment, then MeHg exposed fishes would be more likely to be preyed upon, as they would swim slower and in a less structured manner that would make predator attacks more successful.

Table A. Velocity (SE), cm/sec, recorded for observed behavior. Fractions represent proportion of replicates that exhibited behavior. * Increase in frequency of event from control. ** Decrease in speed at which parameter was observed from control

	control	low exposure	high exposure
Jockeying within school	11.9; 1/3	6.5 (0.0); 2/3	10.1 (2.1); 3/3 *
Critical velocity (burst swimming)	17.87 (2.3); 3/3	17.65 (1.4); 3/3	14.27 (2.0); 3/3 **
speed at which 2 individuals were against screen	19.2 (2.0); 2/3	19.4 (1.2); 3/3	16.6 (0.7); 2/3 **
speed at which school became unorganized	10.3 (1.5); 3/3	8.2 (2.4); 3/3	6.5 (0); 2/3 **
Skittish behavior	3 events; 2/3	5 events; 3/3	5 events; 3/3 *