

# Assessing Mercury Exposure and Biomarkers in Largemouth Bass (*Micropterus Salmoides*) from a Contaminated River System in California

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**Abstract** We evaluated mercury (Hg) exposure and two biomarkers, metallothionein (MT) gene expression and histopathological alterations in a wild fish species, largemouth bass (*Micropterus salmoides*), collected from the Sacramento-San Joaquin Delta, CA, a region polluted with Hg from historic mining activities. Hg is highly toxic and can disrupt multiple physiological systems in vertebrate species, including the immune system. Total mercury (THg) concentration in muscle tissue ranged from 0.12 to 0.98 ppm (wet weight) and was not related to body condition ( $r^2 = 0.005$ ,  $p = 0.555$ ). Using linear regression analysis, we found a positive relationship between MT gene expression (as determined using quantitative polymerase chain reaction) and copper, zinc, manganese, aluminum, and nickel (decreased to one variable by way of principal component analysis) ( $r^2 = 0.379$ ,  $p = 0.044$ ), a negative relationship with selenium ( $r^2 = 0.487$ ,  $p = 0.017$ ), and a weak, negative relationship with THg concentrations ( $r^2 = 0.337$ ,  $p = 0.061$ ). Juvenile largemouth bass collected from Hg-contaminated areas displayed histopathological features of immunosuppression compared with those collected from less contaminated areas as evidenced by significantly lower macrophage density in kidney and liver tissue ( $p = 0.018$  and  $0.020$ , respectively),

greater trematode density in liver tissue ( $p = 0.014$ ), and a greater number of adult trematodes. Our results suggest that largemouth bass may be experiencing sublethal effects from chronic Hg exposure. Furthermore, our findings illustrate the utility of examining multiple sublethal markers of effect to assess the impacts of contaminant exposure on physiological function in wild species.

Mercury (Hg) is a well-known environmental contaminant (National Research Council 2000) with coal-burning power plants and mining activity believed to be the primary sources of anthropogenic pollution (Tchounwou et al. 2003). Hg is particularly problematic in wetlands (Hurley et al. 1995) because wetland sediments are rich in sulfate-reducing bacteria that have been shown to convert inorganic Hg into the highly toxic methylmercury (MeHg) (Compeau and Bartha 1985). One reason for MeHg's toxicity is that it is readily absorbed (Boudou et al. 1991), sequestered, and bioaccumulated within an individual (Mason et al. 1991) as well as biomagnified upward through food webs (Schwarzbach and Adelsbach 2002; Davis et al. 2008). Hg exposure can cause a wide range of toxic effects (e.g., reproductive impairment, immune disruption, neurological damage) in fish (Hammerschmidt et al. 2002; Berntssen et al. 2004; Sweet and Zelikoff 2001) as well as birds (Finkelstein et al. 2007; Wolfe et al. 1998) that rely on aquatic ecosystems. Moreover, humans who consumed fish from areas known to be contaminated with Hg have also had severe physiological dysfunction (Lebel et al. 1998).

For the past 15 years, the state of California has advised women and children to avoid eating sportfish (e.g., largemouth bass, striped bass) from the Sacramento-San Joaquin Delta (bay-delta) in central California due to increased Hg concentrations in those fish (Office of Environmental

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Health Hazard Assessment 1999). The bay-delta region is heavily polluted with Hg transported from mining activities during the 1,800s in the foothills of the Sierra Nevada Mountains. Mine tailings were deposited into various tributaries connected to the Sacramento-San Joaquin Delta (delta) (United States Geological Survey 2005) and the biota in this region is known to contain increased MeHg concentrations (Davis et al. 2002, 2008).

Although multiple studies have evaluated the effects of Hg exposure to fish in laboratory settings (Hammerschmidt et al. 2002; Berntssen et al. 2004; Gehringer 2007), there are limited investigations on the sublethal effects of fish in the wild (Adams et al. 1999). Biomarkers assess physiological function and are thus useful for evaluating the effects of contaminants, such as Hg, on wild populations (Finkelstein et al. 2007; Adams et al. 1999). Production of metallothionein (MT), a protein that binds to and sequesters many divalent transition metals [Hg, copper (Cu), cadmium (Cd), and zinc (Zn)], has been used as a molecular biomarker to assess effects to fish exposed to MeHg under laboratory (Gonzalez et al. 2005; Berntssen et al. 2004) and field conditions (Schlenk et al. 1995). In addition, histopathology can be a useful tool to elucidate tissue damage from MeHg exposure, including potential impacts to immune-related parameters, because Hg is well-known to disrupt immune function in vertebrate species, including fish (Sweet and Zelikoff 2001).

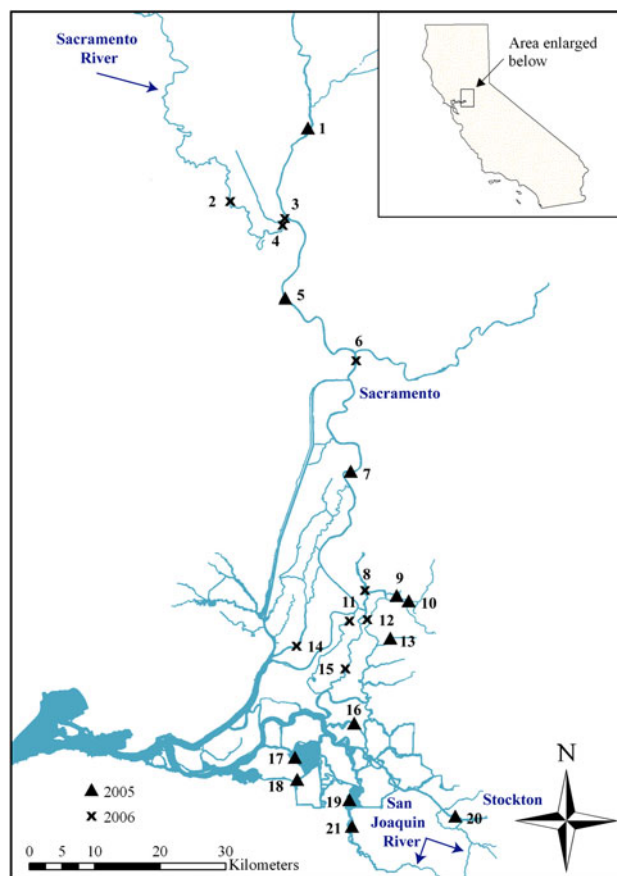
We investigated the effects of Hg exposure in largemouth bass (*Micropterus salmoides*), a high trophic-level sportfish known in the Sacramento-San Joaquin Delta to exceed the human health safety limit for Hg (OEHHA 1999). We evaluated the relationship between Hg concentrations in adult muscle tissue and biological markers of effect, including MT gene expression and various immune-associated histopathological alterations (i.e., macrophage density). A comparison between histopathological alterations in juvenile and adult largemouth bass was also conducted to determine whether juvenile fish may be more sensitive to MeHg exposure, a finding that has been observed in mammals (National Research Council 2000). To better understand the cause for variation in MT gene expression, concentrations of trace metals were analyzed in adult largemouth bass liver samples. Ultimately, we illustrate the utility of examining multiple sublethal markers of effect to assess the impacts of Hg exposure on fish physiological function.

## Materials and Methods

### Site Selection and Sample Collection

The sampling efforts (Fig. 1, Table 1) took place as part of the Fish Mercury Project, a project funded by California

Bay-Delta Authority, with data sharing permitted by San Francisco Estuary Institute. The aims of the Fish Mercury Project are to determine Hg concentrations in fish collected from the Sacramento and San Joaquin watersheds and to provide information required to update fish consumption advisories. Samples were collected in the San Francisco bay-delta complex, which forms the largest estuary (~40,000 ha) on the west coast of the United States and provides essential habitat for many wildlife species. Largemouth bass were selected for this study because of their high trophic level (Moyle 2002), which allows for biomagnification of MeHg. Largemouth bass are abundant and widely distributed throughout the delta (Davis et al. 2008) and were collected during the summer of 2005 and 2006 using boat-based electroshocking methods. Length and weight measurements of adult fish and lengths of juvenile fish were obtained on collection. In 2005, 24 juvenile fish (ages 2–3 years) and 26 adult fish (ages 4–6



**Fig. 1** Largemouth bass sample collection sites in 2005 and 2006. Samples collected in 2005 are represented by triangles, and samples collected in 2006 are represented by the letter X. Sites 1 through 15 are northern or eastern delta sites that represent high (mean 0.471 ppm, SE 0.024 ppm [ $n = 57$ ]) exposure for juvenile largemouth bass. Sites 17 through 21 are central delta sites that represent low (mean 0.218 ppm, SE 0.018 ppm [ $n = 14$ ]) exposure for juvenile largemouth bass. For sample size and THg concentrations, see Table 1

**Table 1** Site number corresponding to Fig. 1 plus mean THg concentration, SE, and sample size (*n*) for adult largemouth bass samples and exposure group (low or high) for juvenile largemouth bass

Site no.	Adult THg (SE), <i>n</i>	Juvenile exposure group; overall mean adult THg (SE), <i>n</i>
1	0.395 (NA), 1	High 0.673 (0.306), 4
2	0.464 (0.045), 4	NA
3	0.389 (0.045), 2	NA
4	0.469 (0.030), 12	NA
5	0.531 (0.121), 2	High 0.818 (0.215), 5
6	0.885 (0.047), 3	NA
7	0.365 (0.016), 4	NA
8	0.497 (0.030), 6	NA
9	0.370 (0.016), 2	NA
10	0.547 (0.037), 2	High 0.758 (0.096), 7
11	0.451 (0.109), 4	NA
12	0.411 (0.061), 4	NA
13	0.296 (0.003), 2	NA
14	0.585 (0.126), 5	NA
15	0.314 (0.027), 4	NA
16	0.305 (NA), 1	Low 0.373 (0.029), 7
17	0.170 (0.012), 4	Low 0.187 (0.021), 6
18	0.166 (0.036), 2	Low 0.244 (0.038), 7
19	0.270 (0.022), 2	NA
20	0.178 (0.061), 2	Low 0.312 (0.030), 8
21	0.279 (0.031), 3	Low 0.186 (0.027), 4
Overall	0.421 (0.023), 71	0.442 (0.047), 48

Mean THg concentration from adult largemouth bass used to confirm the juvenile exposure groups along with SE, sample size is also provided

NA not applicable, juveniles were not collected from this site

years) were collected; in 2006, 43 adult fish (ages 3–8+ years) were collected. Ages were determined from fork length measurements using growth estimates determined by Schaffter (1998). Killing of the fish was performed by delivering a blunt blow to the head (San Jose State University Institutional Animal Care and Use Committee protocol no. 872).

Samples were collected in 2005 at sites where adult largemouth bass (305–450 mm TL) were expected to have average Hg concentrations of <0.3 or >0.9 ppm (Davis et al. 2008). The Fish Mercury Project sampled fewer sites in 2006; thus, adult fish from all sites in that year were included in this study (Fig. 1). Juvenile fish were collected at sites where historical data (Davis et al. 2008) indicated consistently “low” exposure (mean adult THg concentrations <0.4 ppm) or “high” exposure (mean adult THg concentrations >0.4 ppm) (Fig. 1; Table 1). Low-exposure sites were in the central delta, and high-exposure sites were located in the northern and eastern delta (Fig. 1; Table 1).

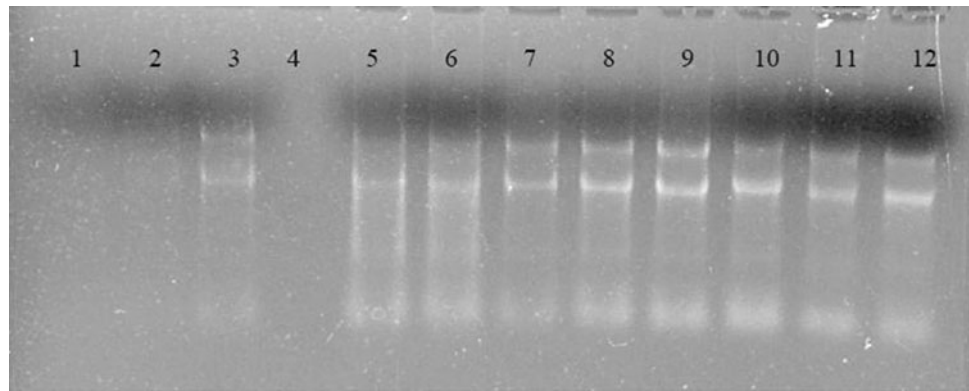
## MT Expression Analysis

Hepatic MT gene expression was analyzed for 13 adult largemouth bass (Table 2) collected in 2006 using real-time quantitative polymerase chain reaction (qPCR). Liver tissue was collected immediately after killing, and half of the tissue was stored in RNAlater (Invitrogen, Carlsbad, CA, USA) at room temperature and subsequently stored at –20 °C. RNA was extracted from tissue (0.015 g ± 0.001 g) using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA), and sample integrity was determined by assessing the quality of the two ribosomal RNA bands present on a 1.2 % formaldehyde agarose gel (Fig. 2). When these ribosomal RNA bands were bright and discrete, RNA concentrations were determined using a Nanodrop spectrophotometer ND-1000, and RNA (mean = 1556 ng [SE = 94.9 ng]) was DNased (Fisher Scientific, Fair Lawn, NJ) to minimize amplification of genomic DNA. Reverse transcription (Reverse-iT First Strand Synthesis Kit; Abgene, Epsom, UK) was performed to synthesize cDNA from equal mass of RNA. MT expression was standardized against a commonly used reference gene, elongation factor 1 $\alpha$ , using the 2<sup>– $\Delta$ Ct</sup> method (Livak and Schmittgen 2001) to normalize for sample integrity and technical error.

**Table 2** Site number and corresponding sample sizes (*n*) for adult largemouth bass MT expression and histopathological analyses plus juvenile largemouth bass histopathological analysis

Site no.	Adult <i>n</i> MT	Adult <i>n</i> histopathology	Juvenile <i>n</i> histopathology
1	0	1	2
2	2	4	0
3	0	2	0
4	5	12	0
5	0	2	3
6	3	3	0
7	0	4	0
8	0	6	0
9	0	2	0
10	0	2	5
11	1	4	0
12	1	4	0
13	0	2	0
14	1	5	0
15	0	4	0
16	0	1	2
17	0	4	4
18	0	2	4
19	0	2	0
20	0	2	1
21	0	3	3
Total	13	71	24

**Fig. 2** RNA for each sample on formaline—1.2 % agarose denaturing gel. No sample was loaded into 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



**Table 3** Primer sequences used for standardized MT gene expression

MT	Sequence (5'–3')	Product size (bp)
Metallothionein		
Percoideian (F)	ACCWCSTGCAAGAAGAGCTG	242
T <sub>17</sub> adapter (R)	GACTCGAGTTCGACATCGATTTTTTTTTTTTTTTTTT	
Largemouth bass (F)	CTGCTCATGCTGCCATC	158
Largemouth bass (R)	TGCAGTTAGTCATTAGTTGTTACAC	
Elongation factor 1 $\alpha$		
Perciform (F)	CATYGAYATCGCTCTGTGG	240
Perciform (R)	ACTCCAACGATGATCTGCTT	

Forward and reverse primers indicated by (F) and (R), respectively

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<sub>17</sub> adapter (Hastings et al. 1999) primer (Table 3). The resulting PCR products were cloned using TOPO TA Cloning Kit for Sequencing (Invitrogen) and sequenced (Table 3). Primers for the elongation factor 1 $\alpha$  were designed based on highly conserved regions in several perciform and percoideian sequences (Table 3).

The conditions for the qPCR reactions were optimized as follows: MT, elongation factor 1 $\alpha$  : 1x Sybr Green Master Mix with Fluorescein (Abgene), 0.5  $\mu$ M primers, and 30 ng cDNA. Reactions were run in a Bio-Rad iCycler iQ thermocycler in opaque 96-well plates sealed with optical film (Fisher-Thermo Scientific). Cycling parameters for both genes were as follows: initial 95 $^{\circ}$  denaturing step (15 min) followed by 40 cycles of 95  $^{\circ}$ C (30 s), 55  $^{\circ}$ C (30 s), and 72  $^{\circ}$ C (45 s). Determination of the crossing threshold (Ct) was performed automatically in BioRad thermocycler software using cycles 2 through 10 to define baseline fluorescence level. We monitored qPCR over 50 PCR cycles. Thirty nanograms of cDNA was determined to be appropriate for this study because cDNA >60 ng inhibited reactions. PCR products were visualized on a 1.2 % gel to confirm a single PCR product of correct size and little or no primer-dimer. Assessment of genomic DNA contamination in cDNA was examined using 5 ng DNase

RNA without reverse-transcription as template, and no-template controls were performed for each PCR experiment (Fig. 2).

#### Histopathological Analyses

The histopathology of liver, spleen, and kidney tissue were evaluated because these tissues experience high blood flow and are thus potentially exposed to MeHg after dietary intake (Keating et al. 1997). Juvenile and adult largemouth bass were examined to discern differences in sensitivity to MeHg exposure between age classes. Juvenile ( $n = 24$ ) and adult ( $n = 71$ ) largemouth bass (Table 2) liver, kidney, and spleen tissues were fixed in 10 % buffered formalin immediately after the fish were killed, and histological slides were prepared and stained with hematoxylin and eosin using standardized techniques (Histo-Tec in Hayward and the Community Hospital of Monterey Peninsula in Monterey [both CA]). One section (sample) was prepared from each tissue per fish and was analyzed under blind conditions. Tissue damage was quantified using multiple histopathological features (Table 4) that were chosen based on ease of identification, presence in our samples, and findings from Adams et al. (1999). Immune parameters included melanomacrophage centers (hereafter “macrophages”), inflammatory events (eosinophilic granulocytes and lymphocytes aggregates), and granulomas.

**Table 4** Histopathological features examined in kidney, liver, and spleen tissues from juvenile and adult largemouth bass

Histopathological features	Kidney		Liver		Spleen	
	Juvenile fish	Adult fish	Juvenile fish	Adult fish	Juvenile fish	Adult fish
Mean no. of macrophages in 10 fields of view (200×)	X	X	X	X	X	X
No. of granulomas (adjusted to no. of 200× fields)	NA	X	X	X	NA	X
No. of mature trematodes (adjusted to no. of 200× fields)	NA	X	X	X	NA	X
No. of inflammatory events (adjusted to no. of 200× fields)	X	X	X	X	NA	NA
No. of larval trematodes (adjusted to no. of 200× fields)	X	X	NA	NA	NA	NA
Mean no. of protozoan parasites ( <i>Eimeriidae</i> -like) in 10 fields of view (400×)	X	X	NA	NA	NA	NA
Mean percent coverage of eosinophilic droplets in 10 fields of view (400×)	NA	X	NA	NA	NA	NA

X feature was observed, NA feature was not observed in  $\geq 66$  % of samples

## Hg Analysis

### Adult Fish

Total mercury (THg) concentrations were determined in adult largemouth bass muscle tissue by the Marine Pollution Studies Laboratory at Moss Landing Marine Laboratories with the EPA Method 7473 and a direct mercury analyzer (Milestone DMA-80) as part of a larger assessment by the Fish Mercury Project. Total Hg in muscle tissue was assumed to be representative of the MeHg body burden because >95 % of THg in largemouth bass has been shown to be MeHg (Bloom 1992) and >80 % of THg found in fish tissues have been found in muscle tissue (Berntssen et al. 2004). All concentrations reported are wet-weight concentrations.

### Juvenile Fish

Due to budget constraints, juvenile largemouth bass samples were not analyzed for THg but instead were categorized into low MeHg- and high MeHg-exposure groups based on the mean adult THg concentration for all adult largemouth bass collected for each site for the Fish Mercury Project, which included a greater number of individuals than those analyzed for this study (Table 1). Our categorization of juvenile exposure groups is consistent with previous findings that juvenile and adult largemouth bass tissues are known to contain high concentrations of Hg when collected from the northern, southern, and eastern delta tributaries compared with those collected from the central delta (Foe et al. 2003; Davis et al. 2008). Furthermore, a significant positive relationship between the concentrations of THg in adult and juvenile largemouth bass collected from the same location has been previously documented (Melwani et al. 2009).

## Trace Metal Analyses

Largemouth bass liver samples were processed and analyzed for trace metals (silver [Ag], aluminum [Al], arsenic [As], Cd, chromium [Cr], Cu, manganese [Mn], nickel [Ni], lead [Pb], selenium [Se], and Zn) by the California Department of Fish and Game according to United States Environmental Protection Agency (USEPA) method 200.7 on a Perkin-Elmer Sciex Elan 9000 inductively coupled plasma mass spectrophotometer. Standard reference material [Dorm3 (fish protein homogenate) and 2976] and sample (largemouth bass liver) spike recoveries were within 25 % of all elements evaluated.

## Data Analysis

All analyses were performed in SYSTAT (11th ed; Systat, San Jose, CA) using parametric tests (simple linear regression, two-sample Student *t* tests), and statistical significance was set at  $\alpha \leq 0.05$ . When necessary, data were log transformed to fit parametric model assumptions, or data were evaluated using nonparametric equivalents (Mann–Whitney *U* test).

### General Physiological Assessment

Body condition [ $100 \times (\text{body weight}/\text{body length}^3)$ ] for each adult largemouth bass was compared with THg concentration in muscle tissue using linear regression.

### MT Expression Analysis

Adult largemouth bass analyzed for MT gene expression were selected based on THg concentrations (lowest = 0.27 to 0.37 ppm [ $n = 7$ ] and highest = 0.63 to 0.97 ppm [ $n = 6$ ]) and sufficient RNA integrity (Fig. 2). To validate



that there was no treatment effect on elongation factor 1 $\alpha$  expression, the Ct was examined by treatment using two-sample Student *t* test ( $p = 0.625$ ). Gene expression of elongation factor 1 $\alpha$  was stable ( $SD = 0.92$ ) as determined using BestKeeper (Pfaffl et al. 2004).

Principal component analysis (PCA) was performed to avoid the confounding effects of colinearity among independent variables (contaminants) (Quinn and Keough 2002). Principal components were used in analyses if their eigenvalues were  $>1$  (Quinn and Keough 2002). Variables with a PC loading of  $>0.71$  or  $<-0.71$  (the correlation coefficient of the relationship between the component and original variable) were determined to be excellent and exceptionally well represented by the PC (Kevin et al. 2000). Because one of the goals of our study was an exploratory investigation of relationships between MT expression and metal exposure to help guide further study, we examined the relationship between each individual metal (THg, Se, Cd, and Pb), or PC1 (Al, Mn, Ni, Cu, and Zn) and MT expression [ $\log(\text{standardized MT expression} + 1)$ ] using linear regression analysis.

### Histopathological Analyses

Histopathological data from largemouth bass samples collected in 2005 and 2006 were combined because preliminary analysis found no difference in the distribution and trends between years. Statistical relationships were only examined for parameters that were detected in  $\geq 66\%$  of a particular tissue type (Table 4). Histopathological alternations in adult fish were analyzed using linear regression analyses, whereas those in juvenile fish were analyzed using two-sample Student *t* test.

Although up to 14 statistical analyses were performed to examine the relationship between MeHg exposure (in juvenile fish) or THg concentration (in adult fish) and histopathology; alpha was not adjusted for multiple comparisons because this procedure can result in false rejection of real effects in studies with typical sample sizes (Rothman 1990; Gotelli and Ellison 2004). As such, recent studies that examined relationships between multiple contaminant exposures and biological effects in wildlife did not perform multiple comparison adjustments (Verreault et al. 2004; Finkelstein et al. 2007).

## Results and Discussion

### Metal Concentrations

Adult largemouth bass THg concentrations ranged from 0.12 to 0.65 ppm in 2005 and 0.27 to 0.97 ppm in 2006, with 72 % of the samples collected exceeding the USEPA

screening value of 0.3 ppm (Table 5). However, we found no evidence that body condition was affected by THg concentration ( $r^2 = 0.005$ ,  $p = 0.555$ , linear regression). Previous studies reported measurable toxicity when THg concentrations in muscle tissue were near 1 ppm (Adams et al. 1999; Berntssen et al. 2004), and  $\sim 9\%$  of our samples had THg concentrations between 0.8 and 0.97 ppm (wet weight). The mean THg concentration in adult fish collected from low-exposure sites was 0.290 ppm wet weight ( $SE = 0.019$  [ $n = 32$ ], range = 0.117–0.494) and 0.755 ppm wet weight ( $SE = 0.102$  [ $n = 16$ ], range = 0.302–1.587) collected from fish at high-exposure sites (Table 1; Fig. 1), thus confirming our prediction that these sites would fall into low and high categories and validate the assignment of juvenile fish from these sites into low- and high-exposure groups.

The geometric means of several trace metal (Cu, Cd, THg, and Se) concentrations (Table 5) in largemouth bass livers were greater than the national geometric mean concentrations from whole-body tissue analyses in largemouth bass (Schmitt and Brumbaugh 1990), suggesting that the samples in our study represent fish collected from a polluted environment. Because  $>50\%$  of liver tissues had Cr, Ag, and As concentrations lower than the detection limit (Table 5), these metals were not examined for a relationship with standardized MT expression. D PCA reduced five (Cu, Zn, Mn, Al, and Ni) of the nine remaining metals into one principal component (PC1) that explained 46 % of the variation. The independent variables [PC1 (Cu, Zn, Mn, Al, and Ni), THg, Se, Pb, and Cd] were evaluated for relationships with MT gene expression using simple linear regression analysis.

### MT Expression

Linear regression analysis indicated a significant positive relationship between standardized MT gene expression and PC1 (representing Cu, Zn, Mn, Al, and Ni) (Table 5), a finding supported by previous studies on the relationship between MT gene expression and Cu and Zn exposure (Hamilton and Mehrle 1986). However, unlike previous studies, Cd concentrations were not significantly correlated with MT gene expression and may have been too low in our samples (Table 5) to elicit induction (Hollis et al. 2001). MT expression also showed a significant negative relationship with Se concentration (Table 5). Se, a sulfur analog, can bind to the divalent form of Hg because MeHg is demethylated in the liver; this interaction between Se and Hg may impact MT production (Chmielnicka and Brzezniacka 1978).

Although previous laboratory and field-based studies observed a positive relationship between MT and MeHg (Berntssen et al. 2004; Gonzalez et al. 2005; Schlenk et al.

**Table 5** Sample size and concentrations (average, geometric mean, range, and SE) of THg from muscle tissue and of trace metals in liver tissue of largemouth bass analyzed for MT expression (ppm, wet weight)

Sample size and concentrations	THg	Al	Cr	Mn	Ni	Cu	Zn	As	Se	Ag	Cd	Pb
Sample size	11	11	4	11	11	11	11	5	11	5	11	11
Average	0.577	39	0.538	2.24	0.121	10.4	25.5	0.295	1.16	0.031	0.268	0.055
Geometric mean	0.517	15.7	0.536	1.85	0.074	6.6	24.9	0.265	1.07	0.028	0.228	0.016
National geometric mean <sup>a</sup>	0.10	ND	ND	ND	ND	0.65	21.7	0.14	0.42	ND	0.03	0.11
Range	0.289, 0.974	1.90, 157	0.517, 0.603	0.707, 4.11	0.019, 0.411	1.98, 33.0	19.1, 38.1	0.108, 0.431	0.516, 1.94	0.012, 0.048	0.124, 0.703	0.006, 0.485
SE	0.081	14.7	0.031	0.419	0.038	3.05	1.75	0.06	0.144	0.005	0.054	0.043
<i>p</i> for MT	0.061	0.044 <sup>b</sup>	NA	0.044 <sup>b</sup>	0.044 <sup>b</sup>	0.044 <sup>b</sup>	0.044 <sup>b</sup>	NA	0.017	NA	0.594	0.985

Tissue samples containing concentrations of Cr, As, and Ag were lower than the reporting limit and were not examined for a relationship with MT gene expression (NA)

*p* values were generated using linear regression analysis with either the individual metal as the independent variable (THg, Se, Cd, Pb) or PC1 (representing Al, Mn, Ni, Cu, Zn) and MT expression [ $\log(\text{standardized MT expression} + 1)$ ] as the dependent variable

ND no data

<sup>a</sup> Data from Schmitt and Brumbaugh (1990)

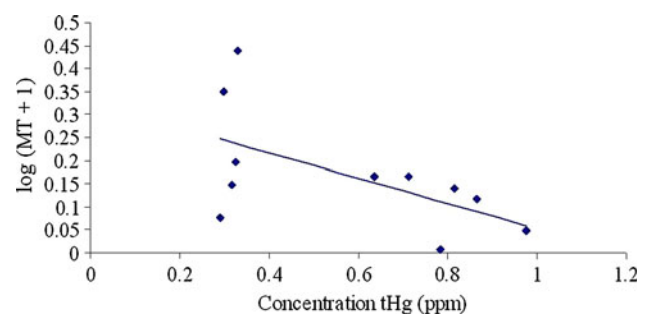
<sup>b</sup> These values represent PC1 and are not individual values

1995), we observed a weak negative relationship (Table 5). It is noteworthy that adults fish with low THg concentrations (mean =  $0.312 \pm 0.008$  SE ppm [ $n = 5$ ]) had greater variability of MT gene expression (mean =  $0.828 \pm 0.287$  SE) than those with high THg concentrations (THg mean =  $0.797 \pm 0.048$  SE ppm [ $n = 6$ ]; MT mean =  $0.292 \pm 0.0764$  SE) (Fig. 3). Furthermore, previous laboratory studies (Berntssen et al. 2004; Gonzalez et al. 2005) administered doses of MeHg to generate significantly greater tissues concentrations (5–13.5 ppm dry weight) than those observed in our study.

### Histopathological Analyses

Although some histopathological alternations were significantly related to THg, there was high variability within each category suggesting the influence by other unknown factors (Table 6). We found that for both juvenile and adult fish, MeHg had a negative association with macrophage density as evidenced by significantly lower mean macrophage densities in juvenile kidney and liver tissues from the high THg-exposure group, a trend toward significance in juvenile spleen tissue, and a significant relationship between THg and macrophage density in adult kidney tissue.

Our results also indicate that inflammation in kidney tissue may be influenced by increased Hg exposure because adult fish had significantly lower densities of inflammatory events with increased Hg exposure ( $r^2 = 0.06$ ,  $p = 0.041$ , linear regression), and this relationship was marginally apparent in juvenile fish ( $p = 0.066$ , two-sample Student *t* test). Although the occurrence of granulomas was not



**Fig. 3** THg in largemouth bass muscle tissue had a negative (nonsignificant) association ( $r^2 = 0.337$ ,  $p = 0.061$ ) with MT expression [ $\log(\text{standardized MT expression} + 1)$ ].  $y = (-0.274)x + 0.326$

significantly related to Hg exposure in adult fish, a greater incidence of granulomas in spleens from juvenile fish in the high- versus low-exposure groups (granulomas in 25 % [ $n = 4$ ] of low-exposure individuals vs. 60 % [ $n = 5$ ] of high-exposure individuals) was detected.

Larval trematode density was not significantly associated with Hg concentrations; however, high-exposure juvenile fish with larval trematodes had a greater incidence of mature trematode occurrence than that of low-exposure juvenile fish. Of the low-exposure juvenile fish with larval trematodes present (55 % [ $n = 11$ ], kidney tissue), 17 % had mature trematodes in kidney tissue, and 17 % had mature trematodes in liver tissue. In contrast, of the high-exposure juvenile fish with larval trematodes present (50 % [ $n = 8$ ]), 100 % of the individuals had mature trematodes in the kidney, and 50 % had mature trematodes present in the liver. Spleen tissue was not evaluated for trematode presence trends due to an insufficient number of samples

**Table 6** Summary of histopathological relationships with Hg exposure in juvenile and adult largemouth bass kidney, liver, and spleen tissues as observed from performing Student *t* test (juvenile fish) or linear regression analysis (adult fish)

Tissue type		Macrophages	Granulomas	Adult trematodes	Inflammatory events	Larval trematodes	Protozoan parasites	% Eosinophilic droplets
Kidney								
Juvenile fish	<i>p</i>	0.018 (–)	NA	NA	0.066 (–)	0.748	0.241	NA
	Low	0.56 (0.099)			0.76 (0.17)	0.27 (0.16)	20.1 (3.48)	
	High	0.20 (0.067)			0.30 (0.13)	0.13 (0.077)	13.2 (5.07)	
Adult fish	<i>p</i>	0.016 (–)	0.422	0.479	0.041 (–)	0.463	0.055 (–)	0.192
Liver								
Juvenile fish	<i>p</i>	0.020 (–)	0.48	0.014 (+)	0.808	NA	NA	NA
	Low	0.23 (0.12)	0.359 (0.207)	0.097 (0.007)	0.13 (0.045)			
	High	0.10 (0.00)	0.066 (0.034)	0.19 (0.028)	0.11 (0.052)			
Adult fish	<i>p</i>	0.772	0.100 (+)	0.689	0.540	NA	NA	NA
Spleen								
Juvenile fish	<i>p</i>	0.087 (–)	NA	NA	NA	NA	NA	NA
	Low	0.76 (0.250)						
	High	0.19 (0.106)						
Adult fish	<i>p</i>	0.401	0.0141	0.726	NA	NA	NA	NA

Mean densities and SE are provided for juvenile low- and high-exposure groups

(–) and (+) direction of the relationship with increased Hg exposure, NA feature was not observed in  $\geq 66$  % of samples

collected (low-exposure [ $n = 4$ ], high-exposure [ $n = 5$ ]), only some of which had larval trematodes present. These findings indicate that although both exposure groups appear to be equally infected with the parasite, the trematodes may be more successful at reaching adulthood in high-exposure juvenile fish. Furthermore, the high-exposure juvenile fish appear immunosuppressed because they displayed significantly lower densities of macrophages and inflammatory events, which should increase with foreign bodies, despite the increased incidence of mature trematodes. The potential for Hg to disrupt immune function in fish, including at the cellular level, has been well documented (Barst et al. 2011; Sweet and Zelikoff 2001).

Mature trematode density was greater in juvenile liver tissues from the high-exposure group ( $p = 0.014$ , two-sample Student *t* test). This pattern was also consistent in spleen (mature trematodes present in 25 % [ $n = 4$ ] of low exposed and 60 % [ $n = 5$ ] of high exposed juvenile fish) and kidney (mature trematodes present in 18 % [ $n = 11$ ] of low-exposed and 63 % [ $n = 8$ ] of high-exposed juvenile fish) tissues. Adult largemouth bass had lower densities of larval and mature trematodes in kidney and liver tissues (mean =  $0.21 \pm 0.05$  SE [ $n = 27$ ]; mean =  $0.08 \pm 0.02$  SE [ $n = 32$ ], respectively), than juvenile largemouth bass (mean =  $0.40 \pm 0.16$  SE [ $n = 10$ ]; mean =  $0.23 \pm 0.13$  SE [ $n = 9$ ], respectively). This trend was also observed with lower protozoan parasites densities found in adult fish (mean =  $8.5 \pm 0.98$  SE [ $n = 68$ ]) compared with juvenile fish (mean =  $17.2 \pm 2.9$  SE [ $n = 19$ ]).

In addition, we observed a marginally significant negative association between protozoan parasite density and THg concentration in adult fish ( $r^2 = 0.055$ ,  $p = 0.055$ , linear regression), a relationship that was not observed in juvenile fish nor with mature trematode density. Therefore, increased tissue THg concentrations may be not only be affecting the fish's physiological function, but it may also be impacting the survival of the fish's parasites because adult largemouth bass collected in the delta can have 2–3 times greater Hg concentrations than juvenile fish (Davis et al. 2008).

## Conclusion

Biomarkers may provide insight into the health of a population as chemical-induced alteration of physiological processes may indirectly lead to decreases in fitness and survival (Fossi 1998; Huggett et al. 1992; Connon et al. 2011). To our knowledge, we provide one of the few studies that examined biomarkers of effect in contaminated fish collected from the Sacramento-San Joaquin Delta (Spearow et al. 2011; Ostrach et al. 2008) and one of the first to examine the effect of Hg exposure even though the delta supports several sensitive and declining fish species and has a long history of Hg pollution. Our histopathology data suggest that juvenile largemouth bass may be experiencing sublethal effects (immunosuppression) and may be more sensitive than adult fish to increased MeHg exposure.



MT gene expression appears to be driven by Cu and Zn exposure, and the relationship with MeHg exposure is not well understood and may be complex if existent. Our results reflect the importance of performing field-based studies because quantifying parasites, a stressor found in the natural environment, was a useful indicator of fish health. Furthermore, our findings support the use of histopathology as a tool for assessing health status changes due to Hg exposure.

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