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Evaluation of the use of metallothionein as a biomarker for detecting physiological responses to mercury exposure in the bonnethead, *Sphyrna tiburo*

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Abstract Previous studies have demonstrated that sharks, perhaps more so than any other fishes, are capable of bioaccumulating the non-essential toxic metal mercury (Hg) to levels that threaten the health of human seafood consumers. However, few studies have explored the potential effects of Hg accumulation in sharks themselves. Therefore, the goal of this study was to examine if physiological effects occur in sharks in response to environmentally relevant levels of Hg exposure. To address this goal, the relationship between muscle Hg concentrations and muscle/hepatic levels of metallothionein (MT), a widely used protein biomarker of toxic metal exposure in fish, was examined in bonnetheads, *Sphyrna tiburo*, from three Florida estuaries. Total Hg concentrations in bonnethead muscle, as determined using thermal decomposition and atomic absorption spectrometry, ranged from 0.22 to 1.78 μg/g wet weight and were correlated with animal size. These observations were consistent with earlier studies on Florida bonnetheads, illustrating that they experience bioaccumulation of Hg, often to levels that threaten the health of these animals or consumers of their meat. However, despite this, MT concentrations measured using Western blot analysis were not correlated with muscle Hg concentrations. These results suggest that either environmentally relevant levels of Hg exposure and uptake are below the physiological threshold for inducing effects in sharks or MT is a poor biomarker of Hg exposure in these fishes. Of these two explanations, the latter is favored based on a growing body of evidence that questions the use of MTs as specific indicators of Hg exposure and effects in fish.

Keywords Metallothionein · Biomarker · Mercury · Sharks · *Sphyrna tiburo*

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Introduction

Mercury (Hg) is a highly toxic prevalent non-essential metal that is commonly found in aquatic environments (see reviews by Chen et al. 2008; Kim and Zoh 2012; Driscoll et al. 2013). It is deposited in its inorganic form into the environment primarily by anthropogenic activities such as mining, waste incineration, and the combustion of Hg-rich fossil fuels. Once Hg is

deposited into an aquatic system, it often undergoes bacterial methylation to produce the organic compound, monomethylmercury (MeHg). MeHg is more readily absorbed by aquatic organisms than inorganic forms of Hg; in fact, up to or greater than 90 % of the total Hg content in some aquatic species can be in the form of MeHg (Scheuhammer et al. 2007). It is also known to bioaccumulate and biomagnify in aquatic organisms and, as a result, MeHg levels in some aquatic taxa, particularly large terminal consumers such as marine mammals, sharks, and large teleosts can range from one to ten million times greater than MeHg levels present in the ambient environment (Chen et al. 2008). Because of its high rate of uptake, distribution, and accumulation in exposed organisms, its slow excretion and its tendency to bind to and disrupt the normal function of sulfhydryl-containing proteins, MeHg is considered to be the most toxic form of Hg, capable of eliciting adverse effects such as neurotoxicity, behavioral abnormalities, reproductive impairment, immunosuppression, and death (Scheuhammer et al. 2007). Therefore, it is critical to examine potential health effects of MeHg and other forms of Hg in aquatic species, particularly those that have been found to accumulate substantial quantities of this metal.

Based on a 2010 review by Gelsleichter and Walker and more recent studies (De Boeck et al. 2010; Escobar-Sánchez et al. 2010, 2011; Pethybridge et al. 2010; Nam et al. 2011; Barrera-García et al. 2012; Hurtado-Banda et al. 2012; Maz-Courrau et al. 2012; Bosch et al. 2013; Vélez-Alavez et al. 2013), Hg is the most widely studied toxicant in elasmobranchs (sharks and rays), with levels having been examined in close to 100 species. A sizeable number of these studies observed muscle Hg concentrations that were near to or above the maximum recommended limit for human consumption in most countries (i.e., 0.3 ppm, US. Environmental Protection Agency [EPA] 2001). Furthermore, as in some other taxa, MeHg can often make up a sizeable proportion of the total Hg burden in certain elasmobranchs (i.e., >95 %), particularly largely piscivorous species. However, despite the multitude of studies that have shown the presence of elevated Hg levels in sharks and their relatives, few studies to date have considered the possible biological effects of Hg exposure in these animals as opposed to human consumers of their meat/fins. In contrast, the effects of Hg on bony fish have been studied more extensively in both past and recent years, showing impaired reproduction, growth, and health as well as neurological alterations that could result in behavioral abnormalities in species exposed to elevated, but still ecologically relevant, Hg levels (Scheuhammer et al. 2007; Adams et al. 2010; Cambier et al. 2012; Batchelar et al. 2013a, b; Gehringer et al. 2013; Ho et al. 2013; Rhea et al. 2013; Stefansson et al. 2013). Given that Hg concentrations in elasmobranchs generally rival, if not exceed, those observed in even the largest bony fish species, it is important to determine if similar effects or physiological responses of any kind occur in these fish as a result of Hg exposure.

Previous studies have shown that metallothioneins (MTs), a group of metal-binding proteins, can serve as useful indicators for detecting physiological responses to metal exposure in fish and other aquatic organisms (see reviews by Nordberg and Nordberg 2009; Shariati and Shariati 2011). MTs are low molecular weight (6–7 kDa), cysteine-rich, intracellular proteins that are present in many invertebrate and vertebrate species and appear to play roles in the homeostasis and detoxification of metal ions by binding to and sequestering several divalent transition metals such as copper (Cu), zinc (Zn), cadmium (Cd), and Hg. MTs also appear to function in other important physiological processes including the scavenging of reactive oxygen species and the regulation of cell proliferation and apoptosis (Chiaverini and De Ley 2010). In general, MT expression increases in response to elevated metal exposure, a property that has led to its widespread use as a biomarker for detecting toxic metal effects in both human and wildlife populations. Since Hg has been shown to induce MT gene transcription and protein synthesis in various fish species (e.g., spotted scat *Scatophagus argus*, Sinaie et al. 2010; barbel *Barbus graellsii*, Quirós et al. 2007), it is a potentially useful biomarker for exploring whether ecologically relevant levels of Hg uptake in sharks are associated with physiological alterations. A few laboratory-based studies have confirmed that MT is present in elasmobranchs and can be induced by exposure to some toxic metals in certain species (Hidalgo et al. 1985; Hidalgo and Flos 1986a, b; Betka and Callard 1999; Cho et al. 2005; De Boeck et al. 2010); however, they did not focus on Hg and MT has not been extensively put to use in field ecotoxicology studies on elasmobranchs.

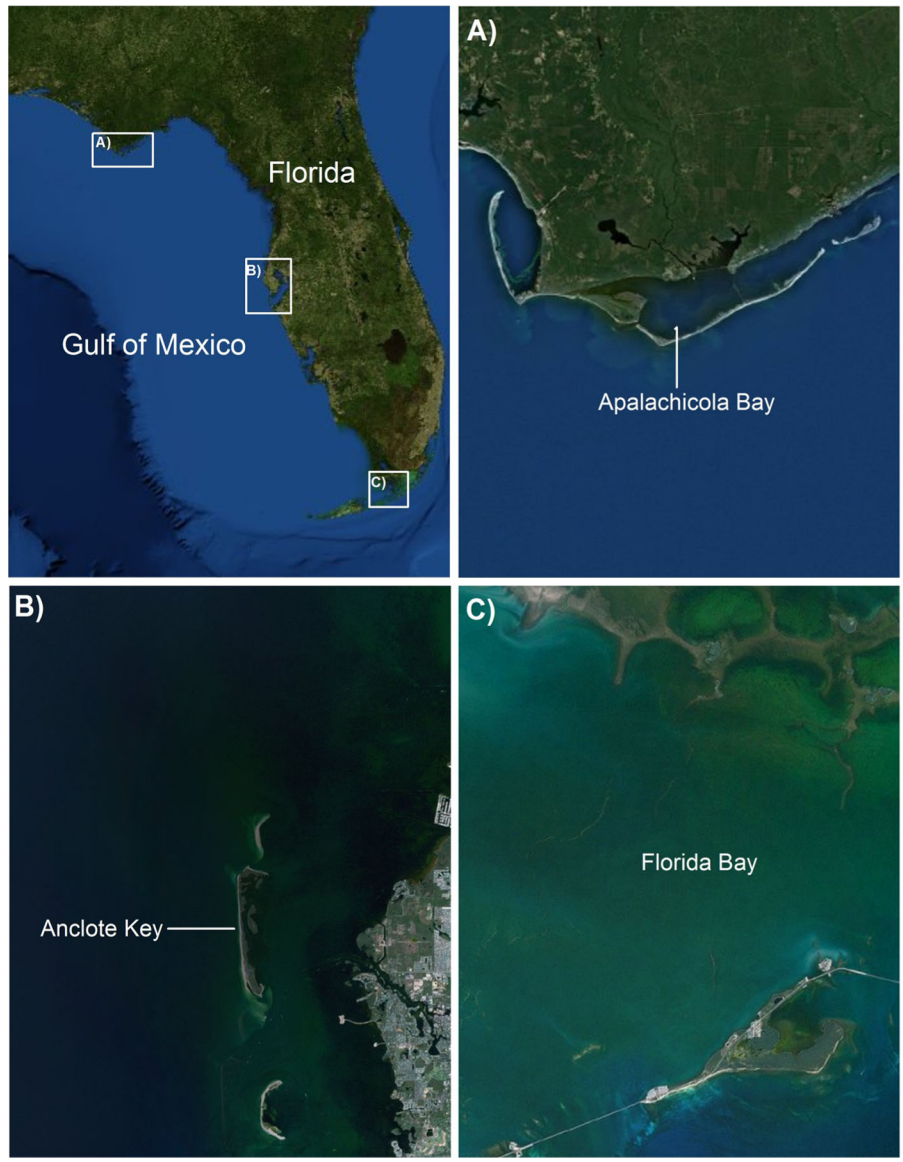

Fig. 1 Map of Florida showing the location of the three study sites used for the collection of samples in this study: **A** Apalachicola Bay, **B** Anclote Key, and **C** Florida Bay

Therefore, the goal of this study was to evaluate the use of MT as a biomarker for detecting physiological responses to toxic metal exposure in sharks in a field setting. In particular, this study examined the reliability of using MT as an indicator of Hg exposure and accumulation in the bonnethead, *Sphyrna tiburo*. The bonnethead was selected as the target species for this study based on the available evidence for high Hg uptake in this species (Adams and McMichael 1999; Adams et al. 2003), as well as the extensive body of knowledge on the biology of this shark from the areas sampled (Carlson and Parsons 1997; Cortes et al. 1996; Lombardi-Carlson et al. 2003). The suitability of MT as a biomarker for Hg exposure was assessed by determining if muscle or liver MT levels were correlated with *S. tiburo* muscle Hg concentrations. Hg and MT levels were also compared between populations of *S. tiburo* from three estuaries on Florida's Gulf coast. It was hypothesized that based on the evidence for MT induction in response to toxic metal exposure in sharks and other fish, MT levels would increase proportionally along with increased Hg uptake. Furthermore, we anticipated that *S. tiburo* would exhibit a site-associated difference in MT levels with bonnetheads from more Hg-contaminated sites

having higher MT levels than their counterparts from less Hg-contaminated locations.

Methods

Animal collection

Bonnetheads ($n = 50$) were collected using set gill nets between 1998 and 2001 from three Florida Gulf coast locations (Fig. 1): Anclote Key ($n = 15$), Florida Bay ($n = 20$), and Apalachicola Bay ($n = 15$). Sharks were weighed to the nearest 0.1 kg, measured in total length (i.e., measuring from the tip of the snout to the tip of the upper lobe of the caudal fin in a natural position; TL) to the nearest 1.0 cm, and examined in order to determine sex.

Biological sample collection

Following capture, sharks were rinsed with ambient seawater and packed in ice until arrival at the laboratory. Once at the laboratory, each shark was rinsed with running local tap water and a 1-g subsample of muscle was removed from the lateral musculature just below the dorsal fin and placed in a cryovial, which was subsequently placed in liquid nitrogen in order to prevent moisture loss during the freezing process. Samples were stored at −80 °C until time of analysis in 2009. Sub-samples of liver were also collected (~1 g) from the ventral edge of the right lobe of the liver, placed in cryovials, frozen with liquid nitrogen, and stored at −80 °C.

Mercury analysis

Muscle samples were dried for 48 h at 60 °C in an oven using aluminum weight boats to minimize cross-contamination, homogenized using a glass mortar and pestle, and stored at 4 °C until analysis. Samples were weighed before and after drying in order to monitor water content and reduction throughout the process. Percent moisture was determined using the formula,

$$\%\,\text{moisture} = 100 - \left(\frac{W_\text{d}}{W_\text{w}}\right) \times 100$$

where W_d = weight of dry sample (g) and W_w = weight of wet sample (g).

Total Hg (THg) was measured in μg/g dry weight (d.w.) in *S. tiburo* muscle samples by the Florida Fish and Wildlife Conservation Commission's Indian River Field Laboratory (Melbourne, FL, USA) using thermal decomposition (combustion), amalgamation, and atomic absorption spectrometry [EPA Method 7473] (EPA 1998). The analysis was completed with a calibrated DMA-80 Direct Mercury Analyzer (Milestone Inc., Shelton, Connecticut) according to Tremain and Adams (2012). Quality control procedures included analysis of laboratory method blanks, duplicate or triplicate tissue samples, and certified reference material (TORT-2 and DOLT-4 obtained from the National Research Council of Canada) for each group of 10 samples analyzed. In addition, a duplicate matrix spike was completed during the analytical run.

THg was presented in μg/g d.w. in all figures. However, because the majority of previous studies on THg in elasmobranchs have reported concentrations in μg/g wet weight (w.w.) (Gelsleichter and Walker 2010), d.w. measurements have been converted to their w.w. equivalents and are reported for comparative purposes.

Metallothionein analysis

Muscle and liver samples were homogenized in 3 volumes of buffer (100 mM Tris–HCl with 5 mM β-mercaptoethanol, pH 8.1, Hylland et al. 1995) using the FastPrep-24 bead homogenizer (MP Biomedicals, Inc., Santa Ana, CA, USA). Homogenates were centrifuged at 18,000*g* at 4 °C for 1 h, and the resulting supernatant was stored at −80 °C in three 200-μL aliquots until the time of analysis. A standard Bradford protein assay, using 1/50 sample dilutions, was used to determine protein concentrations (mg/mL) of each sample (Bio-Rad Laboratories, Hercules, CA, USA). Several samples were centrifuged a second time at 100,000*g* to attempt to reduce the occurrence of immunoreactive high molecular weight proteins, which have been observed in earlier studies on elasmobranchs in which they were assumed to represent MT associated with membrane components (Hylland et al. 1995) or oligomeric forms (Hidalgo et al. 1988).

Proteins (90 μg/sample/well) were separated via SDS-PAGE gel electrophoresis under denaturing and reducing conditions using 15 % polyacrylamide gels and the Laemmli buffer system. Gels used for the

visualization of protein content were fixed in a standard fixation solution (40 % methanol, 10 % glacial acetic acid) and stained with a 0.03 % Coomassie blue in fixation solution.

For immunoblotting, proteins were electrotransferred to PVDF membranes (Bio-Rad Laboratories), which were afterward blocked in 10 % non-fat dried milk (NFDM) in Tris-buffered saline (TBS) overnight at 4°C to prevent non-specific binding. Immunoreactive MT was detected using a polyclonal rabbit anti-cod MT antibody (KH-1, Cayman Chemical Co., Ann Arbor, MI, USA) diluted 1:500 in 1 % NFDM in TBS containing 0.05 % Tween 20 via overnight incubation at 4 °C. This antibody has been previously shown to cross-react with putative MT from several elasmobranchs (Hylland et al. 1995), including *S. tiburo* in preliminary studies (Gelsleichter, unpublished data). Goat anti-rabbit IgG (whole molecule)-alkaline phosphatase conjugate (Sigma-Aldrich Corporation, St. Louis, MO, USA) was used as secondary antibody (1:5,000 in 1 % NFDM in TBS containing 0.05 % Tween-20, 1 h incubation at room temperature), and 5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt/nitro-blue tetrazolium chloride (BCIP/NBT) (Vector Laboratories, Burlingame, CA, USA) was used as chromogen. Membranes were washed five times for 5 min each in TBS containing 0.05 % Tween 20 between each incubation. Following color reaction, membranes were rinsed in deionized water and air-dried. Band intensity for each sample was acquired and calculated using the Gel Logic Imaging System and Kodak Molecular Imaging Software (Carestream Heath, Inc., Rochester, NY, USA).

Statistical analysis

Correlations between THg and TL and MT band intensity in liver (MT_{liver}) and muscle (MT_{muscle}) were analyzed using Spearman's rank order correlation. Pearson's product–moment correlation was used to determine if MT_{liver} and MT_{muscle} were significantly correlated. Differences in mean THg levels associated with animal gender and location of capture were analyzed using Student's *t* test and one-way ANOVA followed by Tukey's post hoc test, respectively. Last, differences in MT_{liver} and MT_{muscle} associated with site of capture were also analyzed via one-way ANOVA followed by Tukey's post hoc test.

Results

Total Hg analysis

Specimens ranged in TL from 56 to 103 cm (range of free-swimming *S. tiburo* in these locations was 34–119 cm based on an n = 423, Manire, unpublished data). THg concentrations in *S. tiburo* muscle ranged from 0.86 to 6.58 μg/g d.w. (Mean ± SD = 3.00 ± 1.82 μg/g d.w.), which corresponded to w.w. values of 0.22–1.78 μg/g (Mean ± SD = 0.79 ± 0.49 μg/g w.w.) (Table 1). Of all samples analyzed, 86 % were found to have THg concentrations above 0.3 ppm, the US. EPA's fish tissue residue criterion (US. EPA, 2001). All results of quality assurance procedures were found to be within EPA standards.

There were no significant differences in mean THg levels associated with animal gender (Student's *t* test, $p > 0.05$). Because of this, sexes were combined for comparisons of THg levels between sites, but no significant differences were observed (one-way ANOVA, $p = 0.603$) (Table 1). A significant positive correlation between THg and TL was observed (Spearman's rank order correlation, $\rho = 0.628$, $p < 0.001$) (Fig. 2).

Metallothionein analysis

Western blot analysis of *S. tiburo* muscle and liver consistently resulted in the observation of two immunoreactive protein bands, corresponding to molecular weights of ~34–39 and ~14 kDa (Fig. 3). The latter of these two bands was equivalent to the expected molecular weight of MT, as reported in earlier studies (~12–14 kDa, Hidalgo and Flos 1986b; Hylland et al. 1995). Despite multiple attempts at separating this band from the larger band using ultracentrifugation as successfully employed by Hylland et al. (1995), the high molecular weight band remained present. Although there is strong evidence to suggest that the high molecular weight band represented polymerized forms of MT based on previous studies (e.g., Hidalgo and Flos, 1986b; Hylland et al. 1995), optical intensity of only the low molecular weight band was analyzed for comparative purposes.

Relative levels of MT in muscle and liver were not significantly correlated (Pearson's product–moment correlation coefficient, $r = -0.119$, $p = 0.438$). There was no significant correlation between THg and either MT_{muscle} (Spearman's rank order correlation,

Table 1 Range and mean ± SD of total mercury (THg) in *Sphyrna tiburo* muscle reported in μg/g dry weight (d.w.) and μg/g wet weight (w.w.) from three Florida Gulf coast locations (*AK* Anclote Key, *FB* Florida Bay, *AB* Apalachicola Bay), including the percentage of samples that contained THg levels that met or exceeded the maximum recommended limit for consumption in the USA (0.3 μg/g w.w.)

Location	Sample number	THg (μg/g d.w.)		THg (μg/g w.w.)		
		Range	Mean ± SD	Range	Mean ± SD	% ≥ 0.3 μg/g
AK	15	1.17–6.14	2.94 ± 1.68	0.31–1.53	0.77 ± 0.43	100.0
FB	20	0.95–6.58	3.29 ± 1.86	0.25–1.78	0.88 ± 0.51	90.0
AB	15	0.86–6.36	2.67 ± 1.94	0.22–1.64	0.71 ± 0.51	66.6
Totals	50	0.86–6.58	3.00 ± 1.82	0.22–1.78	0.79 ± 0.49	86.0

Mean THg concentrations did not differ significantly by location (one-way ANOVA, $p = 0.603$)

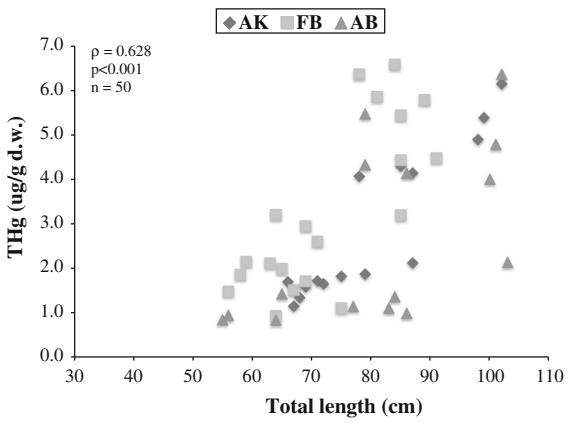

Fig. 2 Total mercury concentrations (THg; dry weight mg/kg) measured in *Sphyrna tiburo* muscle compared with the total length (TL) for each specimen ($n = 50$). A significant positive correlation between length and THg was observed (Spearman's rank order correlation; $\rho = 0.628, p < 0.001$). *AK* Anclote Key, *FB* Florida Bay, *AB* Apalachicola Bay

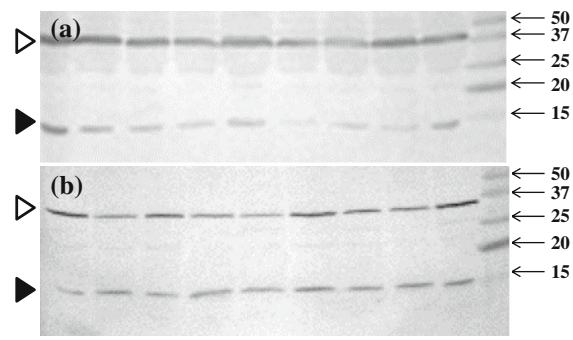

Fig. 3 Western blot analysis of *Sphyrna tiburo* metallothionein (MT) in **a** muscle and **b** liver tissue. *Arrows* indicate size of molecular weight markers in kDa. The low molecular weight bands (*black arrowhead*) represent a protein of the expected size for MT, whereas high molecular weight bands (*open arrowhead*) likely represent MT associated with membrane components or oligomeric forms. Due to the inability to reduce the high molecular weight bands by ultracentrifugation, only the lower weight bands were used for MT analysis

$\rho = -0.18$, $p = 0.899$) or MT_{liver} (Spearman's rank order correlation, $\rho = 0.055$, $p = 0.721$) (Fig. 4). There were no statistically significant differences in MT_{liver} associated with site of collection (one-way ANOVA, $p = 0.256$), but MT_{muscle} varied significantly between *S. tiburo* collected from Anclote Key and Apalachicola Bay (One-way ANOVA with Tukey's post hoc test, $p < 0.05$).

Discussion

Muscle THg concentrations measured in *S. tiburo* examined in this study (0.22–1.78 μg/g w.w. with a mean of 0.79 ± 0.49 μg/g w.w.) were consistent with those observed in Florida bonnetheads in prior investigations. Adams and McMichael (1999) reported mean muscle THg concentrations of 0.50 ± 0.36 μg/g w.w. in *S. tiburo* from nearshore areas on the southeast Florida coast. More recently, Adams et al. (2003) reported muscle THg concentrations ranging from 0.03 to 1.60 μg/g w.w. in bonnetheads collected throughout Florida with site-specific levels in sharks from the Florida Bay (0.28–1.60 μg/g w.w.) and Tampa Bay (0.03–1.60 μg/g w.w.) regions that were similar to those observed in individuals collected from the same locations in the current study (0.25–1.78 and 0.31–1.53 μg/g w.w. for *S. tiburo* from Florida Bay and Anclote Key, respectively). Like Adams and McMichael (1999) and Adams et al. (2003), we also found a significant positive relationship between muscle THg concentrations and shark length, demonstrating that bioaccumulation of Hg

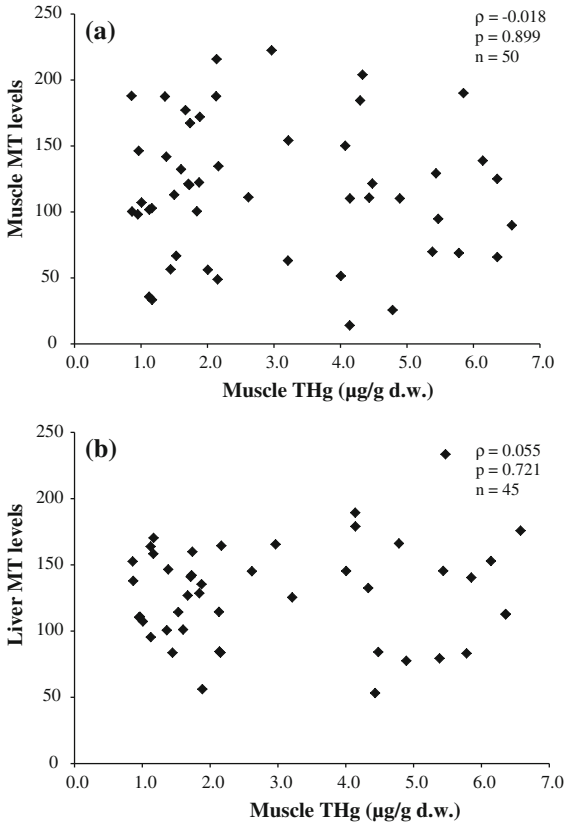

Fig. 4 Levels of *Sphyrna tiburo* metallothionein (MT) in **a** muscle ($n = 50$) and **b** liver ($n = 45$) compared with total mercury (THg; µg/g dry weight) measured in muscle. Relative MT levels were calculated by subtracting the mean background intensity from the mean intensity of 14-kDa immunoreactive bands observed using Western blot. No significant relationship was found between THg and relative MT levels in muscle (Spearman's rank order correlation; $\rho = -0.018$, $p = 0.899$) or liver (Spearman's rank order correlation; $\rho = 0.055$, $p = 0.721$)

occurs in this species. Last, like both previous studies, we observed that a sizeable proportion (86 %) of our samples had muscle THg levels that exceeded the federal fish tissue residue criterion in the USA. The occurrence of such high levels of Hg in this and other species of sharks, of course, formed the basis for our investigation on the physiological responses to Hg exposure in this group.

Despite the high levels of THg commonly observed in Florida *S. tiburo*, there was no evidence for elevated quantities of MT in such individuals and, in general, muscle THg concentrations and MT levels in muscle and liver were not positively correlated. Therefore, these results suggest that MTs is not likely to be a useful biomarker for Hg exposure in this species and perhaps other sharks. While these results seemed surprising based on longstanding and widespread use of MT as a specific indicator of metal exposure in aquatic organisms, they agree with a growing body of evidence that challenges its suitability as a biomarker for Hg exposure due to lack of positive correlations between endogenous Hg levels and MT content in several species (Rotchell et al. 2001; Monserrat et al. 2007; Miero et al. 2011; Gehringer et al. 2013; Sevcikova et al. 2013). As suggested in these studies, this may be due to the fact that MeHg, the most ecologically relevant and abundant Hg species found in wildlife, appears to be less capable of inducing MT than inorganic forms of Hg. It is also possible that differences in exposure to other metals or factors such as animal gender, stage of maturity, time of capture, and environmental conditions (e.g., salinity) at the time of capture influence MT production in ways that obscure possible relationships between the levels of this protein and Hg exposure. It is also feasible to consider that, while occasionally high, Hg levels in *S. tiburo* and some other elasmobranchs may still fall below the physiological threshold for inducing MT in these fishes. Last, it is possible that the semi-quantitative nature of MT analysis used in this study was not sensitive enough to detect fine differences in MT expression that may have been associated with THg.

The premise that MT content may not accurately reflect a MeHg-dominated Hg burden in some species because MeHg is generally less capable of inducing MT expression than inorganic forms of Hg is supported by previous research. For example, MeHg has been shown to be ineffective or less effective than inorganic Hg at inducing MT expression in both in vitro (e.g., cultured mouse neurons and astrocytes, Kramer et al. 1996a, b) and in vivo (e.g., mouse brain, liver, and kidney, Saijoh et al. 1989; Yasutake et al. 1998; Yasutake and Nakamura 2011; zebrafish (*Danio rerio*) liver, skeletal muscle, and brain, Gonzalez et al. 2005) animal models. Furthermore, in the few cases in which MeHg has been shown to successfully induce MT expression in vertebrate tissues (e.g., mouse brain, liver, kidney, and testis, Dufresne and Cyr 1999; Yasutake and Nakamura 2011), this effect has been largely attributed to increased presence of inorganic mercury following demethylation of MeHg (i.e., which occurs in mammals and birds, but is not thought to occur in fish) or increased production of reactive

oxygen species (Aschner et al. 2006). This may explain species-specific differences in the relationship between MT content and THg concentrations in various vertebrates as both the contribution of MeHg to total Hg burden and the ability to demethylate MeHg vary considerably among taxa. Therefore, it is possible that MT is a suitable biomarker for Hg in cases when inorganic Hg represents a large contribution to total Hg uptake in a species and/or when the species is capable of demethylating MeHg and that prior evidence for positive relationships between MT content and THg concentrations (e.g., Sinaie et al. 2010) reflects such instances and/or perhaps evidence for oxidative stress. This could explain why Hg in water, but not sediment was positively correlated with hepatic MT concentrations in golden gray mullet (*Liza aurata*) from a metal-contaminated site on the Portugal coast, as inorganic forms of Hg would clearly represent a greater contribution to water-borne levels of this metal compared with those in sediment (Oliveira et al. 2010). This could also explain the positive association between THg and MT concentrations in *L. aurata* but lack of such a relationship in European sea bass (*Dicentrarchus labrax*) from the same area on the northwest Portugal coast, as inorganic Hg is believed to represent a greater contribution to THg in the pelagic detritivore *L. aurata* in comparison to the demersal benthivore *D. labrax* (Miero et al. 2011). However, data on the proportion that MeHg comprises of THg in *S. tiburo* is needed to confirm or refute this hypothesis.

The possibility that MT content may reflect exposure to other metals to a greater extent than Hg is also well supported by previous research. Numerous studies have demonstrated that metals vary in their effectiveness at inducing MT expression in many vertebrates, including sharks. For example, Cho et al. (2005) found that zinc (Zn) was more effective than copper (Cu) and cadmium (Cd) at inducing hepatic and renal MT expression in the cloudy catshark, *Scyliorhinus torazame*, whereas De Boeck et al. (2010) observed positive MT induction in gill and liver of spotted dogfish, *Scyliorhinus canicula*, in response to exposure with Cu, but not Cd, lead (Pb), or silver (Ag). Furthermore, positive relationships between concentrations of some of these metals (i.e., Cd, Cu, Zn) and MT levels in fish tissues have been observed more often than those between MT and THg. As a recent example, Gehringer et al. (2013) observed significant positive correlations between hepatic MT expression and muscle concentrations of Cu, Zn, manganese (Mn), aluminum (Al), and nickel (Ni) in largemouth bass, *Micropterus salmoides*, but only a weak negative relationship between MT content and THg. However, it is also possible that physiological differences between individuals and/or environmental variables other than metal uptake may influence MT production to a greater extent than Hg, as they have been hypothesized to obscure relationships between MT content and even some of the most effective MT-inducing metals in prior studies (Creti et al. 2010). For example, previous studies have reported that factors such as nutritional status, temperature, salinity, dissolved oxygen, metabolic rate, stress, immune function, gender, stage of maturity, and reproductive stage can often alter MT expression in some species, resulting in high natural variability in levels of these proteins (Baer and Thomas 1990; Monserrat et al. 2007; Dragun et al. 2009).

Given that *S. tiburo* is a relatively small shark species with a low trophic position, compared with many other sharks (Bethea et al. 2007), it is logical to also consider that Hg accumulation in this species may fall below the levels necessary to induce MT expression in elasmobranchs and that larger, higher trophic-level sharks may be better subjects for this research. However, while it is true that some higher trophic-level shark species have been shown to accumulate fivefold to 10-fold higher levels of Hg than *S. tiburo* (e.g., smooth hammerhead, *Sphyrna zygaena,* gulper shark, *Centrophorus granulosus*, kitefin shark, *Dalatias licha*, Storelli et al. 2002, 2003), it is also important to note that the range in THg levels observed in *S. tiburo* in both this study and prior investigations overlap with those observed in many other shark species in which Hg accumulation has been surveyed including a number of larger species (Gelsleichter and Walker 2010). Therefore, if induction of MT is unlikely to occur in response to typical levels of Hg accumulation experienced by most sharks, it holds minimal value as a biomarker for Hg effects in this group.

As a final point, it is possible that the use of a semi-quantitative approach such as Western blot rather than more quantitative methods more commonly used in MT studies such as electrochemical techniques (e.g., Dragun et al. 2009; Oliveira et al. 2010; Sevcikova et al. 2013) or enzyme-linked immunosorbent assays

(ELISA) (e.g., Sinaie et al. 2010) may have limited our ability to detect subtle differences in MT content that could have been correlated with THg concentrations. This was an initially unplanned component of the present study made necessary by our inability to eliminate high molecular weight immunoreactive bands (i.e., presumed to be polymers), which could have resulted in overestimation of MT content using ELISA (Shariati and Shariati 2011). However, as reported in the surprisingly large number of reviews describing methodology for MT determination (Dabrio et al. 2002; Nordberg and Nordberg 2009; Ryvolova et al. 2011; Shariati and Shariati 2011), immunological approaches including Western blot are generally considered to provide the highest sensitivity and specificity for detecting MTs, provided that antibody probes successfully cross-react with target molecules. Furthermore, prior studies have successfully used comparable approaches to detect differences in MT content associated with factors that would induce its expression. For example, Ronco et al. (2005) used Western blot to demonstrate that MT levels are increased in human placenta from pregnant female smokers compared with that of non-smokers.

In conclusion, while Hg does accumulate in *S. tiburo* muscle to levels that exceed the maximum recommended limits for monthly human consumption, these levels do not appear to correlate with MT levels in either muscle or liver. Therefore, although other explanations for these findings have been considered, the results of this study suggest that there may be limitations for the use of MT as a biomarker for Hg exposure and effects in this species and perhaps other elasmobranchs. As mentioned, this conclusion is consistent with a sizeable and growing body of literature that question the use of MT as a specific biomarker for Hg exposure in vertebrates. Notwithstanding these results, future studies should continue to explore the relationship between Hg exposure and MT expression in sharks, particularly in species that have been shown to accumulate sizeable quantities of inorganic as well as organic forms of Hg. Future work should also focus on developing alternative biomarkers for detecting Hg effects in sharks, such as oxidative stress indicators, some of which have been shown to be potentially useful in recent studies (i.e., protein carbonyl concentrations, Barrera-García et al. 2012).

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