



Identification and response to metals of metallothionein in two ancient fishes: White sturgeon (*Acipenser transmontanus*) and lake sturgeon (*Acipenser fulvescens*)



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ABSTRACT

White sturgeon (*Acipenser transmontanus*) are among the most sensitive species of fishes to Cu, Cd, and Zn, but there is no information about sensitivity of lake sturgeon (*Acipenser fulvescens*). To begin to elucidate molecular mechanism(s) of sensitivity of sturgeons to metals a cDNA encoding metallothionein (MT) was amplified from livers of white sturgeon (WS-MT) and lake sturgeon (LS-MT), and expression in response to Cu, Cd, or Zn was characterized in liver explants from each species. The primary structure of WS-MT and LS-MT contained 20 cysteine residues, which is the same as MTs of teleost fishes. However, the primary structure of WS-MT and LS-MT contained 63 amino acids, which is longer than any MT identified in teleost fishes. Abundance of transcripts of WS-MT in explants exposed to 0.3, 3, 30, or 100 µg/L of Cu was 1.7-, 1.7-, 2.1-, and 2.6-fold less than in controls, respectively. In contrast, abundances of transcripts of WS-MT were 3.3- and 2.4-fold greater in explants exposed to 30 µg/L of Cd and 1000 µg/L of Zn, respectively. Abundance of transcripts of LS-MT was not significantly different at any concentration of Cu, Cd, or Zn. MT is hypothesized to represent a critical mechanism for detoxification of metals. Therefore, results of this study suggest that sensitivity of sturgeons to exposure to Cu, Cd, or Zn might be a result of the relatively lesser maximal response of MT to metals. The study also suggests lake sturgeon might be more sensitive than white sturgeon to metals.

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1. Introduction

Sturgeons (Acipenseridae) are ancient species of fishes with recognizable fossils dating back more than 65 million years (Wilimovsky, 1956). Today, all of the 24 species of sturgeons found in Europe, Asia, and North America are protected under the Convention on the International Trade of Endangered Species of Wild Fauna and Flora (CITES) (CITES, 2015). There are several hypotheses for the cause of these declines in populations of sturgeons, including alterations to habitat, over-harvest, predation by or competition from introduced species, exposure to pathogens, and pollution (LeBreton et al., 2004; Pikitch et al., 2005; Lenhardt et al., 2006). Metals represent one class of pollutants of concern to populations of sturgeons due to past and present activities of

mines, metallurgical facilities, pulp and paper mills, and other industrial and municipal input sources (Pikitch et al., 2005). Metals have been implicated in declines in populations of sterlet (*Acipenser ruthenus*) in the Danube River in central Europe (Hensel and Holcik, 1997; Poleksic et al., 2010); beluga sturgeon (*Huso huso*), stellate sturgeon (*Acipenser stellatus*), and Russian sturgeon (*Acipenser gudeldenstaedtii*) in the Caspian Sea in eastern Europe (Khodorevskaya et al., 1997); and white sturgeon (*Acipenser transmontanus*) in North America (Bortleson et al., 2001; Coutant, 2004; Besser et al., 2007; Hildebrand and Parsley, 2013). However, pollution by metals likely represents a concern to other populations of sturgeons worldwide. This has raised questions about adverse effects of pollution by metals of concern, such as copper (Cu), cadmium (Cd), and zinc (Zn), on sturgeons.

There is uncertainty about relative sensitivities of sturgeons to metals. Early life-stages of white sturgeon are among the most sensitive species of fishes to waterborne exposure to Cu (Vardy et al., 2011, 2013; Little et al., 2012). In particular, white sturgeon are very sensitive to Cu

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during the transition to exogenous feeding, which takes place at approximately 15 to 40 days post hatch (dph) (Vardy et al., 2013). Other species of sturgeons such as Atlantic sturgeon (*Acipenser oxyrinchus*), shortnose sturgeon (*Acipenser brevirostrum*), and shovelnose sturgeon (*Scaphirhynchus platyrhynchus*) are also among the most sensitive species of fishes to Cu (Dwyer et al., 2005; Vardy et al., 2013). Although little information exists regarding sensitivities of different species of sturgeons to Cd, Zn, or other metals, white sturgeon were found to be among the most sensitive known species of fishes to Cd and Zn (Vardy et al., 2011; Vardy et al., 2014). However, no information exists for the majority of other species of sturgeons. This raises questions as to why species of sturgeons appear to be more sensitive to metals than other species of fishes, especially during early life-stages, and what the underlying mechanism for this difference is. One hypothesis is that sturgeons, being ancient species, might have more primitive compensatory response mechanisms that impair their ability to cope with exposure to metals.

One protein that is crucial to the ability of an organism to cope with exposure to metals is metallothionein (MT). MTs are a superfamily of low molecular weight proteins that are rich in cysteine (C) (Kägi et al., 1984). MTs have been conserved throughout evolution and are expressed in prokaryotic and eukaryotic cells (Kägi et al., 1984). Four isoforms of MT (MT1 to MT4) have been identified in mammals (Babula et al., 2012), whereas one or two MTs have been identified in teleost fishes (Bargelloni et al., 1999). These proteins are thought to be important for several processes including detoxification of metals such as Cd and mercury (Hg) by sequestration, scavenging of superoxide radicals, and homeostasis and transport of Cu and Zn (Roesijadi, 1996; Babula et al., 2012). Induction of expression of MT genes is caused by binding of the metal responsive transcription factor 1 (MTF1), a zinc-finger transcription factor belonging to the Cys₂His₂ family, to metal response elements (MRE) located in the proximal promoter of MT (Maur et al., 1999; Saydam et al., 2002). Because of the correlation between increasing expression of MTs and increasing concentrations of metals such as Cu, Cd, and Zn, MTs are used as biomarkers of exposure of fishes to anthropogenic pollution by metals (Kille et al., 1992; van der Oost et al., 2003).

In this study MT in white sturgeon and lake sturgeon (*Acipenser fulvescens*) were cloned and characterized to begin elucidating mechanism(s) of sensitivity of sturgeons to metals such as Cu, Cd, and Zn. The white sturgeon was chosen due to its greater sensitivity to Cu, Cd, and Zn while the lake sturgeon was chosen because there is currently no information regarding sensitivity to metals of this species. Because a limited number of individuals of both species were available, an in vitro liver explant assay was used as an initial step to compare expression of MT transcript in livers exposed to a range of concentrations of Cu, Cd, or Zn. Characterizing molecular responses to exposure to metals in sturgeons will enhance understanding of potential mechanisms for the observed sensitivities of these ancient fishes to metals.

2. Materials and methods

2.1. Cloning and sequencing of metallothionein

Initial attempts to isolate MT of white sturgeon by the use of degenerate primers designed by aligning nucleotide sequences of MT from teleost fishes were unsuccessful. This result is consistent with previous studies that were unsuccessful in developing gene specific primers for MT of white sturgeon based on nucleotide sequences of MT from teleost fishes (Kruse and Webb, 2006). A full-length MT gene was later identified as a secondary product of rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR) of the aryl hydrocarbon receptor in cDNA of liver of white sturgeon (Doering et al., 2014). Gene-specific primers (Invitrogen, Burlington, ON) that flanked the start and stop codons were designed from this sequence (Table 1) and used to

amplify the full-length coding sequence of MT of both white sturgeon (WS-MT) and lake sturgeon (LS-MT).

Full-length cDNAs of WS-MT and LS-MT were amplified by the use of a LongRange PCR kit (Qiagen, Toronto, ON, Canada) and the polymerase chain reaction (PCR) products were purified by use of the QIAQuick PCR purification system (Qiagen) according to the protocol provided by the manufacturer. The purified PCR products were cloned into pGEM-T easy vectors by use of a DNA ligation kit (Invitrogen) and transformed into competent JM109 *Escherichia coli* cells, according to the protocol provided by the manufacturer (Promega, Madison, WI, USA). Plasmids were isolated by use of a plasmid purification kit (Qiagen) and products were sequenced at the University of Calgary's University Core DNA Services (Calgary, AB, Canada). Consensus sequences for WS-MT and LS-MT were determined by aligning sequences of at least six PCR products by use of CLC Genomics Workbench v.4.7.2 (Katrinebjerg, Aarhus, Denmark).

2.2. Phylogenetic tree and multiple sequence alignment

A phylogenetic tree showing the relationship of WS-MT and LS-MT to MTs from other species of fishes, mammals, amphibians, and birds was constructed by use of CLC Genomics Workbench v.4.7.2. A subset of sequences that were used to create the phylogenetic tree were aligned by use of CLC Genomic Workbench v.4.7.2. Accession numbers of MTs used for these analyses are: *Homo sapiens* MT1 (CAA45516), MT2 (CAA65915), MT3 (AAH13081), and MT4 (AAI13445); *Canis lupus familiaris* MT1 (NP_001003173), MT2 (NP_001003149), MT3 (AB001388), and MT4 (NP_001003150); *Rattus norvegicus* MT1 (NP_620181), MT2 (NP_001131036), MT3 (NP_446420), and MT4 (NP_001119556); *Xenopus laevis* MT4 (NP_001081042); *Gallus gallus* MT (BAF51974); *Danio rerio* MT1 (NP_571150) and MT2 (NP_001124525); *Cyprinus carpio* MT1 (AAV52385) and MT2 (AAV52384); *Salmo salar* MTA (NP_001117149) and MTB (NP_001117141); *Oncorhynchus mykiss* MTA (CAA42038) and MTB (CAA42037); *Esox Lucius* MT (CAA42035); *A. transmontanus* MT (KP164836); *A. fulvescens* MT (KP164837).

2.3. Fish

Juvenile white sturgeon, ranging in mass from 685 to 1287 g (approximately 4 years of age), were randomly selected from an in-house stock that was grown from fertilized eggs acquired from the Kootenay Trout Hatchery (Fort Steele, BC, Canada). Juvenile lake sturgeon, ranging in mass from 174 to 401 g (approximately 2 years of age) were randomly selected from an in-house stock that was grown from larva acquired from the Wild Rose State Fish Hatchery (Wild Rose, WI, USA). White sturgeon and lake sturgeon were maintained in the Aquatic Toxicology Research Facility (University of Saskatchewan, Saskatoon, SK, Canada) in separate flow-through systems maintained at approximately 15 °C. Culture water has been demonstrated to have negligible background concentrations of Cu, Cd, or Zn as reported in previous studies (Vardy et al., 2011, 2012). Fish were maintained on a diet of commercial trout feed (Martin Classic Sinking Fish Feed, Martin Mills Inc., Elmira, ON, Canada).

2.4. Dosing solutions

Stock solutions of copper(II) sulfate pentahydrate (Chemical Abstracts Service [CAS] 7758-99-8; purity 99.995%), cadmium chloride hemi-pentahydrate (CAS 7790-78-5; purity 99.999%), and zinc chloride (CAS 7646-85-7; purity 98%) (Sigma-Aldrich, Oakville, ON, Canada) were prepared in laboratory reverse osmosis water. Serial dilutions from each stock solution were prepared in laboratory reverse osmosis water. Concentrations were selected based on a prior range finding study with white sturgeon and lake sturgeon and represent a range of potentially environmentally relevant doses.

Table 1

Sequences, efficiencies, annealing temperatures, and corresponding target gene GenBank accession numbers of white sturgeon and lake sturgeon oligonucleotide primers used in the sequencing of full-length cDNA and in quantitative real-time PCR.

Species	Assay	Target gene	Accession #	Primer sequence (5'–3')	Efficiency (%)	Annealing temp (°C)
White sturgeon	Full	MT	KP164836	Forward: ACTCGTCACCGGAAACAAAGC Reverse: CGTTTGCCTCCAGACATAGGGG	NA	67
Lake sturgeon	Full	MT	KP164837	Forward: ACTCGTCACCGGAAACAAAGC Reverse: CGTTTGCCTCCAGACATAGGGG	NA	67
White sturgeon	qPCR	β -actin	FJ205611	Forward: ACTGCAAGTGCACAGACTG Reverse: AGGAGCAGCAGCTTTTCTTG	96	60
	qPCR	MT	KP164836	Forward: CCGAGCACAAATGAAAATGA Reverse: ACATCTGCTGGAAGGTGGA	89	60
Lake sturgeon	qPCR	β -actin	FJ205611	Forward: ACTGCAAGTGCACAGACTG Reverse: AGGAGCAGCAGCTTTTCTTG	90	60
	qPCR	MT	KP164837	Forward: CCGAGCACAAATGAAAATGA Reverse: ACATCTGCTGGAAGGTGGA	104	60

2.5. In vitro exposure

Exposure of liver explants was performed according to methods described for gonad explants (Beitel et al., 2014) with modifications. In brief, white sturgeon and lake sturgeon were euthanized by blunt trauma and livers were excised and placed in ice cold supplemented Leibovitz L-15 media (13.8 g of L-15 powder per L medium, 420 mg NaHCO₃/L, 10% fetal bovine serum, 1% antibiotic–antimycotic solution [100 units penicillin, 0.1 mg streptomycin and 0.25 μ g amphotericin B per mL], pH 7.6). The liver tissue was sliced into 1 mm³ sections, rinsed several times with supplemented L-15 media, and two or three pieces of liver were added to each well of a 24-well plate containing 1 mL of L-15 media. Nominal concentrations of Cu, Cd, or Zn were dosed in duplicate for white sturgeon (n = 6 individuals) and lake sturgeon (n = 5 individuals). Plates were incubated at 15 °C for 24 h on a platform rocker with gentle shaking. Upon termination of the exposure, explants were removed from each well and snap frozen at –80 °C.

2.6. Quantitative real-time PCR

Total RNA was extracted from liver explants by use of the RNeasy Lipid Tissue Kit (Qiagen), according to the protocol provided by the manufacturer. Concentrations of RNA were determined by use of a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and samples of RNA were stored at –80 °C. First-strand cDNA was synthesized by use of the QuantiTect reverse transcription kit (Qiagen) with 1 μ g of total RNA and according to the protocol provided by the manufacturer. Samples of cDNA were stored at –20 °C.

Quantitative real-time polymerase reaction (qPCR) was performed in 96-well plates by use of an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). A 70 μ L reaction mixture of 2 \times concentrated Power SYBR Green master mix (Qiagen), 3.5 μ L cDNA, 10 pmol of gene-specific qPCR primers, and nuclease free water were prepared for each sample of cDNA and primer combination. Primers for amplification of β -actin of white sturgeon (Doering et al., 2012) were determined to be suitable for amplification of β -actin of lake sturgeon (Table 1). A single gene-specific primer pair for MT was designed in conserved regions of WS-MT and LS-MT by use of Primer3

software (Table 1) and synthesized by Invitrogen. Reactions were conducted in triplicate with 20 μ L reaction volumes per well. The reaction mixture for qPCR was denatured at 95 °C for 10 min followed by a thermal cycle profile consisting of denaturing at 95 °C for 10 s and extension for 1 min at 60 °C for a total of 40 PCR cycles. Abundances of transcripts of WS-MT and LS-MT were quantified by normalizing to β -actin according to methods described previously (Simon, 2003).

2.7. Statistical analysis

Statistical analyses were conducted by use of SPSS 19 software (SPSS, Chicago, IL, USA). Normality of each dataset was evaluated by use of the Kolmogorov–Smirnov test and homogeneity of variance was determined by use of Levene's test. Data were log-transformed when necessary to meet assumptions of normality and homogeneity of variance. Significant differences were evaluated by use of either one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test or a two-sample t-test. Data are shown as mean \pm standard error of the mean (S.E.M.).

3. Results and discussion

3.1. Sequence comparisons and phylogeny

A cDNA encoding an MT was amplified from livers of white sturgeon (WS-MT) and lake sturgeon (LS-MT). The sequences of nucleotides and the putative sequence of amino acids are shown (Fig. 1). The coding regions of WS-MT and LS-MT are 192 nucleotides and encode 63 amino acids. The molecular mass of WS-MT is predicted to be 6304 g/mol whereas the molecular mass of LS-MT is predicted to be 6348 g/mol. The putative sequences of amino acids of WS-MT and LS-MT are 97% identical and both MTs contain 20 C residues, a feature that is characteristic of all MTs identified to date (Capasso et al., 2003). The only difference in their primary structure is at position 11 where WS-MT had an alanine (A) residue but LS-MT had a glycine (G) residue, and at position 41 where WS-MT has a G residue but LS-MT has an aspartic acid (D) residue.

Sturgeons are estimated to have diverged from the lineage leading to teleost fishes approximately 300 million years ago following divergence of the Actinopterygii (which includes both sturgeons and teleost fishes) from the Sarcopterygii (which includes the tetrapods) (Kumar and

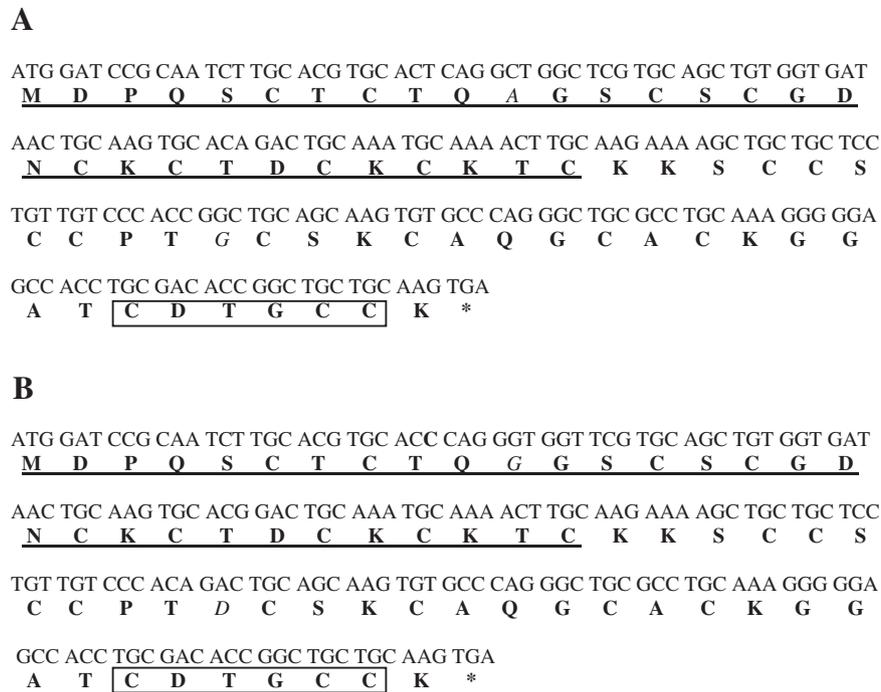


Fig. 1. Nucleotide sequence of the protein coding region of MT that was cloned and sequenced from the liver of white sturgeon (A) and lake sturgeon (B). Amino acids are shown below each codon. Amino acids that are underlined form the β -domain and those not underlined are the α -domain. The CXXXCC motif, which is a characteristic of MTs from fishes is enclosed in a box. The stop codon is indicated by an asterisk. Differences in amino acids are indicated in italics.

Hedges, 1998; Blair and Hedges, 2005). Therefore, to our knowledge, WS-MT and LS-MT are the earliest forms of MT identified to date in bony fishes. The phylogenetic relationship between WS-MT, LS-MT, and MTs from teleost fishes, amphibians, birds and mammals is shown (Fig. 2). Recently, four major clades of MTs have been identified. These include: fish MT, eutherian MT1 and MT2, tetrapod MT3, and amniote MT4 (Serén et al., 2014). The WS-MT and LS-MT were identified as being most closely related to MTs of fishes (Fig. 2). Features of the primary structure of WS-MT and LS-MT that are common to MTs from fishes support this conclusion. For example, the presence of a CXXXCC motif in the α -domain of WS-MT and LS-MT (Fig. 1), resulting from a shift in the position of the ninth C residue of the α -domain, is a characteristic of MTs from fishes whereas MTs from other clades contain a CXCC motif in this region (Scudiero et al., 1997; Capasso et al., 2003).

Several features of the putative primary structure of WS-MT and LS-MT proteins are unique from MTs in species of teleost fishes. A specific feature used to distinguish between MTs from fishes and mammals is the number of cysteine–lysine (CK) or lysine–cysteine (KC) amino acid pairs. In MTs from mammals the number of these pairs is 5 to 7, which is greater than in fishes (D'Auria et al., 2001; Scudiero et al., 2005). There are 4 CK (KC) pairs in each MT shown in the alignment (Fig. 2B). The exception to this is the MT from common carp (*C. carpio*), which has 5 pairs. However, 7 CK (KC) pairs are present in WS-MT and LS-MT, and these extra pairs are located at positions 25–26, 27–28, and 62–63. Another feature of WS-MT and LS-MT that is unique from MTs of other species of teleost fishes is the length of the protein. MTs of teleost fishes contain 60 amino acids, except for metallothionein A (MTA) from rainbow trout (*O. mykiss*) and Atlantic salmon (*S. salar*) that contain 61 amino acids (Cho et al., 2005). However, WS-MT and LS-MT have 63 amino acids, which is longer than any MT identified in teleost fish. Compared to other species of fishes, the WS-MT and LS-MT have amino acids inserted at positions 4 (glutamine; Q), 5 (serine; S), and 55 (A). An MT identified in the cloudy catshark (*Scyliorhinus torazame*), which is a cartilaginous fish, contains 68 amino acids (Cho et al., 2005). In mammals, most MT1 and MT2 contain 61 amino acids, while MT3 consists of 65 to 68 amino acids, and MT4 of

62 amino acids (Cho et al., 2005). The length of WS-MT and LS-MT is identical to that of MTs from several species of birds (Richards, 1984; Wei and Andrews, 1988; Lin and Huang, 1990; Lee et al., 1996; Nam et al., 2007). This finding is similar to results of studies of the aryl hydrocarbon receptor (AhR) and hypoxia inducible factor 1 α (HIF1 α) where sequences of proteins in sturgeons were more similar to those of birds and other tetrapods than those of other fishes (Rytönen et al., 2007; Doering et al., 2014). Functional significance, if any, of this similarity to tetrapods is unknown. However, it could be a remnant of the relatively close evolutionary relationship of sturgeons to the earliest tetrapods (Kumar and Hedges, 1998; Blair and Hedges, 2005).

3.2. Effect of copper, cadmium, or zinc on expression of MT in liver explants

Liver is an important organ for accumulation of some metals, particularly Cu and Cd (McGeer et al., 2000; Hollis et al., 2001; Kraemer et al., 2005). Therefore, liver explants were used to assess expression of WS-MT and LS-MT following exposure to metals. Relative to the abundance of β -actin, abundances of transcripts of WS-MT were approximately 33-fold greater than LS-MT under basal conditions (Fig. 3). After 24 h of exposure abundances of transcripts of WS-MT in liver explants exposed to 0.3, 3, 30, or 100 $\mu\text{g/L}$ of Cu were 1.7-, 1.7-, 2.1-, and 2.6-fold less than in controls, respectively (Fig. 4A). In contrast, abundance of transcripts of WS-MT were 3.3-fold greater in liver explants exposed to 30 $\mu\text{g/L}$ of Cd (Fig. 4B). Although there was no statistically significant alteration at any other concentration of Cd, including 100 $\mu\text{g/L}$, a trend of greater abundance of WS-MT began at concentrations as little as 0.03 $\mu\text{g/L}$ (Fig. 4B). Abundance of transcripts of WS-MT was 2.4-fold greater in liver explants exposed to 1000 $\mu\text{g/L}$ of Zn, but was not altered at any other concentration (Fig. 4C). Abundance of transcript of LS-MT was not significantly different at any concentration of Cu, Cd, or Zn (Fig. 5A, B, C).

In general, response of WS-MT to Cd and Zn in vitro was consistent with the response in other species of fishes both in vitro and in vivo. In all species of fishes studied to date, abundances of transcripts of MT were greater following exposure to Cd. In livers, up-regulation of

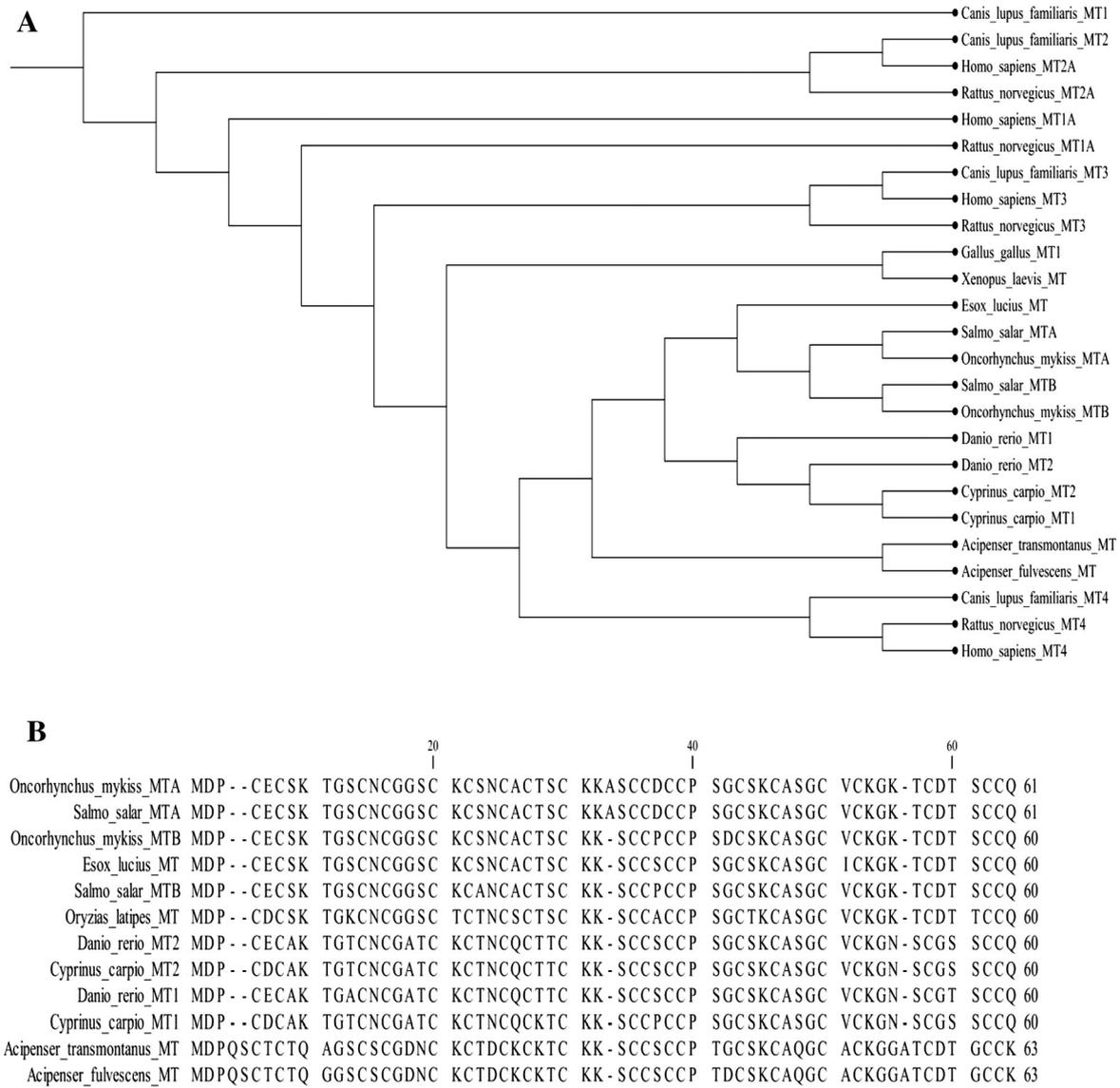


Fig. 2. Comparisons of sequences of amino acids of MTs from different species of vertebrates. Phylogenetic tree for relatedness of MT from mammals, birds, amphibians, and teleost fishes (A). Branch lengths represent bootstrap values based on 1000 samplings. Alignment of sequence of MTs from teleost fishes (B). Accession numbers of MTs are given in the Materials and methods section.

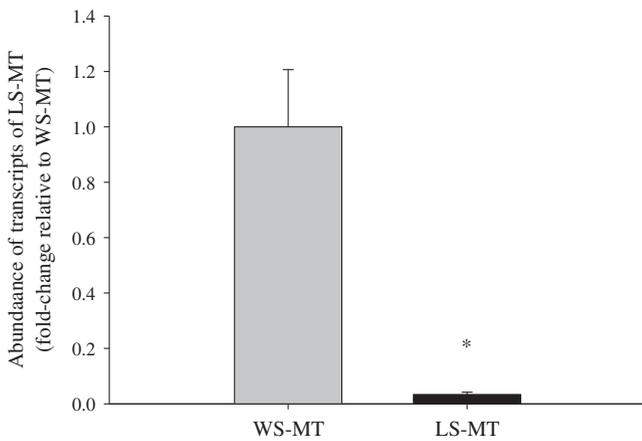


Fig. 3. Comparison of basal abundance of transcripts of MT relative to β -actin in the liver explants of white sturgeon (n = 7) and lake sturgeon (n = 5). Data represent the mean \pm S.E.M. Statistical difference indicated by an asterisk (*) (t-test; p \leq 0.05).

transcripts ranged from 1.8-fold in Atlantic cod (*Gadus morhua*) to 35-fold in striped sea bream (*Lithognathus mormyrus*) following exposure to Cd (Tom et al., 2004; Softeland et al., 2010). Response of MT following exposure to Zn is less clear than with Cd. The promoter of the MT-A gene from rainbow trout is activated by Zn (Mayer et al., 2003), and greater abundance of transcripts of MT have been quantified in livers of several species of fishes exposed to Zn, including the yellow catfish (*Pelteobagrus fulvidraco*) (Woo et al., 2006; Kim et al., 2012). However, several studies found little or no change in abundances of transcripts of MT in livers of several species of fishes, including cloudy catshark, chub (*Leuciscus cephalus*), Crucian carp (*Carassius carassius*), and Nile tilapia (*Oreochromis niloticus*) following exposure to Zn (Hayes et al., 2004; Atli and Canli, 2008; Cho et al., 2008; Ding et al., 2014). Unlike in other fishes, abundances of transcripts of LS-MT were not greater following exposure to Cd or Zn.

Lesser abundance of transcripts of WS-MT, and the trend towards lesser abundances of transcripts of LS-MT, in explants exposed to Cu is not consistent with results from studies with species of teleost fishes in vivo. It is not clear why exposure to Cu causes down-regulation of MT in liver explants of white sturgeon or lake sturgeon. Greater

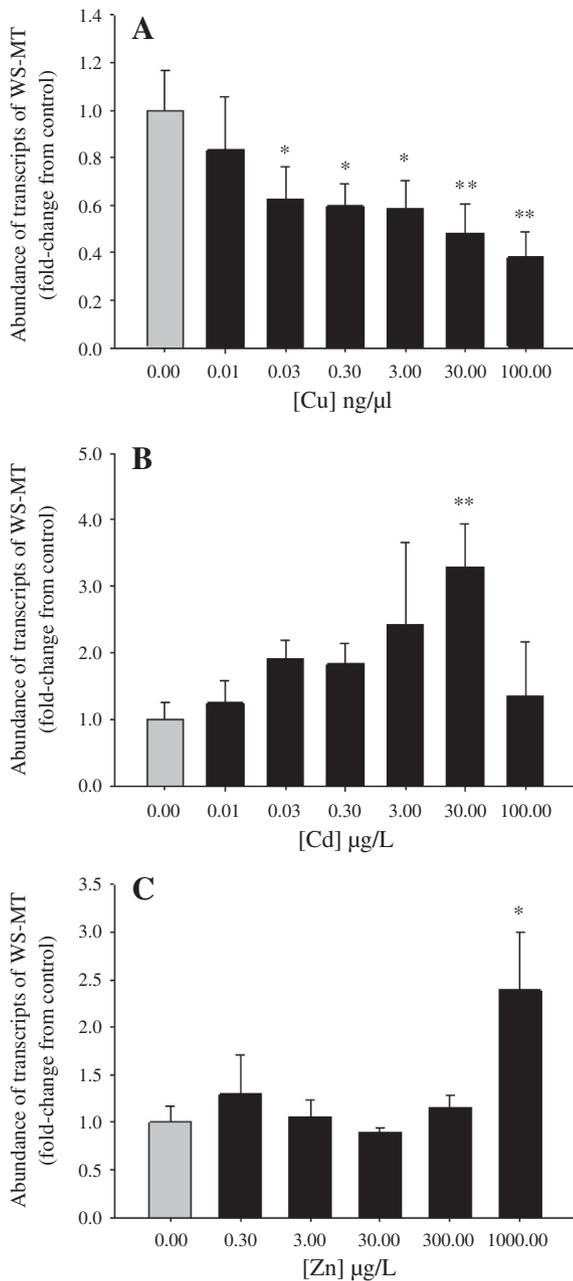


Fig. 4. Abundance of transcripts of WS-MT in liver explants from white sturgeon following exposure to copper (A), cadmium (B), or zinc (C). Concentrations are presented in $\mu\text{g/L}$. Data represent the mean \pm S.E.M. ($n = 6$ individuals). An asterisk (*) represents a significant alteration relative to 0 $\mu\text{g/L}$ (one-way ANOVA; $p \leq 0.1$) while a double asterisk (**) represents a significant alteration relative to 0 $\mu\text{g/L}$ (one-way ANOVA; $p \leq 0.05$).

abundances of transcripts by as much as 23-fold have been reported in livers of other fishes exposed to Cu (Kim et al., 2012). However, as with Zn, some studies found little or no change in abundance of transcripts in livers of fishes following exposure to Cu (Hayes et al., 2004; Cho et al., 2008). A down-regulated response in livers following exposure to Cu might be unique to sturgeons and could represent one reason for the great sensitivity of sturgeons to Cu, as discussed below (Vardy et al., 2011, 2012, 2014; Little et al., 2012).

3.3. Potential mechanisms for sensitivity of sturgeons to metals

Compared to other fishes studied to date, based on lethality of early life stages, white sturgeon are more sensitive to waterborne exposure to

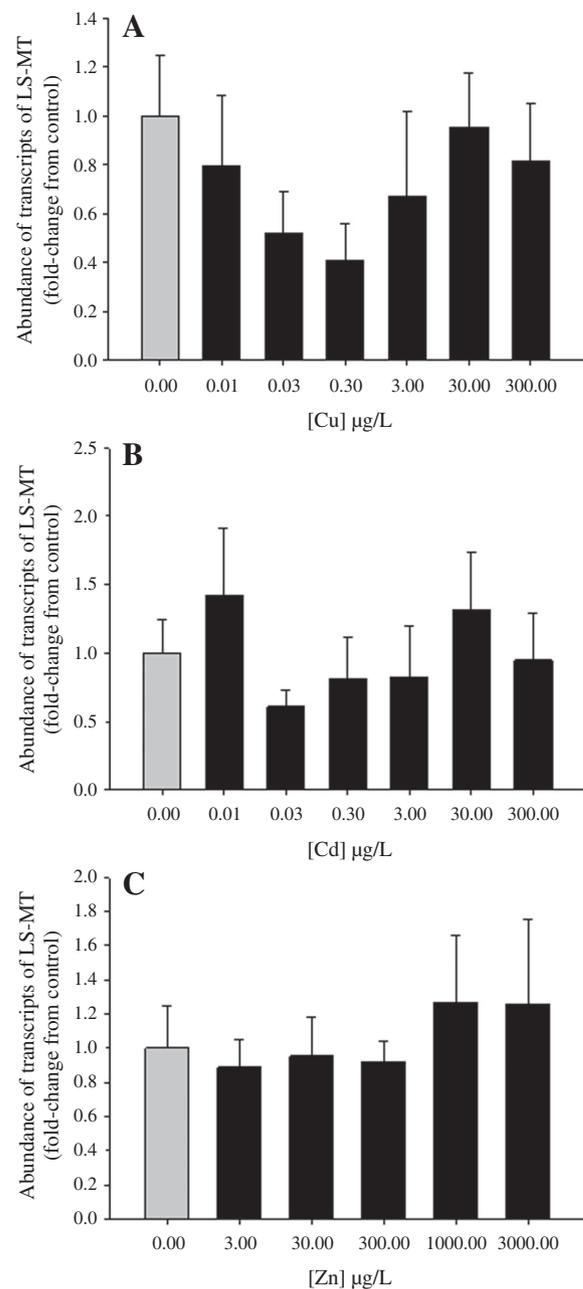


Fig. 5. Abundance of transcripts of LS-MT in liver explants from lake sturgeon following exposure to copper (A), cadmium (B), or zinc (C). Concentrations are presented in $\mu\text{g/L}$. Data represent the mean \pm S.E.M. ($n = 5$ individuals). No significant alteration relative to 0 $\mu\text{g/L}$ (one-way ANOVA; $p \leq 0.1$) was observed at any of the tested concentrations.

Cu, Cd, and Zn (Vardy et al., 2011, 2012, 2014; Little et al., 2012). Sensitivity of lake sturgeon to these metals is not known. However, all species of sturgeon that have been tested to date rank among the more sensitive species of fishes to Cu, Cd, and Zn (Dwyer et al., 2005; Vardy et al., 2014). The mechanism(s) of greater sensitivity is not known. One hypothesis is that MT protein expressed in sturgeons is inefficient or incapable of binding metals. Although binding of metals by WS-MT or LS-MT was not determined, the primary structures of WS-MT and LS-MT contain 20 C residues that bind metals via their thiolate groups (Vasak and Meloni, 2011). This would suggest that WS-MT and LS-MT are capable of binding metals. However, the primary structures of WS-MT and LS-MT are unique from MTs in other species of fishes, and it is not known if this affects the function of the mature proteins.

Another hypothesis that might explain the relatively great sensitivity of sturgeons to metals is a reduced capacity to mount compensatory responses following exposure to metals. Metallothioneins protect cells and organisms from adverse effects associated with exposure to metals, and there is some evidence that greater expression of MT confers greater tolerance to metals. For example, mammalian cell lines that cannot produce MT have greater sensitivity to metals relative to cells that over-produce MT (Beach and Palmiter, 1981). In another study a natural population of fathead minnows (*Pimephales promelas*) collected from a pond contaminated with metals was more tolerant to Cd than another natural population of fathead minnows collected from a reference pond, and the greater tolerance was attributed, in part, to greater levels of MT protein as a response to metals (Benson and Birge, 1985). In a study of rainbow trout, common carp, and gibel carp (*Carassius gibelio*), there appeared to be a relationship between expression of MT protein, concentrations of Cu in tissues, and sensitivity of these species (De Boeck et al., 2003). For rainbow trout, which was the most sensitive species, there was no relationship between concentrations of Cu in tissues and levels of MT. However, in gibel carp, which was the least sensitive species, there was a strong positive relationship between concentrations of Cu in tissues and levels of MT. The relationship in common carp, which was intermediate in terms of sensitivity, was intermediate of the relationship between concentrations of Cu in tissue of rainbow trout and gibel carp. However, in another study that used Cd the relationship between sensitivity and expression was not clear (Olssen and Ellie, 1997). A comparison of expression of MT from rainbow trout, which is sensitive to adverse effects of Cd, with MTs from northern pike and stone loach, which are less sensitive than rainbow trout to adverse effects of Cd, failed to find a relationship between induction of expression of MT and sensitivity (Olssen and Ellie, 1997). It is not known if greater sensitivity of sturgeons to metals is related to the lesser magnitude of up-regulation, or lack thereof, of expression of MT. Responses of MT in liver explants from white sturgeon exposed to Cd and Zn occurred only at very great concentrations, and maximal change in abundance of transcripts of MT was less than 4-fold. Abundance of transcripts of MT was not altered in liver explants from lake sturgeon, even at concentrations 3 × greater than those used in liver explants from white sturgeon. If the capacity of responses of MT to metals is important to the sensitivity of a species to metals, it is hypothesized that white sturgeon and lake sturgeon would be more sensitive to Cd and Zn than those species in which up-regulation of MT is greater than in white sturgeon. Also, white sturgeon would be hypothesized to have greater sensitivity to Cu relative to other fishes as expression of WS-MT was significantly lesser in explants exposed to Cu relative to control. By the same reasoning, lake sturgeon would be hypothesized to be more sensitive than white sturgeon to Cd and Zn as LS-MT did not respond to exposure to these metals. However, lake sturgeon would be hypothesized to be less sensitive than white sturgeon to Cu as expression of LS-MT showed a downward trend but not as great as that of white sturgeon. Also, if basal abundance of transcripts is an indicator of basal levels of proteins then lake sturgeon might be significantly more sensitive to exposure to all metals than white sturgeon given the significantly lesser basal expression of MT in this species.

Although this study identified a MT in white sturgeon and in lake sturgeon and characterized differences in response to exposure with Cu, Cd, and Zn in liver explants, it needs to be acknowledged that additional studies are necessary to test the hypothesis that MT has a role in the great sensitivity of sturgeons to metals. Although limited alterations in abundance of transcripts of MT were observed in white sturgeon and no alterations were observed in lake sturgeon, other species of fishes are known to express 1 or 2 forms of MT (Bargelloni et al., 1999). Sturgeons have up to at least a hexadecaploid (16n) genome, and therefore, it is likely that they express additional forms of MT that might be up-regulated in response to exposure to metals. However, numerous attempts to identify additional forms of MT in white sturgeon or lake sturgeon were unsuccessful, including by use of transcriptome sequencing

by use of RNAseq (Doering et al., unpublished data). Further, white sturgeon are known to display unique life-stage specific sensitivities to Cu in particular, but likely for other metals as well (Vardy et al., 2011, 2012). This study used juveniles of both white sturgeon and lake sturgeon that were greater than 1 year of age, but studies have reported that white sturgeon have greatest sensitivity to Cu, and likely other metals, during the transition to exogenous feeding stage at around 15 to 40 dph with juvenile fish that were 100 dph or older being more tolerant to exposure to Cu (Vardy et al., 2013). Therefore, future research should investigate life-stage specific differences in the responses of MT and other metal compensatory genes to identify the molecular mechanism for the greater sensitivity of white sturgeon to Cu, or other metals, at the transition to exogenous feeding stage. Lastly, the molecular basis for differences in responses between WS-MT and LS-MT are not known but might be a result of differences in binding and activation of MTF1 in these species. However, differences among species in the activation of MTF1 and how this regulates expression of MT have not previously been explored.

4. Conclusions

In summary, this study identified MT genes for the first time in two members of the Acipenseridae: white sturgeon and lake sturgeon. The putative primary structures of these MTs were similar to MTs from other fishes and they contained structural characteristics required for binding of metals. However, other features of the MT from these species were different from other fishes, and the functional implications of these differences are not known. White sturgeon displayed an up-regulation of transcripts of MT in liver explants following exposure to Cd or Zn, but a down-regulation of MT following exposure to Cu. No alteration in abundance of transcripts of MT was observed in liver explants of lake sturgeon following exposure to Cu, Cd, or Zn. Great sensitivity of sturgeons to exposure to metals, such as Cu, Cd, and Zn, is likely not a result of MT proteins that are inefficient or incapable of binding metals, but might be a result of the relatively lesser maximal response of MT following exposure to metals. Future research should investigate other metal compensatory responses in sturgeons, responses in other target organs of metals, and life-stage specific differences in responses of MT.

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