Development of methods to assess metallothionein expression in lake trout (*Salvelinus namaycush*) during a reproductive cycle and the effects of cadmium and ethynylestradiol

By

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A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
In partial fulfillment of the requirements of the degree of

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Abstract

Metallothioneins (MT) are low molecular weight proteins rich in cysteine whose physiological role is thought to include the regulation of essential metals Cu and Zn and protection against heavy metal toxicity and oxidative stress damage. MT has been isolated from a wide variety of terrestrial and aquatic organisms and, because its production increases markedly in the tissues of organisms exposed to certain metals, has been proposed for use as a bioindicator. Specifically, MT is a good indicator of environmental exposure to group IB and IIB metals for fish.

However, before MT can be routinely applied as a bioindicator, the influences of other biotic and abiotic factors and methodological considerations must be addressed. In particular, it has been suggested that MT can vary widely with the reproductive stages of fish as a secondary consequence of intracellular metal mobilization.

Four methods for measuring MT were developed and applied to tissues from lake trout (*Salvelinus namaycush*). High performance liquid chromatography (HPLC) and western blot analysis were used to measure MT protein concentrations while quantification of mRNA for MT-I and MT-II was performed with Northern blot analysis and real-time PCR. These techniques were used to examine MT expression and protein production throughout a reproductive cycle in lake trout as well as to examine the effects of chronic exposure to Cd in a field setting or the potent estrogen mimic, ethynylestradiol (EE2) in the field and laboratory exposures.

Based on the idea that MT changes through the reproductive cycle of fish and that MT changes when the fish are exposed to Cd and EE2, two hypothesis were estrablished. a) Concentrations of the metal binding protein metallothionein will vary in female lake

trout throughout the year as a function of reproductive stage. b) Normal seasonal variations in MT concentrations will be disrupted in lake trout chronically exposed to Cd or to 17α-Ethynylestradiol.

MT protein and mRNA were elevated during the fall, at the time of spawning, relative to winter, spring and summer. Induction was greater for the MT-I isoform than MT-II. The elevated levels of MT-I during the fall are likely a secondary result of the release of Zn from organelles after the process of vitellogenesis. Higher concentrations of free Zn would enhance production of MT protein and bind and retain the metal away from sites of toxic action.

It has been proposed that contaminants found in complex effluents have the potential to disrupt normal MT regulation. For example, environmentally relevant concentrations of the synthetic estrogen, ethynylestradiol (EE2) can induce production of vitellogenin outside the normal reproductive window. This could affect Zn mobiliztion and MT expression. However, exposures at environmentally relevant EE2 concentrations were not sufficient to alter seasonal expression patterns of MT protein or mRNA in lake trout. Only when EE2 concentrations exceeded 40 ng/L, was a decrease in liver MT protein observed. The hevay metal Cd, also found in industrial effluents, can induce MT expression. Chronic exposure therefore has the potential to disrupt normal MT regulation.

Lake trout exposed to elevated waterborne concentrations of Cd in an experimentally treated lake were found to exhibit an increase in MT protein. However, no differences were observed in mRNA content of fish from the treated lake. It has been suggested that mRNA may be elevated soon after exposure, but that in chronic exposures

elevated protein with no increase mRNA production can occur. This dynamic should be considered when applying MT analysis to fish from a specific location for biomonitoring purposes.

The techniques that have been developed for measuring MT and its isoforms will allow for more widespread application of MT as a bioindicator. Differences in the levels of expression of the two isoforms indicate their different roles in regulating essential metals during the reproductive cycle. Several areas of research must be expanded before MT can be effectively used as a bioindicator. For example, more long term exposures of environmentally relevant concentrations of EE2 in mature fish as well as the effects on juveniles and larval fish are warranted. Juvenile and larval fish stages may be more dramatically affected by hormonal changes compared to adult fish.

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Abbreviations

Cadmium	Cd
Chinook salmon embryonal tissue cells	CHSE-214
Complementary DNA	cDNA
Condition Factor	CF
Copper	Cu
Cystein	Cys
Diethyl Pyrocarbonate	DEPC
Diethylaminoethyl	DEAE
Enzyme linked immunosorbent assay	ELISA
Ethydium Bromide	EtBr
Ethylenediaminetetraacetic acid	EDTA
Ethynylestradiol	EE2
Förster or Fluorescence resonance energy	FRET
Gonadal Somatic Index	GSI
High Performance Liquid Chromatography	HPLC
Interleukin	IL
Lead	Pb
Liver Somatic Index	LSI
Lysine	Lys
Manganese Superoxide Dismutase	Mn-SOD
Metal Response Elements	MRE
Metal Transcription Factor	MTF
Metal Transcription Inhibitors	MTI
Metallothionein	MT
Mercury	Hg
Poly(vinylidene fluoride)	PVDF
Reverse Transcriptase Polymerase Chain Reaction	RT-PCR
Radio Immuno Assay	RIA

Rainbow Trout Gonadal cell line	RTG-2
Silver	Ag
Size-exclusion chromatography	SEC
Sodium dodecyl sulphate	SDS
Sodium dodecyl sulphate polyacrylamide gel electrophoresis	SDS-PAGE
Tricane Methanesulphonate	MS222
Trifluoroacetic acid	TFA
Tumor Necrosis Factor	TNF
Zinc	Zn

Chapter 1

Literature review

Introduction

The cellular mechanisms involved in regulating essential metals like copper (Cu) and zinc (Zn), and in detoxifying heavy metals like cadmium (Cd), mercury (Hg) and silver (Ag), have been increasingly examined over the last three decades. Metallothioneins (MT), proteins with high affinity for group IB and IIB metal ions have been studied in both of these capacities. Metallothioneins are a low molecular weight proteins with a high percentage (~30%) of cysteine amino acid residues (Couillard and St-Cyr, 1997) that confer metal binding capabilities to the molecule. Because of this property, MTs are believed to represent the most important metal detoxification pathway in a variety of species.

MTs have been isolated in all species of fish studied to date (Gerpe et al., 2000), and has been used as a biomarker for metal contamination in fish collected from lakes, rivers and marine environments. The precise biological functions of metallothionein are still being intensely investigated, but three biological functions have been postulated 1) the regulation of intracellular concentrations of essential metals (Cu and Zn) (Kägi and Schaffer, 1988; Olsson et al., 1989; Roesijadi, 1992), 2) detoxification of non-essential metals (Roesijadi, 1994; Roeva et al., 1999; Gerpe et al., 2000; Cheung et al., 2004) and 3) protection against oxidative stress (Kägi and Schaffer, 1988; Bremner, 1991; Olsson et al., 1996b; Coyle et al., 2002). Many factors are known to induce metallothionein, including changes in free Zn or Cu ions, the presence of heavy metals, hormones and second messengers, growth factors, inflammatory agents, cytokines and stress conditions (Kägi and Schaffer, 1988; Olsson et al., 1996b; Vašák, 2005).

Biochemical properties of metallothionein

Metallothionein is a low molecular weight protein (7-8 Kda), with an amino acid sequence that is highly conserved between species. It has a high proportion of cysteine residues (30%) that facilitate the binding of metal ions. These metal ions are bound to MT in oligonuclear complexes called clusters. Metallothioneins have been divided into three classes on the basis of their structural differences. Class I MT includes the mammalian forms and MTs from other phyla with related primary structures. Class II comprises polypeptides displaying no, or only a very distant, correspondence to the mammalian forms. Nematodes, yeast and cyanobacteria have been found to express Clas II MTs. Class III, are not proteins at all, but short atypical non-translationally synthesized metal-thiolate-polypeptides built up from repetitive -glutamyl cysteinyl isopeptide units (Kojima, 1991; Kägi, 1993). These polypeptides have been identified in plants and some fungi (Roesijadi, 1992).

Metallothioneins are usually single chain proteins containing 61-62 amino acids in vertebrates or 57-58 amino acids in crustaceans (Uchida et al., 1991; Palmiter et al., 1992). Included in this structure are 20 cysteine residues that can bind bivalent metal ions. The most common structural motif of cysteine distribution is a repeated Cys-X-Cys trypeptide sequence, where X stands for any other amino acid (Kägi, 1993). The absence of disulfide bonds within the protein means that the structure of the metal free MT is that of a random chain (Couillard and St-Cyr, 1997). Another defining characteristic of metallothioneins is the complete absence of aromatic amino acids (e.g. tyrosine) and the presence of very few bulky hydrophobic residues.

The three dimensional structure of MT indicates that the metal ions are clustered in two different domains. These domains are isolated from the external medium by the peptide chain except for two deep crevices which provide direct access to the metal-thiolate structures (Couillard and St-Cyr, 1997). The clusters are located as "mineral cores" in the interior of the equally sized globular domains. Each is formed by two large helical turns of the respective halves of the polypeptide chain. In the amino terminal beta domain the chain fold is right handed, while in the carboxyl terminal alpha domain it is left handed. These domains are connected by the residues at position 30 and 31 (Kägi, 1993).

A series of loops, which make up the major portion of the protein, allow for a structural flexibility that confers the ability to accommodate metals of widely differing size (Kägi, 1993). Besides the ability to accommodate different metals, metallothionein can accommodate different numbers of ions. For example, each MT molecule is capable of binding 7 metal ions for Hg(II), Cd(II), Zn(II), 12 for Cu(I), and 12, 17 or 18 for Ag(I) (Couillard and St-Cyr, 1997). Finally, different metal ions are also bound to MT with varying affinity. For example, Zn (II) and Cd (II) bind more strongly to the alpha domain than the beta domain, while Cu (I) and Ag (I) exhibit the opposite preference (Briggs and Armitage, 1982; Nielson and Winge, 1984; Otvos et al., 1993). It has been shown that the affinity of MT for the different metals is also different, where Hg shows the highest affinity, followed by Ag, Cu, Cd, Pb and Zn (Couillard, 1991; Dallinger et al., 2004).

Metallothionein has been proposed as a free radical scavenger. It has been shown that Cd-tolerant cells, which have increased ability to synthesize MT also have an increased ability to resist oxidative stress (Mello-Filho et al., 1988). One possible

mechanism is that Fenton-mediated radical damage is eliminated through iron chelation or exchange of iron with MTs (Templeton and Cherian, 1991). A second proposed mechanism involves MTs scavenging free radicals through their binding to MT's thiol cluster (Bremner, 1991; Kling and Olsson, 2000).

Heat stability is another important defining characteristic of MT; this thermal stability might be partly due to the absence of well-defined secondary structures such as α-helices and β-strands (D'Auria et al., 2001). The metal cluster structure also confers the molecule a particularly high structural firmness. The relatively small size and dumbbell-like shape of MT can also account for its high thermo stability (Dallinger et al., 2004).

Synthesis and degradation of metallothionein

Induction of MT has been attributed to many physiological and external factors. Perhaps the most important inducer of MT is cellular metal concentrations (Stillman, 1995; Couillard and St-Cyr, 1997). It has been proposed that MT induction might result from elevated levels of intracellular Zn, which may be displaced from cellular ligands by other metals (Hamilton and Mehrle, 1986). The Zn displaced from the MT protein becomes available to bind to metal transcription inhibitors (MTI) in the cell nucleus, MTIs are bound to the metal transcription factors (MTF) keeping the metallothionein gene inhibited. The release of the MTF (Couillard and St-Cyr, 1997) induces the transcription and translation of the MT protein by the binding of the MTF to metal response elements (MRE) on the MT gene. MTF is a protein composed of six zinc fingers that are responsible for DNA binding, therefore, an increase in Zn concentration

allows for the binding of MTF to DNA that subsequently initiates transcription of the MT gene (Vašák, 2005).

The metal-ligand complexes formed by the binding of heavy metals to cellular ligands represent toxic interactions (George et al., 1996). Tese are reduced by metal exchanges from the cellular ligands to newly induced Zn-MT. It has been shown that the affinity of MT for different metals varies. Mercury and Cd bind with higher affinity compared to Cu or Zn (Coyle et al., 2002).

Metallothionein synthesis is induced only by certain metals, including Ag, Au, Cd, Cu, Hg, Ni and Zn. Metals such as Cd, Cu, and Hg, which induce MT, do not activate metal transcription factors directly (Roesijadi, 1996). Instead, induction of MT by these metals follows a pathway that results in the increase of intracellular free Zn levels. Zinc is displaced by the metal from Zn binding ligands in the cell. The displaced Zn is then available to bind to the MTI, releasing the transcription factors from inhibition. Once released from inhibition the transcription factor (MTF-1) is able to bind to the MRE in the MT gene to induce transcription (Couillard and St-Cyr, 1997; Miles et al., 2000; Coyle et al., 2002; Vašák, 2005).

The presence of metal responsive elements (MREs) in the regulatory sequences of MT genes represents evidence for specific metal-induced transcription of MT. In turbot (*Scopthalmus maximus*) Cd induces MT mRNA and MT protein in a dose and time dependent manner (George et al., 1992). Low doses of metals can confer resistance to a subsequent and higher metal exposure (Roesijadi, 1992) due to the induction and increased binding capacity of MT. Moreover, induction of MT synthesis by one metal, such as Zn, can induce tolerance to another metal. Induction of MT by metals is specific

and metal-dependent, but the ability of MT to engage in rapid intra and interprotein metal exchange, facilitates tolerance to several metals (Cherian and Chan, 1993; Roesijadi, 1996).

In RTG-2 cells (rainbow trout gonad cell line) exposed to varying concentrations of $ZnCl_2$ (50 – 250 μM), $CuCl_2$ (50 – 250 μM) and $CdCl_2$ (5 – 50 μM), Zn was the most effective inducer of MT mRNA synthesis while Cu was the least efficient. At 10 μM $CdCl_2$ detectable amounts of mRNA were observed 24 hrs after treatment. Zinc Chloride ($ZnCl_2$, 100 μM) induced a response 3 hrs after treatment, the induction of MT by Zn reached a peak at 48 hrs with a 172 fold induction observed. The induction by Cd was 10 fold at 48 hrs (Zafarullah et al., 1990). The two isoforms present in rainbow trout are also differentially expressed by the three different metals studied. Cadmium chloride and $ZnCl_2$ are better inducers of MT-I compared to MT-II; on the other hand, $CuCl_2$ induced low levels of both mRNAs, with MT-II being slightly higher compared to MT-I.

Inflammatory responses can also induce MT. Compounds like endotoxin lipopolysaccharide (Sobocinski and Canterbury, 1982) and organic solvents (Onoska et al., 1988) release cytokines that induce MT. Specifically, interleukin (IL)-1, IL-6, tumor necrosis factor (TNF) and interferon (De et al., 1990) induce the protein in liver tissue. Tumor necrosis factor also induces MT synthesis in lung and heart. Administration of IL-1 increases hepatic concentrations of Zn-MT in rats (DiSilvestro and Cousins, 1984). The increase in MT synthesis induced by IL-1 has been shown to be associated with a tissue specific redistribution of Zn. Specifically, there is a transient depression of Zn in the plasma and a concomitant uptake of Zn by the liver, bone marrow and thymus (Cousins adn Leinart, 1988; Kyong-son et al., 1993).

In addition to metals and inflammation, MT synthesis can be stimulated in many organs by non-metallic compounds like alkylating agents, alcohols, chelators and glucocorticoid hormones. Some of these inducers produce reactive oxygen radicals, such as superoxide- and hydroxy- radicals and hydrogen peroxide (Sato et al., 1993). *In vitro* studies have demonstrated that MT can scavenge free hydroxyl and superoxide radicals produced by the xanthine-xanthine oxidase reaction (Thornalley, 1985). These radicals are known to induce damage to cellular and sub-cellular membranes via a damaging and self-propagating process called lipid peroxidation (Kyong-son et al., 1993). Oxidative stress induction of MT is thought to occur via the production of cytokines that respond to the presence of oxidative radicals. For example, TNF administration induces MT synthesis, and also increases the activity of the antioxidant enzyme, manganese-superoxide dismutase (Mn-SOD). Thus Mn-SOD and MT may cooperatively exert antioxidative roles in tissues like liver, lung and heart under the direction of TNF (Sato et al., 1993).

Recently, the induction of MT by hormones has been investigated. The elevation of hepatic MT levels observed in mature female fish might be induced by a re-distribution of intracellular Zn. This redistribution occurs just before spawning and results from an increase in the reproductive hormone estradiol (Olsson et al., 1989). On the other hand, Nishiyama et al. (1987), in a study using rats, suggested that female sex hormones can induce MT synthesis in liver and kidney, which in turn protects these animals against Hg-induced renal toxicity. By pre-treating male rats with estradiol, renal toxicity due to Hg administration was ameliorated. Hg-MT concentration in kidney of rats treated with Hg and estradiol was significantly higher than the corresponding value

in rats treated with Hg alone (Nishiyama et al., 1987).

Degradation of MT is thought to take place in lysosomes. The greatest degradative activity of the MT molecule and the metal-MT complex resides in lysosomes (Klaassen et al., 1993). When MT is bound to metal ions the complex is more resistant to hydrolysis (Bremmner, 1983) than MT alone, suggesting that metals stabilize the MT structure.

Inside the lysosome, metals dissociate from the MT protein under acidic conditions. That is, hydrogen ions displace metals from MT and the release of metals causes MT structure to change from a cluster to a random configuration. Lowering the pH increases the rate of metal release from MT. Zn content rapidly diminishes at a pH between 5 and 4.5, with zinc being fully released at pH 4.0. Lysosomes are acidic, with a pH in the range of 3.6 and 5. Therefore Zn can easily dissociate from MT in the lysosomes. On the other hand, Cd is only fully released at pH 1.0 (Miles et al., 2000). These changes take place before MT is broken down by protease enzymes in the lysosome (Cherian and Chan, 1993). The dissociation enables the lysosome to completely digest MT (Klaassen et al., 1993). Cathepsins B and/or L are the proteases that degrade MT (Cherian and Chan, 1993). The end products of MT degradation are eliminated from the cell by exocytosis of polymerized forms accumulated in the lysosomes (Hogstrand, 1991; Couillard and St-Cyr, 1997). The metal atom bound to the protein determines the rate of degradation of MT. Cadmium-MT has a half-life of 80 hrs, and in contrast the half-lives of Zn- and Cu-MT are 20 and 17 hrs respectively (Miles et al., 2000).

Metals taken up in the diet or across the gut epithelium are delivered to internal

organs (eg. Liver, kidney) via the blood. In mammals, the Cd-MT complex can be released from the liver and redistributed to the kidney through the blood system for long term storage (Nordberg and Nordberg, 1987; Barron et al., 2000). This internal mobilization of MT may be an important factor in the restoration phase through detoxification and storage of metals in tissues (Hogstrand and Haux, 1991b). The liver is thought to be the critical organ for detoxification in response to acute loading in fish (Schultz et al., 1996). An increase in Cd concentration in bile suggest that hepatobiliar excretion of cadmium is the route for some of the Cd accumulated (Chowdhury et al., 2003). When chronic exposure of Cd exists, the liver synthesizes the metal binding protein metallothionein that binds Cd, the Cd-MT complex is then released from the liver and redistributed to the kidney where long-term storage can occur. Once the metal reaches the blood stream, 72% of the injected Cd is delivered from the plasma to tissues in 2 hrs, suggesting an efficient detoxification or homeostatic regulation of these metals (Chowdhury et al., 2003).

Genetic regulation of metallothionein

The MT gene has a tripartite structure of three exons interrupted by two introns at amino acids 9 and 31 or 32, a structure that has been conserved among higher eukaryotes (Gedamu et al., 1993). This strongly suggests that metallothioneins have been evolutionarily conserved, and that the differences observed in the different species are possibly due to gene duplication that leads to the separation of subgroups (Kägi, 1993).

Regulation of MT expression occurs mainly at the transcription level. Metal responsive elements (MRE) are recognized by regulatory proteins during the induction of

MT transcription by heavy metal ions such as Cd, Cu and Zn (Seguin, 1991; Kille et al., 1995). Metal regulatory transcription factors (MRFs) act in a positive fashion to initiate expression of the MT gene (Roesijadi, 1996). The mechanism by which these transcription factors are activated differs among lower and higher eukaryotes. In yeast, metal activation of a constitutively inactive transcription factor causes a conformational change that in turn confers the ability to interact with the appropriate MRE and initiate gene expression. In mice, MT gene expression is regulated by Zn-mediated release of an inhibitor that is bound to a constitutively active transcription factor in uninduced conditions (Roesijadi, 1996). Zinc is needed in the Zn-fingers that form part of the transcription factor structure (Radtke et al., 1993; Brugnera et al., 1994). Direct activation by Zn of the MRFs that will bind to DNA is likely due to Zn binding to the Zn-finger binding sites. Other metals such as Cd, Cu and Hg, which induce MT, do not activate the MRFs directly (Seguin, 1991; Otsuka et al., 1993). Instead, it is believed that induction of MT by these metals takes place by an increase in the intracellular Zn pool due to tis metal's displacement from ligands. In turn, the released Zn activates the MRFs. Cadmium, Cu and Hg, have greater affinities for ligands than Zn and would be expected to displace Zn from other Zn-binding sites through a metal-metal exchange reaction. The displaced Zn is then available for binding the inhibitor, releasing the transcriptional factor from inhibition, and initiating MT expression. The metal-exchange reactions that result in the initial release of Zn from target molecules are considered toxic interactions that precede induction of MT (Roesijadi, 1996).

There are two MRE isoforms for induction of MT, MREa and MREb. Both MREs are located at the proximal 100 base pairs of the gene. Deletion of MREb results

in severe loss of MT activity, whereas deletion of MREa abolishes metal inducibility (Zafarullah, 1983).

Factors influencing metallothionein expression in fish

Metal exposure

Significant research has been focused on the induction of MT by environmental metal exposures in fish. Many studies have shown that an increase in metal exposure will increase MT concentration in tissues such as liver and kidney of fish and in tissues of other aquatic organisms (George et al., 1996; Linde et al., 1999; Hermesz et al., 2001; Vasconcelos et al., 2002; Dallinger et al., 2004). Specifically, MT can be induced by heavy metals, like Cd, Cu, Pb, Zn (Farag et al., 1995), Hg and even by metalloids like arsenic (Pedlar and Klaverkamp, 1999).

Schlenk et al., (1999) showed that exposure of juvenile catfish (*Ictalurus punctatus*) to 1.7 mg/L copper sulfate for 24 hrs under flow through conditions, led to a time-dependent increase in hepatic MT concentrations. Similarly, Galvez et al. (1998) documented that chronic exposure to Ag (28 day, waterborne exposure) induced MT in the livers of juvenile rainbow trout (*Oncorhynchus mykiss*) when Ag concentration was above 2.0 μg/L. When the Ag concentrations were lower (0.5 μg/L), MT levels were not elevated. Some studies suggest that there is a threshold concentration of metals where MT will be induced. For example, Dethloff et al. (1999) showed that at sublethal concentrations of copper and zinc, hepatic metal concentrations and MT levels are not altered compared to control values in rainbow trout (*Onchorhynchus mykiss*).

Male and female catfish (*Ictalurus punctatus*) exposed to waterborne $CuCl_2$ for 60 days with different concentrations (0.6, 1.2, 2.5, 5 and 10 μg/L) showed no mortality at low concentrations and no changes in MT concentrations were observed (Marr et al., 1996). However, when Cu concentrations were increased to 354 μg/L and above, an increase in mortality was observed concurrent with an increase in MT levels. This study also showed that males were more susceptible than females to changes in Cu (Perkins et al., 1997).

Administration of Cd (George et al., 1996; Roesijadi et al., 1997) or Zn to rainbow trout (*Onchorhynchus mykiss*) induced an increase in liver MT concentration (Olsson et al., 1995). It has also been shown that a slight elevation of Cd concentrations in liver induced the sequestration of this metal by displacement of Zn from MT binding sites.

Studies in cultured cells have also shown the inducibility of MT by heavy metals. Using a turbot (*Scophthalmus maximus*) fibroblast cell line, MT mRNA and protein were induced by exposing the cells to 50 μM Cd for 4 days (George et al., 1992). After the exposure period the cells were assayed for MT content using the ^{109}Cd saturation method. At this concentration an increase in MT levels (6 fold) was observed. There was also a significant increase in MT mRNA levels in the Cd exposed cells compared to the controls. The relative increase in MT mRNA was much more marked compared to the changes observed at protein level at the same Cd concentration.

The promoter region of the MT-A gene by Ag and Zn has also been examined using two fish cell lines differing in their ability to produce MT. RTG-2, a cell line derived from rainbow trout (*Oncorhynchus mykiss*) gonadal tissue, express MT while

CHSE-214 derived from Chinook salmon embryonal tissue lacks functional expression of MT. Both cell lines were transfected with a plasmid construct that contained a truncated rainbow trout MT-A promoter region fused upstream of a reporter gene and exposed to various concentrations of Zn (75-250 μM for CHSE-214 and 75-750 μM for RTG-2) or Ag (0.01-2.5 μM for CHSE-214 and 0.5-25 μM for RTG-2). A luciferase activity assay was used to measure the rainbow trout MT-A promoter activity. Both cell lines responded in a dose-dependent manner to either metal exposure. Maximal MT induction was observed at 250 μM Zn and 2.5 μM Ag in RTG-2 cells, whereas in CHSE-214 cells maximum induction was obtained at 125 μM Zn and 1.5 μM Ag. Concentrations beyond the maximal induction showed a decline in luciferase activity, this decline probably indicates cell death or decreased viability of the cells (Mayer et al., 2003).

Common carp (*Cyprinus carpio*) exposed to 1 or 10 mg/L Cd-acetate showed an increase in both MT-mRNA isoforms. MT-II mRNA was induced in liver in a dose-dependent manner. In contrast, MT-I mRNA was induced maximally at 1 mg/L Cd. When the fish were exposed to 10 mg/L, MT-I mRNA induction did not increase beyond the induction observed with the low concentration. There were also differences in the basal levels of both isoforms, where MT-II mRNA was significantly less compared to MT-1 mRNA levels (Hermesz et al., 2001). The differences observed in the basal levels, as well as the induction, of the two isoforms have also been observed in rats injected with 8.9 μmol/Kg $CdCl_2$, where Cd induced more MT-I that MT-II in the liver (Vasconcelos et al., 2002). This suggests that the MT-I gene respond very rapidly and significantly to Cd in the liver.

Stress

In addition to metal exposure, other non-toxicological factors can influence the expression of MT. In experiments where air pumping stress was induced, a time dependent induction of MT in liver and kidney of crucian carp (*Carassius cuvieri*) was observed. The hepatic content of MT after 6 days of air pumping stress was elevated to twice the basal levels measured after 24 hours. The renal content increased less dramatically. This suggests that crucian carp have the ability to produce MTs in response to environmental stress conditions. Production of MT might have been through the release of endogenous factors, such as glucocorticoids (Muto, et al. 1999).

Rearing density, a factor for aquaculture operations, has been shown to induce an increase in stress-related genes in sea bass (*Dicentrarchus labrax*, L.). Fingerling sea bass reared at nominal densities of 80 and 100 Kg/m^3 induced a significant increase in MT mRNA compared to fish reared at lower densities, as shown by northern blot analysis. The induction in MT mRNA coincides with changes in plasma cortisol levels.

The effects of stress on MT induction have also been observed in mice where restraint stress induced as much as a 30 fold increase in MT concentration. In this study, mice were restrained in a well-ventilated polypropylene tube for 12 hrs each day. After just one daily restraint cycle the levels of MT-I and MT-II mRNA were elevated as much as 10-20 fold. Pre-treatment of the mice with RU 486, a glucocorticoid receptor antagonist, reduced MT induction by at least 50%. The results obtained with RU 486, suggest that activation by glucocorticoids plays an important role in MT transcription during stress situations. When restraint cycles were increased to nine, a decrease in MT-I and MT-II mRNA was also observed, pointing to habituation of the animals to the stress

(Jacob et al., 1999).

Chemical stress induced by exposure of roach (*Rutilus rutilus*) to the fungicide procymidone [N-(3,5 dichlorophenyl)-1,2 dimethylcyclopropane-1,2 dicarboximide] increased in MT content in livr tissue. This fungicide is used in the control of *Botrytis* and *Sclerotinia* species in various vegetables, fruits, flower crops and ornamentals. Procymidone has the potential to reach aquatic ecosystems after run-off events. It induces hepatotoxicity at a concentration of 4 mg/L (Paris-Palacios et al., 2003). At lower concentrations (0.4 mg/L and 4 days water exposure) this fungicide induced an increase in MT protein (10 fold induction compared to control animals) and an increase in total hepatic glutathione (2 fold induction compared to control fish). When the fish were placed into clean water a decrease in MT protein was observed after 14 days of depuration. It is not clear what the mechanism is, but it is possible that MT elevation is the result of induction through activation of glucocorticoid response elements in the MT gene (Paris-Palacios et al., 2003).

Age, season, sex and reproductive status

Muller and Prosi (1978) observed an age-dependent metal accumulation of Cd in liver and kidney in the roach (*Rutilus rutilus*). But when fish were collected from an area contaminated with Zn, no age-dependent accumulation of Cd was observed. The Cd accumulated in the older fish exceeded the tolerance limit for human health, and the accumulation of this metal showed a differentiation in organ accumulation (muscle < liver< kidney). In the Zn pollution site, fish also showed a difference in organ accumulation following the same distribution as what was observed with Cd. Douben

(1989) did not find an age-dependent metal accumulation in stone loach (*Noemacheilus barbatulus*) exposed to Cd (80 μg/L). In brown trout (*Salmo trutta*) no differences in metal (Cu, Cd, Zn and Pb) and MT contents between individuals of 1 year and 2 years of age were found (Toledo, 1996; Linde et al., 1999). MT significantly increased in all the fish examined but no differences were found when the data was examined for differences across age groups. The differences observed when comparing these studies can be attributed to the wide range of variation in MT and metal concentrations according to species and organs, depending on age, development status, and other physiological factors (Kägi and Schaffer, 1988; Linde et al., 1999).

In the mollusks *Mytilus galloprovincialis* exposed to Cd (0.5 μg/ml sea water) for a 7-day period, MT was found to be induced in gills, viscera, mantle and the adductor muscle. The induction changed depending on the time of year that the bioassays were performed. MT concentrations reached high levels during the spawning season (May-June for this species) and then showed a sharp decrease in August. This correlated with the differential accumulation of Cd, reaching a peak in May and June (Serra et al., 1999).

The relationship between increasing MT and the reproductive status of fish has been hypothesized to involve the proteins that regulate essential metals such as Zn. During vitellogenesis MT may regulate the pool of available Zn at an appropriate concentration (Olsson et al., 1987). Availability of Zn is important during vitellogenesis because this metal is required for a variety of hepatic enzymes that are active during vitellogenesis. Furthermore, Zn is also needed to stabilize membrane bound organelles which proliferate in the liver tissue of female fish during the production and export of vitellogenin (Olsson et al., 1989).

MT concentrations change in mature female squirrelfish (*Holocentrus adsencscionis*) during a reproductive cycle availability (Thompson et al., 2002). An increase in MT mRNA and MT protein were observed at the onset of sexual maturation compared to immature females. Moreover, a greater proportion of MT protein was found in the nuclear fraction of matured females, compared to immature females where MT was found in the cytosolic fraction. These changes have been attributed to changes in Zn availability (Thompson et al., 2002). Hepatic zinc and circulating levels of zinc in the bloodstream also increase in mature females, as a result of sexual maturity. These changes have not been observed in male fish, where MT levels remain more constant.

Normal variations in hepatic levels of MT and zinc were studied during an annual reproductive cycle in both sexes of rainbow trout (*Oncorhyncus mykiss*) (Olsson et al., 1987). Variations in hepatic levels of MT were found when rainbow trout were studied during a reproductive cycle. This strain of rainbow trout spawns in January-February and vitellogenesis occurs from late September to January. In female fish, the total hepatic zinc levels were closely correlated with circulating levels of estradiol 17β. Zinc levels rose in September (vitellogenesis), peaked in December and dropped in January (ovulation). In male fish, no dramatic peaks were observed when Zn levels were measured, but there was a slight increase in liver MT during the time of spermatogenesis (September).

It is thought that MT may be involved in the cellular localization of Zn during the period of vitellogenin induction. When hepatic zinc content returns to lower levels (March to August) in rainbow trout, a redistribution of Zn at a cellular level is observed, moving from the microsomal and mitochondrial fractions to the cytosolic fraction

(Olsson et al., 1987; Valencia et al., 1998). This corresponds to the time of maximum levels of hepatic MT, suggesting that MT bind the Zn released to the cytosolic fraction. An increase in MT concentration was observed in January (beginning of the spawning), when the redistribution of Zn was also observed. Thus, cytosolic MT levels increase concomitantly with the increase in free Zn that correlated with the end of exogenous vitellogenesis (September).

In squirrelfish (*Holocentrus adsencscionis*) a marine fish of the family Holocentridae, seasonal shifts in the subcellular localization of MT in females were observed by Thompson (2002). Metallothionein was primarily located in the liver cell cytosol in late spring, but co-precipitated with the nuclear fraction during the winter months. These changes were not observed in male squirrelfish, suggesting that this gender specificity is due to the dynamics of zinc and MT accumulation due to changes during the reproductive cycle. Conversely, MT in male squirrelfish liver cells is maintained in the cytosol throughout the year (Hogstrand et al., 1996, Thompson et al., 2002).

Oxidative stress

It is well accepted that MT is involved not only in metal detoxification and homeostasis, but also can mediate oxidative stress (Couillard et al., 1997). It has been shown *in vitro* that MT captures reactive oxygen species through the thiolate cluster to prevent oxidative damage to macromolecules such as lipids, proteins and DNA (D'Auria et al., 2001; Feng et al., 2005). It is also well known that MT releases Zn under oxidative stress conditions in cultured cells. The release of this essential metal causes structural

changes in the protein, specifically by allowing the formation of disulfide bonds (Aizenman et al., 2000; Feng et al., 2005). These bonds are likely intramolecular with both the α- and β-domains being involved in their formation, although the α-domain appears to be more easily oxidized that the β-domain. It is important to note that the release of zinc from MT and the formation of the disulfide bonds occur under physiological conditions, but it has been shown that under oxidative stress more disulfide bonds are found (D'Auria et al., 2001). The mechanism that may lead to the transcriptional activation of the MT gene involves the Zn released from MT protein by H_2O_2. Once released, Zn acts through metal responsive elements (MRE) and metal responsive transcription factor-1 (MTF-1) to increase expression of MT (Chung et al., 2005). The 5' regulatory region of the rainbow trout MTA and MTB genes contains six and four MRE, respectively. MREs in turn provide the binding site for MTF-1, which is known to initiate MT expression.

In rainbow trout (*Oncorhynchus mykiss*) gonadal (RTG-2) and Chinook salmon embryonic (CHSE-214) cells, Kling (2000) showed an increased resistance to H_2O_2 damage when the cells were exposed to 150 μM Zn that induced the expression of metallothionein. In mouse (Dalton et al., 1994) and in catfish, *Ictalurus punctatus* (Schlenk et al, 1997) the promoting regions of the MT-I gene have been shown to possess antioxidant response elements (AREs) that mediate the induction of the MTs by hydrogen peroxide.

Cai (1999) also suggested a possible role of radiation for MT induction. Induction of MT has been considered as one of the mechanisms involved in adaptation to low dose radiation. Induction of MT has been suggested as one of the mechanisms involved in the

adaptation to low dose radiation where MT acts as a free radical scavenger (Cai et al., 1996). MT mRNA expression and MT protein synthesis were induced by *in vitro* exposure of rodent cell lines (Chinese hamster V79 lung fibroblasts) or rat tissues (liver and kidney) to ionizing or UV radiation. It has been shown that radiation-induced MT synthesis is not mediated either by metal redistribution or by the release of glucocorticoids from radiation-damaged tissues (Cai et al., 1999). Several mechanisms have been proposed to mediate radiation induced MT, including formation of lipid peroxides and oxidative cell damage (Hidalgo et al., 1988), the production of cytokines such as interleukin-1, 6 and 8, tumor necrosis factor and interferon (Sato et al., 1993) and induction of protein synthesis mediated by protein kinase C (Haimovits-Firedman et al., 1996).

Temperature

Another factor that has been suggested to influence MT concentration in fish tissues is water temperature. For example, common carp (*Cyprinus carpio*) exposed to 1 hr hypothermia (5°C for 1 to 5 hrs) followed by 1 hr recovery or 2 hr of direct cold shock, showed an increase in MT-I and MT-II gene expression. MT-I was induced to a higher extent than MT-II and the elevated levels of MT-I mRNA persisted longer than the levels of MT-II. In contrast to what was found with the hypothermia, when fish were exposed to hyperthermia (29°C for 1 to 5 hrs) a small change was observed in the expression of both MT genes. Specifically, a two fold induction of MT-I compared to 4-6 fold induction in hypothermia (Hermesz et al., 2001). No changes in MT induction were found when Chinook salmon embryonic cells were incubated at elevated

temperatures (from 20 to 24°C) (Heikkila et al., 1982).

Changes in MT levels in liver and kidney of juvenile rainbow trout (*Oncorhyncus mykiss*) were followed for 8 months concurrent with changes in water temperature (Olsson et al., 1996). Hepatic and renal MT levels were found to increase during the first 4 months as the water dropped from 15 degrees to 4 degrees Celsius. At 2 degrees, MT levels drop even further in both tissues. Rainbow trout (*Oncorhynchus mykiss*) hepatocytes grown at 9°C responded to Zn treatment with an increase in MT protein levels (Hyllner et al., 1989; Olsson et al., 1996b).

Killifish (*Fundulus heteroclitus*) exposed to temperature stress (10°C and 26°C) for 8 days, compared to normal rearing temperature (19°C), had increased MT mRNA in liver, gill and intestine at the high temperature. On the other hand, there were no differences in MT mRNA content in the fish kept at 10°C (Van Cleef-Toedt et al., 2001).

MT induction by exposure to cold stress has also been detected in rat and mouse brown fat tissue (Beattie et al., 2000). The timing of MT induction by heavy metals also appears to be affected by temperature. In mouse, a warm blooded animal, a subcutaneous administration of 2.5 mg/Kg $CdSO_2$ induced maximal induction of MT mRNA within 4 hours of cadmium administration (Durnam and Palmiter, 1981). Furthermore, rats exposed to 4°C for 24 hrs showed an increase in MT in brown adipose tissue (Beattie et al., 2000). Changes in the metabolism of brown adipose tissue allow for the generation of heat. An increase in metabolic rate and utilization of fat depots result in the generation of free radicals due to increased fatty acid oxidation during thermogenesis, the increase in oxidative stress could be the mechanism involved in the upregulation of MT.

The difference in response time might be due to the metabolic rate of the

organism, which may be governed by temperature (Zarafullah, 1989). The differences in induction due to temperature changes between fish and mammals may be explained by the hydropathic index (a parameter that is inversely proportional to flexibility). This parameter is lower in fish MT than in mammalian MTs. Therefore a decrease in the index equals higher flexibility of the molecule and this allows for an increase in conformational changes at low temperatures (Vergani et al., 2005).

Use of metallothionein in biomonitoring

Because of its responsiveness to metals, MT has been proposed as a biomarker for aquatic organisms exposed to environments contaminated with metals. The relevance of this practice has been defended on the basis that toxicity occurs only when compensatory mechanisms are overwhelmed, saturated or damaged by metals. MT actively binds metals, but when the protein's capacity to bind is overcome, a phenomenon termed *spillover* will lead to metal toxicity (Couillard and St-Cyr, 1997). Spillover results when MT sites are completely occupied by metals, so that metal binding to ligands in other intracellular pools increases (Roesijadi, 1996; Couillard and St-Cyr, 1997; Campbell et al., 2005). Binding to these other ligands may induce cellular toxicity. Also, binding of toxic metals to MT implies that constitutive metals, such as essential metals have been removed from the MT molecule (Couillard et al., 1995). Based on this, it has been postulated that high levels of metallothionein in an organism at a metal contaminated site reflect prior exposure, potential toxicity, or acclimation or adaptation of this organism to chronic metal contamination of its habitat (Stegeman et al., 1992).

Roch and McCarter (1984) found a dose dependent increase in hepatic MT

concentration in fish from contaminated lakes, compared to fish from a reference site. In this study Cu was the primary contaminant of interest and MT was almost exclusively bound to the metal. In the most contaminated lake (539 μg/g dry weight) MT was significantly higher (269 nmol/g wet weight). The other lakes showed Cu concentrations of 496 and 196 μg/g, and MT concentrations were 164 and 94 nmol/g respectively. The reference lake had a Cu concentration of 35 μg/g and an MT concentration of 58 nmol/g.

This dose dependent response of MT illustrates the utility of using this protein as a bioindicator for exposure to Cu. That is, the degree of metal contamination in a watershed where no knowledge of the extent of contamination exists can be evaluated by measuring MT concentration and by defining the metal that is predominantly bound to the protein. It was also shown that MT decreased when the quality of the water improved, concurrent with planktonic and bentonic species also recuperating from the effects of the toxic exposure. The improvement of these species could, in fact, be modeled by keeping record of the changes in MT concentration in fish (Roch and McCarter, 1984).

In a study of Lakes Neso and Nesootao in Flin Flon, a mining development in the province of Manitoba, Canada, Klaverkamp et al., (1991) showed an increase in MT concentration in liver and kidneys of white suckers (*Catostomus commersoni*) concomitantly with Cd increases. A decline in plasma electrolyte levels as well as an increase in ascorbic acid was also found in kidney, suggesting that there was an increase in lipid peroxidation with a simultaneous demand for increased antioxidant activities. In Lake Hamell, the closest lake in the Flin Flon area to the smelter, white suckers (*Catostomus commersoni*) exhibited a high resistance to Cd toxicity because of elevated

hepatic, renal and branchial MT concentrations. Even though there was an increase in tolerance to Cd, it was not enough to maintain the fish population in this lake. A decrease in spawning success, reduced larval and egg survival, smaller egg size and reduced longevity eventually occurred (Klaverkamp et al., 1991). These changes occurred after MT concentrations increased in fish, again demonstrating the utility of MT as a predictive bioindicator for higher level effects.

An experiment in which Cd was added to a whole lake at sublethal concentration was used to evaluate the effect on MT in wild fish. Lake 382 is part of the Experimental Lakes Area (ELA) that constitutes an aquatic preserve of 58 small lakes in the Precambrian shield located in Northwestern Ontario, Canada. These lakes have been designated as a research area to study fate and effects at the ecosystem level of anthropogenic perturbations. Beginning in 1987 and for 5 years thereafter, Cd was added to the lake reaching a concentration of 200 ng Cd/ L. During Cd addition, metallothionein concentrations were monitored in Lake 382. Cadmium was added to this lake in order to follow the response of white sucker (*Catostomus commersoni*) and lake trout (*Salvelinus namaycush*) to see if they responded to temporal increases in Cd exposure through water and sediment. Metallothionein levels in liver; kidney and intestine of the two fish species increased throughout the 5 year duration of the experiment, and remained elevated even after the cessation of Cd addition. Metallothionein concentrations were not elevated in organs of fish from a nearby reference sites during the same period (Palace et al., 1993). Induction of MT at low concentrations in fish from lake 382 shows the utility of MT as a bioindicator of low level metal exposure.

Fish communities found in Peace, Athabasca and Slave rivers and their tributaries are exposed to pulp and paper, municipal and industrial effluents. A study by Klaverkamp et al. (1996) showed that there was a 4 fold increase in MT concentration in the livers of burbot (*Lota lota*) collected downstream from the exposure sites compared to fish collected upstream the exposure sites. There was also a strong positive relationship between hepatic Cd, Cu and Zn and MT concentration. These results are consistent with the idea that concentration of MT in burbot liver respond in a dose-dependent manner as a function of metal bioavailability in the natural environment.

Methods to determine metallothionein concentration

Metal saturation methods

Metal saturation methods are based on the principle that when an excess of high affinity metals are incorporated in to a sample, the added metal displaces the originally bound metals from MT. In these methods, tissues are typically homogenized and then centrifuged to isolate the cytosolic fraction. A high affinity metal is then added to displace all the metal originally present in MT. Any excess non-specifically bound metal is removed from the reaction vessel by adding an excess of an exogenous protein (eg. albumin) for which the affinity for the metal is high. The MT-metal complex is then precipitated by heating or by lowering the pH of the mixture. After centrifugation of the preparation, the supernatant contains MT bound to the added metal. The metal concentration is then measured by flame atomic absorption spectrophotometry or by gamma spectrometry if a radioactive gamma emitting metal isotope has been employed.

A molar binding capacity can be calculated by relating nmol metal binding sites per g of tissue.

Hg-saturation method

For the Hg saturation method, samples are incubated with an excess of ^{203}Hg in the presence of 10% trichloroacetic acid. In acidic conditions, Hg has a higher affinity for MT compared with other metals. Therefore, Hg effectively displaces other metals bound to the MT protein. An exogenous protein (chicken egg albumin or mammalian hemoglobin) is used to scavenge Hg in the vessel that is bound to cytosolic ligands other than MT (Klaverkamp et al., 1991; Couillard et al., 1993; Dutton et al., 1993; Wang et al., 1999) during a cleanup stage. This method has a detection limit of 111 ng of MT and in terms of technical ease; this method has 6 steps that consume only 16 minutes of preparation and assay time. The method is accurate, simple, specific and rapid and can be standardized with commercially available MT (Dutton et al., 1993).

However, the use of radioactive materials always presents some disadvantages. Radioisotopes are usually expensive and may not be available at all times. Moreover, the use of radioisotopes requires special equipment and personal protection, as well as continuous monitoring to prevent contamination inside and outside the laboratory.

Metallothionein is susceptible to oxidation and to degradation by proteases during tissue homogenization, so special care has to be taken when preparing tissue for MT determination by Hg saturation. Whole organisms, or specific organs can be used; a minimum of one gram of fresh tissue is usually necessary to perform MT analysis. Homogenization is performed manually on fresh tissue using a glass homogenizer and

Teflon pestle and a minimum number of strokes in order to disrupt the cellular contents. Homogenization buffers must be isotonic with the tissue and are often homogenized under an atmosphere of N_2 or argon, and on ice. The use of antioxidant agents (DTT, mercaptoethanol) are not recommended as they may cause redistribution of metals among cellular components and dissociation of natural MT dimers during isolation procedures (Klaverkamp et al., 2000).

Tissue homogenates, supernatant fractions or fresh tissue samples are stored at -20°C or lower temperature until analysis by Hg saturation assay. Repeated freeze/thaw cycles should be avoided as the reproducibility of results can be affected. However, homogenates and supernatants can be stored after they are carefully purged with nitrogen. Under adequate storage conditions, MT concentrations are normally stable for months or years (Suzuki, 1992).

Cd-saturation method

Because of its relative affinity for MT, the Cd-saturation method is useful for determining Cu-containing metallothionein (Summer et al., 1991). In this method high molecular weight proteins are precipitated using acetonitrile and removal of MT-bound to Cu is done by adding ammonium tetrathiomolybdate. The excess thiomolybdate and the Cu complex are removed using an anion exchanger like DEAE-Sephadel. The sample is then saturated with ^{109}Cd. Excess Cd is removed from the assay vessel with a cation exchanger (Chelex-100). Oxidation of MT is avoided using N_2-saturated solutions and by performing the test in an atmosphere of argon. The reported detection limit for this assay was 14 ng of MT.

Ag-saturation method

Similar to the Hg and Cd saturation assays, MT can also be incubated with excess Ag at pH 8.5. However complete copper displacement from MT by Ag has not been well documented. As a result, the Ag saturation may yield unreliable results (Gagne, 1991; Del Ramo et al., 1995).

Differential pulse polarography

The basis of this method is the detection of the change in electrical current that occurs when a metal is oxidized or reduced. For MT, the signal arises primarily from the reduction of hydrogen in the protein sulfydryl groups. The advantage over metal saturation assays is the smaller amounts of tissue that can be utilized (100-200 mg of tissue). Moreover, a detection limit of only 7.2 μg MT per g of tissue has been reported for yellow perch liver (Hogstrand and Haux, 1992). Another advantage of this method is that the metal composition of MT does not influence the analysis. Specificity is highly dependent on sample preparation. Heating and centrifugation of the homogenates/cytosol extracts and the use of an ammonium buffer containing Co (Bradicka buffer) make it possible to quantify the thiol groups of MT with negligible interference from other sulfydryl-containing compounds.

Immunological methods

Measuring MT with immunological methods requires the use of antibodies raised against the protein. The antibodies are obtained by injecting the antigen, in this case MT,

into an animal species that is different from the one that is studied. This organism will produce antibodies against this foreign protein. MT is induced in the test organism by injection of $CdCl_2$. Liver MT-1 and MT-2 isoforms are isolated and purified from tissue homogenates, the bulk removal of liver proteins is achieved by heat treatment, organic solvent extraction or by a combination of these treatments. Chromatography (HPLC) is performed to separate the isoforms, and the purity of the isoforms is assessed by both polyacrilamide gel electrophoresis (PAGE) and amino acid compositional analysis. The purified protein is then emulsified with Freund's adjuvant and used to immunize rabbits. Injections are made into multiple sites that include subcutaneous, intramuscular and intraperitoneal routes for both soluble and insoluble polymers; additionally, the intravenous route is used for soluble polymers. The serum obtained from a preinjection bleeding and from each of the bleedings throughout the complete course of initial and booster injections is obtained and kept separated, avoiding contamination or denaturation. The serum is purified using glutaraldehyde and then dialyzed against borate buffered saline. The soluble polymer of MT obtained as the product of the above mentioned reaction is then used as immunogen in the different immunological methods (Garvey, 1991).

Radio Immuno Assay (RIA)

Antibodies raised against MT can be used in Radio Immuno Assays (RIA). Typically rabbit anti-MT globulin, is used as the first antibody, and a goat anti-rabbit immunoglobulin is then bound to the first antibody for detection purposes. ^{125}I-labelled and the native MT both compete for a limited amount of rabbit anti-MT antibodies. As

the number of native MT molecules increases, a decreasing portion of the labeled MT will be bound to the antibodies. Thus, the larger the amount of MT in the unknown sample, the smaller the amount of ^{125}I bound to the rabbit anti-MT antibodies. The antibody-antigen complex is then precipitated with a secondary antibody (goat anti-rabbit immunoglobulin), and analyzed for ^{125}I. RIA is thought to be the most sensitive method for MT detection, with a detection limit of 1 to 300 ng of MT (VanderMallie and Garvey, 1979; Hogstrand and Haux, 1989b). A disadvantage of this method is that it is technically difficult and appreciable amount of the MT to be analyzed has to be purified to produce a ^{125}I-MT tracer. Precise calibration curves are also essential. The assay is also time consuming, including periods of incubation lasting 72 hrs in total. Finally, use of an internal MT standard for quality control is difficult with this assay (Couillard et al., 1993).

Enzyme linked immunosorbent assay (ELISA)

ELISA is another emerging technique for measuring MT. A double-antibody procedure is used, where goat anti-MT IgG serves as the primary antibody binding to MT. Rabbit anti-goat IgG conjugated to horseradish peroxidase is then bound to the primary antibody to allow quantification. Reference MT antigen is plated on wells of a microtiter plate and the plated MT and competing antigen in the tissue being analyzed compete against each other for a limited number of anti-MT antibodies. As the number of native MT molecules increases, a declining portion of reference antigen is bound to the antibodies. Plates are washed with a buffer containing Tween-20 after the first incubation to eliminate excess antibodies. The primary antibody bound to the

immobilized reference antigen is incubated in the presence of the secondary antibody, which is conjugated to the horseradish peroxidase. Finally, a chromophore that reacts with the peroxidase is added, and the disappearance of the substrate is monitored by colorimetry (Roesijadi et al., 1988). This is a very sensitive assay with a working range of 1-21 ng of MT. However, similar to the RIA assay, the ELISA procedure for MT is time consuming and complex. Antibodies are not yet readily available, and the independent production of antibodies is an ardouos process. Calibration curves are normally run in each assay. The assay includes periods of incubation lasting up to 40 hrs in total.

Western blot analysis

Western blot analysis has become a standard method for determination of cellular proteins. This method is based on the migration of proteins through a polyacrylamide gel using electrophoresis, and the detection of the proteins using antibodies raised against them. Once the proteins have been separated by SDS gel electrophoresis, they can be transferred to a nitrocellulose or Polyvinylpyrrolidone (PVDF) membrane that is incubated with the primary antibody (anti MT). The excess primary antibody is washed with a buffer containing tween-20 as a detergent, and the membrane is then incubated with the secondary antibody (anti rabbit conjugated to horseradish peroxidase). The excess secondary antibody is also washed with the same buffer, and the membrane is exposed to a detection reagent that will emit light which is visualized through autoradiography film (Conner and Fowler, 1994). This technique has a detection limit of

0.1 μg, but an advantage to the use of this method is the ability to obtain a separate band for each of the MT isoforms (Aoki et al., 1991).

High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography is a technique widely used to separate compounds from a complex mixture. HPLC separation of MT can be done using a variety of methods, including gel permeation, ion exchange, reversed phase and a combination of gel permeation and ion exchange via column switching (Richards, 1991). Moreover, by directing the effluent from an HPLC column directly into the nebulizer of an atomic absorption spectrophotometer, the metal composition of the separated fractions can be analyzed. Sample preparation and analysis using HPLC is time consuming, a routine HPLC run can take up to 40 minutes per sample. Furthermore, the requirement of adequate purified standards poses another possible problem, as only MT standards obtained form rabbit and horse are currently commercially available. While standards can be prepared from organisms exposed to heavy metals, like Cd or Zn to over-express MT, preparation is costly and time consuming and the proteins need to be separated and purified to be useful as standards.

Detection of metallothionein mRNA

In the last 25 years there has been increasing interest in the quantification of mRNA as a way to detect early changes in gene expression. This is particularly useful when early detection of exposure to contaminants is required. There are at least three methods that have been used to determine MT mRNA abundance in different tissues.

This is possible due to the increasing number of species for whom the metallothionein genes have been sequenced.

Northern blot analysis

This technique takes advantage of the separation of RNA molecules based on charge migration in a denaturing gel. Once separated, the RNA molecules are capillary transfered to a nylon or nitrocellulose membrane. The RNA of interest is then identified by hybridization with a radioactive or chemiluminescent probe and visualized by autoradiography or photography. Northern blot analysis of Tilapia (*Oreochromis mossambicus* and *Oreochromis aureus*) exposed to various metals including Cu, Cd, Ni, Pb, Hg and Zn showed a 4 fold induction in MT mRNA in vivo, with Zn showing the highest induction compared to the other metals (Cheung et al., 2005). Northern blot analysis in Ayu (*Plecoglosus altivelis*) exposed to Zn also showed an increase in induction of MT mRNA in liver, kidney and intestine (Lin et al., 2004). Studies in different species (Carginale et al., 1998; Mayer et al., 2003; Chan et al., 2004) have shown the utility of this technique in the identification and quantification of MT mRNA.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Polymerase chain reaction or PCR is a technique used to amplify a segment of DNA that lies between two regions of a known sequence. Two oligonucleotides are used as primers for a series of synthetic reactions that are catalyzed by a DNA polymerase. The primers have different sequences and are complementary to the sequences that lie on opposite strands of the template DNA and flank the segment of DNA that is to be

amplified. The template DNA is first denatured by heating in the presence of the primers and the four dNTPs. After denaturation the reaction is cooled to allow the annealing of the primers to their target sequence. Once annealed, the primers are then extended by the polymerase. This cycle of denaturation, annealing and DNA synthesis is performed many times, and because the products of one round can serve as templates for the next, each successive cycle essentially doubles the amount of the target gene (Maniatis, 1982).

To study mRNA abundance, once the total RNA is isolated from the tissue, the RNA is reverse transcribed using the murine leukemia virus or the avian myeloblastosis virus to obtain cDNA which then can be used for the amplification of the desired sequence. The PCR products obtained after the cycling are separated in an agarose gel and the amount of target gene quantified by densitometry.

Real-Time Polymerase Chain Reaction (Real-time PCR)

Real time PCR monitors the change in fluorescence emitted during the PCR reaction and functions as an indicator of amplicon production in real time. One of the disadvantages of this method is that amplicon size is not quantifiable, and therefore it does not allow for the differentiation between DNA and cDNA amplification. Because real-time PCR is based on the detection and quantification of a fluorescent reporter, the signal obtained increases in direct proportion to the amount of PCR product obtained in each cycle of the reaction. The higher the starting copy number of the target, the sooner a significant increase in fluorescence will be obtained.

As for PCR, in order to measure the amount of mRNA present in a sample, it is necessary to reverse transcribe the total RNA in the sample to obtain cDNA. Primers

targeting the desired sequence have to be designed, and a fluorescence system needs to be chosen. There are three main fluorescence chemistries: 1) hydrolysis probes; 2) hybridizing probes; and 3) DNA-binding dyes. Hydrolysis probes are oligonucleotides that contain a fluorescent dye on the 5' end and a quenching dye on the 3' end. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching molecule, this is called FRET (Förster or fluorescence resonance energy transfer). The close proximity of the reporter and quencher prevents emission of fluorescence while the probe is intact. When the DNA polymerase replicates a template containing a hydrolysis probe, the 5' exonuclease activity cleaves the probe ending the quencher activity and resulting in the emission of fluorescence by the reporter dye. The DNA binding dyes use non-sequence specific fluorescence using the intercalating agent SYBR green I. SYBR green does not bind to single stranded DNA, but is a fluorogenic minor groove binding dye that exhibits little fluorescence when in solution but emits a strong fluorescence upon binding to double stranded DNA.

Research Needs

Metallothionein has been proposed as a bioindicator for metal exposure in aquatic systems. Although a dose relationship between metallothionein protein and mRNA and metal concentration has been found, there are other factors that will induce MT synthesis including hormones, cytokines, oxidative stress, glucocorticoids, etc. Changes in the reproductive status and differences in developmental stages have also been shown to alter the levels of this protein. Therefore, in order for MT to be useful in environmental

monitoring, it is vital that information on the normal regulation and function of this protein is available.

Important for the development of strategies to define the use of a bioindicator is the development of adequate methods to quantify protein and/or mRNA levels of the bioindicator in question. Many methods have been developed to quantify changes in MT, but the choice of method depends on specificity, ease of use, cost effectiveness, and system availability.

Four methods were developed to evaluate changes in MT protein concentration and MT mRNA induction in lake trout (*Salvelinus namaycush*) exposed to Cd 20 years ago and to the endocrine disruptor ethynylestradiol (EE2). Moreover, this fish were also followed during a reproductive cycle to determine to what extent the changes in MT protein and mRNA are affected during a reproductive cycle and the effect of these changes in the handling of contamination exposure.

The development of the methodology used to quantify MT provides an overview of the advantages and disadvantages associated with the methods developed here.

Chapter 2

Preliminary study.
Waterborne ethynylestradiol induces vitellogenin and alters metallothionein expression in lake trout (*Salvelinus namaycush*)

Abstract

Estrogenic contaminants isolated from waters receiving sewage treatment plant effluents are known to induce the egg yolk precursor vitellogenin (VTG) in male fish. Levels of the metal binding protein metallothionein (MT) have also facilitated transfer of the essential metal Zn to cellular components required for VTG synthesis. To examine the changes in MT and VTG concentrations in fish exposed to an estrogen contaminant, lake trout (Salvelinus namaycush) were exposed to waterborne ethynylestradiol at 0, 4, 40, 400 ng/L^{-1} for 21 days. Blood and tissues were collected after 21 days of exposure to measure circulating levels of VTG as well as MT concentrations in liver and kidney. VTG increased in male and female fish from all three exposure groups compared to control fish. MT in liver significantly decreased in males and females compared to controls, in the two highest exposures. MT in kidney was significantly haigher in both sexes of fish exposed to the two highest concentrations of ethynylestradiol. These data are supportive of a relationship between estrogen exposure and the regulation of MT. Further studies to examine the specific links between estrogen exposure, VTG induction and regulation of essential metals like Zn are required.

Introduction

Contaminants with estrogenic activity including estradiol, estrone, ethynylestradiol, nonylphenols and phytoestrogens (Gagne and Balise, 1998), as well as polycyclic aromatic hydrocarbons (PAH's) (Anderson et al., 1996) have been identified in water bodies that receive effluent from sewage treatment plants (Harries et al., 1996, 1997; Belfroid et al., 1999; Solè et al., 2000). Effluents from a Brazilian sewage

treatment plant had concentrations of 0.021 μg/L 17β-estradiol and 0.04 μg/L estrone while 17α-ethynylestradiol (EE2) has been found at concentrations of 7 ng/L in the UK and up to 17 ng/L in Germany (Belfroid et al., 1999). Canadian sewage treatment plants have been found to contain 8 ng/l estrone, 8 ng/L 17β-estradiol and 0.9 ng/L 17α-estradiol (Ternes et al., 1999). While treatment plants have been shown to eliminate estradiol by 78% and estrone up to 83% (Ternes et al., 1999), significant concentrations of estrogenic compounds are still found in many downstream waters.

Estrogenic compounds are known to have many biological effects in fish. Among these are delayed age to maturity, reduced gonad size, smaller eggs, and altered levels of reproductive hormones (McMaster et al., 1991; Munkittick et al., 1991; Arcand-Hoy and Benson, 1998; Carlson and Williams, 1999; Arukwe et al., 2000; Folmar et al., 2001a; Schultz et al., 2001). An increase in the production of the yolk precursor, vitellogenin (VTG), in male fish has been widely used as an indicator of exposure to environmental estrogens (Sumpter and Jobling, 1995). Vitellogenin is a glycophospholipoprotein produced in the liver of mature females in response to increasing circulating estrogen levels leading up to spawning. Binding of estrogen to the estrogen receptor in hepatocytes initiates this response (Tenniswood et al., 1983; Norberg, 1995; Donohoe and Curtis, 1996; Carlson and Williams, 1999; Arukwe et al., 2000). Once released from the liver cells, VTG is taken up by the maturing oocytes and cleaved to produce lipovitellins and phosvitins, the primary yolk proteins (Islinger et al., 1999). Although male fish do not normally produce VTG, the hepatic estrogen receptor and the gene that encodes for VTG is present (Maitre et al., 1985; LeGuellec et al., 1988). When male fish are exposed to estrogen or estrogenic contaminants, VTG concentrations in plasma can

be induced by several thousand folds (Purdom et al., 1994; Sumpter and Jobling, 1995; Folmar et al., 1996; Harries et al., 1996, 1997; Lye et al., 1997; Orlando et al., 1999; Schultz et al., 2001). However, in male fish VTG can not be incorporated into the gonad, and it has been suggested that VTG accumulation in plasma may lead to liver and kidney damage (Herman and Kincaid, 1988; Gagne and Blaise, 1998; Folmar et al., 2001a).

VTG requires zinc as an essential cofactor for its production. The metal is required for membrane and polyribosome stability and for many components of the protein synthesis mechanisms (Valle, 1991). It is also used for the allosteric regulation of some enzymes (Olsson et al., 1995). At the end of vitellogenesis, Zn is mobilized from high molecular weight proteins (Olsson et al., 1989) to the metal binding protein metallothionein (MT) in the liver. Metallothionein is a low molecular weight protein whose function is thought to include regulation of essential metals, including Zn. Metallothionein concentrations in the liver have been shown to decline prior to spawning and during vitellogenesis (Olsson et al., 1987; Banks et al., 1999), suggesting that the protein is important in mediating the transfer of Zn to and from components that are essential for VTG production. Significant MT concentrations are also present in the kidney, but alterations to the amounts of the MT protein in response to estrogens are less clear for this organ in fish.

In this study we have examined the relationship between VTG induction and the expression of MT protein in fish exposed to estrogens. Specifically, VTG was determined in plasma and MT was measured in liver and kidney of lake trout after 25 days of waterborne exposure to environmentally relevant concentrations of EE2, and estrogenic compound found in waters receiving treated waste water.

Material and Methods

Fish

Immature lake trout (625 ± 15 g) were randomly distributed into eight 200 L fibreglass tanks (8 fish per tank; 2 tanks per dose group), and acclimated for 14 days. During this time, each tank received 1 L of aerated and de-chlorinated Winnipeg City tap water per minute. Temperatures were maintained between 11.5 and 13.1°C and dissolved oxygen was at least 90% saturation at all times. During the acclimation and dosing periods of the experiment, fish were fed a diet of commercial dry pellet feed at a ration of 1% body weight per day and were kept on a 12 hour light : 12 hour dark light schedule.

Ethynylestradiol treatment

17α-Ethynylestradiol (EE2), purchased from Sigma Chemical Company (St. Louis, MO) was used to prepare a stock solution (1mg/ml). Three separate dosing solutions were prepared by diluting an appropriate amount of the stock solution into 2 L of HPLC grade absolute ethanol in glass containers. After thorough mixing of the stock solution into the ethanol, 18 L of distilled deionized water (DDW) was added for a final stock solution volume of 20 L. A solvent control solution consisting of 2 L of ethanol and 18 L of DDW was also prepared for pumping in the control tank. All solutions were maintained at room temperature and protected from light for the duration of the experiment to prevent photodegradation of EE2 (K. Kidd, unpubl. observ.).

Following the acclimation period, 200 ml of the appropriate EE2 dosing solution was introduced into each 200 L tank to immediately bring the EE2 or ethanol to the appropriate concentration, based on the required 1 ml/min^{-1} pumping rate. Pre-calibrated

peristaltic pumps were then simultaneously started to deliver 1 ml/min^{-1} of stock solution into each tank to maintain nominal EE2 concentrations (0, 4, 40 and 400 ng/L^{-1}) continuously for 21 days. Water samples taken from each tank were analyzed twice during the exposure period to determine the actual EE2 concentration in each tank using a standard radio immuno assay technique as previously described (Palace et al., 2001). Mean concentrations from these two measurements were 15, 35 and 373 ng/L^{-1}, respectively. No EE2 was detected in the water from the control tanks.

After the exposure period, fish were anaesthetised by immersion in pH buffered tricaine methanelsulphonate (MS222) (0.8 g/L, pH 7.0). When all fin movements had ceased (<3 min), the fish were removed from the anaesthetic, blotted dry, weighed and measured. Blood was obtained from the caudal vein with a pre-heparinized syringe (50,000 U/ml) and centrifuged at 3,000 x g for 10 min to obtain plasma, which was frozen at -90°C and protected from light until analyzed. Condition factor (CF=[Total Wt/L^3] x 100) was calculated to evaluate if EE2 has a direct effect on growth. Liver and gonad tissues were dissected out and weighed to obtain liver somatic index (LSI=[Liver Wt/Body Wt] x 100) and gonad somatic index (GSI=[Gonad Wt/Body Wt] x 100). Liver and kidney were frozen on dry ice in sterile bags before storage at -90°C until analysed.

Vitellogenin determination

Concentrations of VTG were determined in plasma using a competitive ELISA assay. VTG was purified from plasma of vitellogenic lake trout from the same stock and strain (Molecular Biomarkers Core Facility, University of Florida). Briefly, 96 well microplates were pre-coated with lake trout VTG 5.0 µg/ml in 50 mM carbonate buffer,

pH 9.6, followed by blocking of the unbound sites with 5% normal goat serum. To determine VTG, lake trout plasma samples were incubated with a killifish VTG monoclonal antibody (Molecular Biomarkers Core Facility, University of Florida) diluted 1:500 in phosphate buffer pH 7.3 containing 0.5% Tween-20 (PBS-T) for 1 hour at 37°C. Preincubated samples were added to the VTG-coated wells and incubation continued for 1 hour, followed by washing to remove unbound antibodies. A horseradish peroxidase-conjugated secondary antibody (1:8,000) was added to the plates followed by a 1 hour incubation at 37°C. Detection was performed using TMB substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MA). The reaction was stopped after 5 minutes by the addition of 1M phosphoric acid. The change in color was read at 450 nm using a Cambridge Model 750 microplate reader (Cambridge Technology Inc., Watertown, MA). Amounts of VTG were determined by comparison to a standard curve constructed using purified lake trout VTG (Denslow et al., 1997).

Determination of metallothionein

Metallothionein concentrations were determined using the mercury saturation method described by Klaverkamp et al. (2000). Briefly, liver and kidney samples were homogenized in 0.9% NaCl. One gram of the homogenate was heat treated for 5 min at 95°C, cooled on ice for 4 minutes and centrifuged at 10,000 x g for 5 minutes. The supernatant was retrieved and mixed with the working stock (2 mCi ^{203}Hg in 0.1 N HCl and 50 μg/ml HgCl in 20% TCA) while vortexing at high speed, followed by a 10 min incubation. To remove the unbound ^{203}Hg, a 50% egg white solution was added, followed by centrifugation at 10,000 x g for 3 min. Activity of the bound ^{203}Hg was

determined using a LKB-Wallac 1282 Compugamma γ counter (Fisher Scientific) for 10 min. The equivalent MT in each sample was determined by comparison to a standard curve constructed using commercially available rabbit MT standard (Sigma Chemical Company, St Louis, MO). Kalverkamp et al. (2000) showed strong and consistent correlations between Hg binding in this assay and the content of metallothionein protein. They also showed negligible non-specific binding of Hg to other proteins.

Data analysis

All data are presented as Mean ± SEM. Group means were compared using a one way ANOVA followed by Dunnett's and Tukeys multiple comparison tests. Statistical significance was accepted at $p<0.05$.

Results and Discussion

Somatic parameters

Somatic parameters for control and treated fish are shown in Table 1. There were no significant differences in weight or body length between any of the treatments. Condition factor was similar for all of the groups at the end of the exposures, indicating that EE2 treatment did not affect growth rate during the short duration of the experiments. An increase in liver somatic index (LSI) was observed in both males and females of the highest treatment groups (40 ng/L and 400 ng/L). This increase was more pronounced in males that in females (159% and 290% in males; 88.6% and 135% in

Table 2.1 Mean somatic parameters in control and EE2 treated lake trout. Liver somatic index (LSI), gonad somati index (GSI).

Sex	n	EE2 (ng/L)	Weight (g)	Length (cm)	Condition Factor	LSI	GSI
Male	9	0	634.5 ± 28.1^{a}	38.1 ± 0.7^{a}	1.14 ± 0.03^{a}	0.44 ± 0.03^{a}	0.73 ± 0.15^{a}
Female	7	0	579.1 ± 33.8^{a}	36.6 ± 0.7^{a}	1.17 ± 0.02^{a}	0.70 ± 0.10^{b}	2.87 ± 1.16^{b}
Male	8	4	666.2 ± 56.0^{a}	41.5 ± 1.3^{a}	0.92 ± 0.04^{b}	0.65 ± 0.04^{b}	0.35 ± 0.14^{c}
Female	8	4	670.2 ± 52.2^{a}	42.5 ± 0.8^{a}	0.89 ± 0.10^{b}	0.75 ± 0.04^{b}	4.15 ± 3.46^{d}
Male	10	40	653.9 ± 63.9^{a}	41.3 ± 1.3^{a}	0.94 ± 0.08^{b}	1.14 ± 0.04^{d}	0.31 ± 0.06^{c}
Female	6	40	602.5 ± 38.5^{a}	39.2 ± 0.8^{a}	1.01 ± 0.09^{a}	1.32 ± 0.07^{d}	4.58 ± 3.37^{d}
Male	13	400	604.4 ± 35.0^{a}	39.6 ± 1.3^{a}	1.01 ± 0.09^{a}	1.72 ± 0.04^{e}	0.23 ± 0.03^{c}
Female	3	400	635.3 ± 81.6^{a}	38.6 ± 0.4^{a}	1.10 ± 0.15^{a}	1.65 ± 0.16^{e}	7.29 ± 1.48^{e}

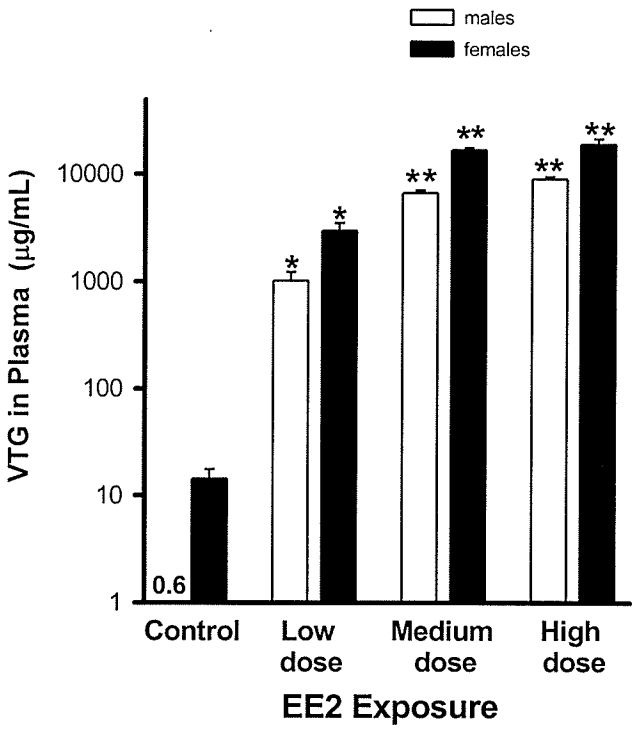
Means with different superscript letters are significantly different from each other based on ANOVA followed by Tukeys comparison test (p=0.05).

females respectively). The increase in liver weight in fish from these groups is likely due to an increase in the synthesis of VTG (Carlson and Williams, 1999; Gerpe et al., 2000). The elevation in LSI in female fish was attenuated relative to males at the higher treatment groups. This attenuation was probably due to the ability of the females to sequester plasma VTG in the oocytes (Van Bohemen et al., 1982; Arukwe et al., 2000, Schultz et al., 2001). It should be noted that there is a significant increase in GSI in females from the low and medium dose groups relative to the controls and an even higher GSI in females from the high dose group (Table 1). No such increase was observed in the males exposed to EE2. In fact, all of the EE2 exposed males had significantly smaller GSI compared to the reference fish. It has been previously reported inhibited testicular development in wild fathead minnows (*Pimephales promelas*) exposed to EE2 (Palace et al., 2002).

Vitellogenin analysis

It is well known that VTG production is induced in fish exposed to estrogenic compounds (Schultz et al., 2001; Folmar et al., 2001a; Arukwe et al., 2000). In this study, the plasma of control male fish had a negligible amount of VTG (0.6 μg/ml) compared to females (14.3 μg/ml) (Fig. 2.1). At the low EE2 exposure, males and females had a significant increase in VTG concentration (1021.6 μg/ml and 2974.1 μg/ml, respectively). VTG concentrations were again significantly higher in fish from the medium and high exposures when compared to the low and control groups, but were not significantly different between these two groups (6672 μg/ml and 9057 μg/ml for males, 16845 μg/ml and 19097 μg/ml for females, respectively).

Figure 2.1 Vitellogenin concentrations in plasma of lake trout exposed to waterborne ethynylestradiol (0, 4, 40 or 400 ng/L respectively). * Denotes significantly different from the control group based on ANOVA followed by Dunnett's and Tukeys (p=0.05). ** Denotes significant differences between dose groups based on ANOVA followed by Dunnett's and Tukeys (p=0.05).


males
females
VTG in Plasma (µg/mL)
10000
1000
100
10
1
0.6
*
*
**
**
**
**
Control
Low dose
Medium dose
High dose
EE2 Exposure

Induction of VTG has been observed in other fish exposed to EE2. In juvenile rainbow trout exposed to a sewage treatment plant effluent containing 105 ng/L EE2, an increase in VTG production was observed, in these fish levels of plasma VTG reached 1.5 mg/ml (Larsson et al., 1999). Sole et al. (2000) also reported a significant increase in VTG when carp (*Cyprinus carpio*) were injected with 500 μg/Kg EE2. VTG induction was observed in males and females, where 90 and 67 fold increases, respectively, were observed.

Metallothionein analysis

Although the induction of VTG by estrogen is well established, the effect of estrogens on metallothionein in fish is not fully understood. Lake trout exposed to the high and medium EE2 concentrations, showed a decrease in MT concentration in the liver compared to the low EE2 exposure and the control group ($p \leq 0.05$) (Fig. 2.2). It has been suggested that MT concentrations decline in the liver of females as spawning approaches (Banks et al., 1999) to facilitate the release of Zn that is required to stabilize membrane structures in the liver. These membranous components are used to produce and export the primary yolk precursor vitellogenin from the liver (Olsson et al., 1989). No effects on MT concentrations were seen at the low exposure dose in this experiment. This may have arisen from the relatively short exposure time of this study or may indicate that the low dose was below the threshold for inducing effects on MT. It should be noted that the increase in liver size associated with vitellogenin production in fish from this study could have contributed to dilution of liver metallothionein. This effect would not be expected in kidney.

Figure 2.2 Metallothionein concentrations in livers of lake trout exposed to waterborne ethynylestradiol (0, 4, 40 or 400 ng/L respectively). * Denotes significantly different from the control group based on ANOVA followed by Dunnett's and Tukeys (p=0.05).

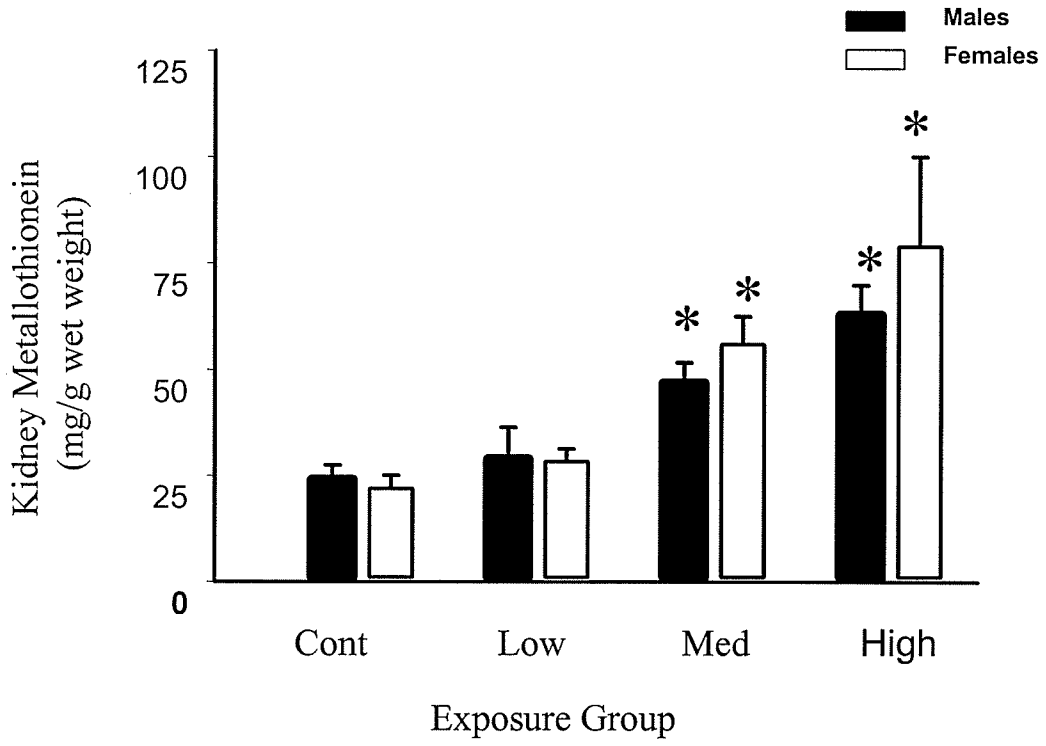

Males
Females
Liver Metallothionein
(µg/g wet weight)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
*
*
*
Cont
Low dose
Med dose
High dose
Exposure Group

Whereas liver MT concentrations were lower in the two groups of fish exposed to the highest concentrations of EE2 in this experiment, kidney levels of the protein were significantly higher in these two groups compared to the control and low exposure groups (Fig. 2.3). Studies have shown that MT concentrations in the kidney of mammals exposed to estradiol are induced indirectly as a result of increased metal accumulation in that organ (Nishiyama et al., 1987). A previous study in arctic char (*Salvelinus alpinus*) showed that kidney MT mRNA was not affected when the fish were exposed to two estrogenic contaminants for 3 days (Gerpe et al., 2000). The differences in our results from that of Gerpe et al. (2000) might be from the different times of exposure to the estrogenic contaminants. The longer exposures in our experiments (25 days) could provide ample opportunity for remobilization of metals from the liver to the kidney, and hence, allowed secondary induction of MT in that organ compared to the relatively short exposure duration (3 days) in the experiments performed by Gerpe et al. (2000).

Summary and Conclusions

The results of these experiments support previous results from our laboratory that showed altered tissue MT levels in fish exposed to an estrogen compound (Palace et al., 2001). In that study lake trout were injected with 5 mg/Kg 17β-estradiol and exhibited a decrease in liver metallothionein with a concomitant increase in kidney metallothionein. Results from this study show that the effects observed with waterborne EE2 exposure are similar to those obtained with the native circulating hormone. We have demonstrated that concurrent with an increase in circulating VTG, there is a significant decrease in liver and increase in kidney MT concentration. However, the mechanism by which

Figure 2.3 Metallothionein concentrations in kidneys of lake trout exposed to waterborne ethynylestradiol (0, 4, 40 or 400 ng/L respectively). * Denotes significantly different from the control group based on ANOVA followed by Dunnett's and Tukeys (p=0.05).


Males
Females
Kidney Metallothionein
(mg/g wet weight)
125
100
75
50
25
0
*
*
*
*
Cont
Low
Med
High
Exposure Group

estrogens alter MT in these two organs is not clear. We are currently examining two possibilities: 1) That binding of EE2 to the ER directly affects MT expression at the transcriptional or translational level, and 2) that an increase in VTG production decreases Zn availability and secondarily down regulated MT protein synthesis.

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Chapter 3

Methodology development

Introduction

Different methods have been used to analyze metallothionein in a variety of organisms including high performance liquid chromatography (HPLC) (Lehman and Klaassen, 1986; Richards, 1991; Morcillo and Santamaria, 1993; Richards and Beattie, 1993; Aït-Oukhatar et al., 1996; Li and Otvos, 1996; Chassigne and Lobinski et al., 1998a, 1998b; Miyairi et al., 1998; Ogra and Suzuki, 1999; Wu et al., 1999), metal saturation assays (Scheuhammer and Cheria, 1991; Dutton et al., 1993; Klaverkamp et al., 2000; Werner et al., 2003), ELISA (Cousins, 1991) and Western blotting (Kimura et al., 1991; Mizzen et al., 1996; Whitacre, 1996). All of these methods measure protein concentrations but there has been recent interest in also measuring mRNA concentrations. Methods for this analysis include northern blot (Carginale et al., 1998; Mayer et al., 2003), RT-PCR (Carginale et al., 1998; Schlenk et al, 2000; Hayes et al., 2004) and real-time PCR. The key challenges for metallothionein analysis are selectivity with regard to the different MT isoforms that bind different metals and sensitivity to be able to detect basal expression of protein or mRNA when levels are not induced (Lobinski et al., 1998).

Metal saturation assays are based on competitive displacement of originally bound metals to MT protein by a metal added in excess. The displacing metal with higher affinity, usually Hg, Cd or Ag, is then determined and because the stoichiometry of bound metals is known, the concentration of MT can be calculated. While methodologically simple, saturation assays lack selectivity with regard to the individual MT-isoforms (Lobinski et al., 1998). It has also been suggested that these methods can overestimate metallothionein concentration by non specific binding of the metal to

cytosolic components that did not fully precipitate with heat treatment (Bienengraber et al., 1995).

High-performance liquid chromatography (also called high-pressure liquid chromatography) techniques can separate metallothionein protein isoforms and sub-isoforms and offer the advantage of being non-destructive. As a result, eluant from the chromatography column can be collected after the MT protein has been quantified so that the metals bound to the protein can also be analyzed. The detection of different MT isoforms relies on the different isoelectric points, the overall net negative charge and the specific amino acid composition of each isoform (Lehman and Klaassen, 1986). Different types of HPLC have been utilized; their nomenclature depends on the characteristics of the column used for chromatographic separation. Reversed-phased (Richards and Beattie, 1993; Aït-Oukhatar et al., 1996; Chassigne and Lobinski et al., 1998a; 1998b; Wu et al., 1999), ion exchange (Wan et al., 1993; Li and Otvos, 1996; Nöstelbacher et al., 2000) and size-exclusion (Morcillo and Santamaria, 1993) HPLC have all been used for the separation of metallothionein from tissues.

Reversed-phase HPLC is based on the ability of the column packing material (stationary phase) to separate compounds delivered in the eluant (also called solvent or mobile phase) based on their hydrophobic character (Richards and Beattie, 1993). The separation is carried out between a non-polar stationary phase (covalently bound C_8 or C_{18} linear hydrocarbon) and a relatively polar mobile phase. Hydrophobicity of the MT polypeptide primarily dictates its retention time; the elution of the isoforms is accomplished by gradually decreasing the polarity of the mobile phase by incorporating

proportionally more methanol or acetonitrile over the course of an analysis (Lobinski et al., 1998).

The mobile phase is clearly an important determinant of MT protein retention time. Acidic and neutral buffer systems have been used to separate metallothionein. The acidic buffer system most commonly used is 0.1% (w/v) trifluoroacetic acid (TFA) with acetonitrile as the organic modifier. Acidic buffers cause bound metals to dissociate from the protein (Aït-Oukhatar et al., 1996; Wu et al., 1999) so that the apothionein is detected. This has lead to a more accurate determination of the concentration of the protein in tissue samples (Richards, 1991; Richards and Beattie, 1993), but the metals bound to the original protein can not be determined. The use of neutral buffer systems is more appropriate when information regarding the metal content in the isoforms is needed (Richards and Beattie, 1993). Tris-HCl is the most widely used neutral pH buffer for the separation of the metallated isoforms (Lobisnki, 1998), but other buffers like sodium phosphate (Richards, 1991; Wu et al., 1999) or sodium acetate (Chassaigne and Lobinski, 1998a, Prange and Schaumloffel, 2002) have also been used.

Acetonitrile is the most widely used organic modifier in reversed-phased HPLC, but methanol has been used. However, methanol may result in incomplete resolution of the MT isoforms (Richards, 1991), and a change to acetonitrile can increase the separation efficiency increasing the number of MT peaks that are resolved (Chassaigne and Lobinski, 1998b). n-Propanol can be used as a modifier but may reduce the lifetime of the column (Van Beek and Baars, 1988; Richards, 1991).

Ion exchange HPLC separates MT proteins based on their overall charges in aqueous solutions, making them suitable for separation using this technique (Prange and

Schaumloffel, 2002). The most suitable columns for separating MT are the weak basic anion-exchangers with diethylaminoethyl (DEAE) functional groups. Aqueous buffers in which the constituents are altered over time in a linear gradient have been successfully used. For example, gradients of Tris-HCL starting at 2 mM and going up to 350 mM have been used (Wan et al., 1993; Li and Otvos, 1996; Lobinski et al., 1998; Nöstelbacher et al., 2000). It should be noted that most HPLC analysis methods detect MT absorbance at 214 nm and to a lesser extent at 254 nm. This poses a problem when using Tris-HCl as mobile phase because Tris-HCl shows considerable UV absorbance at 214 nm. Therefore, care should be taken to perform baseline subtraction manipulations to correct for the absorbance of the mobile phase (Lehman and Klaassen, 1986).

Size-exclusion chromatography (SEC) is based on the molecular sieve effect, meaning that species are separated according to their size, and to a lesser extent, on their shape (Lobinski et al., 1998). The resolution of SEC is not sufficient to discriminate between small changes in amino acid composition. Therefore this technique can separate MT from other proteins, but it cannot separate the different MT isoforms from each other. As with other HPLC techniques, characteristics of the column and the mobile phase play an important role in the separation of MT. Aqueous mobile phases with high ionic strength have been successfully employed, as has Tris-HCl at a pH between 7 and 8 (Lobinski et al., 1998; Ferrarello et al., 2000; Prange and Schaumloffel, 2002; Van Campenhout et al., 2004).

In addition to HPLC, metal saturation assays have been widely used to determine MT concentrations in different organisms and tissues (Dutton et al., 1993; Bienengraber et al., 1995; Dallinger et al., 1997; Klaverkamp et al., 2000; Campbell et al., 2005;

Guevara-Ortiz et al., 2005). Mercury is probably the most appropriate metal because MT has the greatest affinity for this metal especially at low pH and in the presence of chloride (Dutton et al., 1993; Klaverkamp et al., 2000). This becomes important when analyzing MT in fish and mammalian samples because of their high copper content. Because Cu has a higher binding affinity for MT than Cd, employing Cd would not allow for the complete saturation of MT with Cd, leading to the underestimation of MT protein content (Suzuki, 1991; Dutton et al., 1993). Mercury does displace Cu on the basis of higher binding affinity, and would, therefore, be more accurate (Suzuki, 1991; Dutton et al., 1993). Silver, the other metal used in saturation assays would not yield quantitatively accurate MT results due to its ability to precipitate in the presence of halides. Osmoregulatory organs in fish provide a source of interference with the assay due to the elevated chloride concentrations found in these tissues (Dutton et al., 1993; Klaverkamp et al., 2000).

Immunoassay techniques offer the highest degree of sensitivity (1pg) for detecting MT proteins. However, this advantage is sometimes offset by the difficulties encountered in raising high titters of antibodies. Moreover, immunoassays currently cannot provide information about the relative abundance of MT isoforms or their metal composition (Lobinski et al., 1998). Western blotting is one of the techniques used to detect a protein by immunological procedures (Aoki et al., 1991). In this method a protein mixture is separated using sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis. Proteins are separated according to their size as they pass through the gel matrix created by the polyacrylamide. Separation of the protein within the gel is followed by a transfer of the separated proteins onto a synthetic membrane

[poly(vinylidene fluoride) membrane or a nitrocellulose membrane]. The immobilized proteins are then detected by specific binding of antibodies or staining of the membrane (Laemmli, 1970; Aoki et al., 1991; Mizzen et al., 1996; Whitacre, 1996; Regala and Rice, 2004; Xie and Klerks, 2004; Meloni et al., 2005). The MT product obtained by Western blot is a band with a molecular weight of 6 to 15 KDa depending on the antibody used (Mizzen et al., 1996; Regala and Rice, 2004; Xie and Klerks, 2004).

In the last few years, there has been increasing interest in the analysis of metallothionein mRNA and its patterns of induction in aquatic organisms. Three techniques have been successfully used, including northern blot analysis, RT-PCR and real-time RT-PCR. Northern blot analysis separates RNA on a denaturing agarose gel by electrophoresis. After transferring the fractionated RNA to a nylon or nitrocellulose membrane the specific MT-mRNA can be probed with complementary DNA (cDNA) that is either radiolabeled or digoxigenin-labeled. To determine the relative abundance of the genes of interest, a specific probe to an invariant housekeeping gene is also hybridized to the membranes either at the same time or sequentially (Larkin et al., 2003). This technique provides information about mRNA size, alternative splicing and the integrity of RNA samples (Bustin, 2000).

Quantitative real-time PCR has been suggested to be more accurate and sensitive than northern blotting. This is a fairly new technique, developed in the early 1990s (Higuchi et al., 1993; Heid et al., 1996) to measure gene expression. Real-time PCR takes advantage of the 5'→3' exonuclease activity of the *Taq* polymerase enzyme to quantitate levels of gene transcripts. This enzyme acts upon the surface of the template cDNA to catalyze the extension of the annealed oligonucleotide primers and to remove

obstacles downstream of the growing amplicon that may interfere with its generation (Maniatis, 1982). The actions of the enzyme are complemented by the addition of a nucleotide sequence that has FRET (fluorescent resonant energy transfer) activity or a fluorescent dye, therefore allowing for the production of increased levels of fluorescence with increasing amplicon generation. In real time PCR the capability of monitoring in "real-time" the fluorescence produced by the fluorescent dye (SYBR green) or the fluorescence probe exists. Three types of fluorescence probes have been used; molecular beacons, hybridization probes or hydrolysis probes (Bustin, 2000).

DNA binding dyes (e.g. SYBR green) emit little fluorescence in solution, but during elongation of the DNA the dye intercalates to the new double stranded DNA. SYBR green has the ability to intercalate into the minor groove of double stranded DNA. When this intercalation is monitored in "real-time", an increase in the fluorescent signal is observed during the DNA polymerization process (real-time PCR). This signal falls off when DNA denatures. SYBR green does not require complementary probes to the target sequence to be developed, nor does it depend on the proximity of fluorophore and quencher dyes. The presence of any double-stranded DNA will generate a fluorescent signal. Instead, the specificity of real-time PCR depends on the primers used to amplify the target sequence of DNA, in this case the MT genes. Primers that will allow the polymerase to replicate the MT sequences must be developed under stringent conditions to decrease the possibility of amplifying other gene sequences (Morrison et al., 1998, Bustin, 2000; Walker, 2002).

The specificity of the primer sequence can be tested by plotting fluorescence as a function of temperature to generate a melting curve of the amplicon. The shape and

position of the melting curves depend on the GC/AT ratio of the amplified DNA, as well as its length and the specific sequence. Melting curve analysis can distinguish products of the same length that have different GC/AT ratio. It can also differentiate between two products with the same length and GC/AT ratio but with different GC/AT distribution. The method is limited by changes in position and width of the melting curve due to dye concentrations and the temperature transition rate (Ririe et al., 1997).

Molecular beacons are DNA hybridization probes that form a stem-and-loop structure. The loop portion of the molecule is complementary to the MT target sequence and the stem is formed by the annealing of complementary arm sequences on the ends of the probe sequence. A fluorescence dye or high energy dye is attached to one arm, while a quencher dye or low-energy molecule is attached to the opposite arm. In solution, free molecular beacons adopt a hairpin structure where the stem keeps the arms in close proximity, resulting in the quenching of the fluorophore. When the molecular beacon encounters a complementary sequence at the annealing temperature, it undergoes a conformational transformation that forces the stem apart, resulting in the formation of a probe/target hybrid that is longer and more stable than the stem. The separation of the quencher and the fluorophore leads to the fluorescence of the fluorophore. If the sequence of the target and the beacon does not match exactly, hybridization of the probe will not happen and therefore no fluorescence will be emitted (Tyagi and Kramer, 1996; Walker, 2002). The main disadvantage of these molecules is the difficulty in their design. Optimal design of the stem is crucial, as the molecular beacon may fold into alternate conformations that do not place the fluorophore in immediate vicinity to the

quencher. Also, if the stem is too strong, it can interfere with the hybridization (Bustin, 2000).

Hybridization probes use a fluorescein donor at the 3' end of the DNA. The probe emission spectrum overlaps the excitation spectrum of an acceptor fluorophore attached to the 5' end of a second probe. The acceptor probe must be blocked at its 3' end to prevent its extension during the annealing step. Excitation of the donor results in fluorescence resonance energy transfer to the acceptor and the emission of fluorescence. In solution, both dyes are kept apart, therefore only background fluorescence is emitted by the donor probe. After the denaturation step, both probes hybridize to their target sequence in a head-to-tail arrangement bringing the two dyes in close proximity allowing for the transfer of energy from the donor to the acceptor probe that results in an increase in fluorescence from the acceptor probe. As with the molecular beacons, the amount of fluorescence measured depends on the amount of DNA synthesized during the PCR reaction (Bustin, 2000).

Hydrolysis probes or Taqman probes utilize the 5'-nuclease activity of the DNA polymerase to hydrolyze the probe. When using Taqman probes the design of three different primers is needed. Two template specific primers that define the endpoints of the amplicon and an oligonucleotide probe that hybridizes to the amplicon during the annealing/extension phase of the PCR reaction. This oligonucleotide probe contains a fluorescent reporter dye at its 5' end and a quencher dye at its 3'end that inhibits the fluorescence of the reporter when the oligonucleotide is intact. After the denaturation step the probe hybridizes to the amplicon, remaining hybridized while the polymerase extends the primers until it reaches the probe. The polymerase cleaves the probe

resulting in the separation of the reporter and quencher dyes, the release of the quencher from the reporter allows for increased fluorescence from the reporter dye (Ririe et al., 1997; Bustin, 2000; Bustin, 2002; Walker, 2002; Larkin et al., 2003). As with molecular beacons and hybridization probes, the amount of fluorescence emitted by the hydrolysis or Taqman probes depends on the amount of amplicon.

Methods for quantifying MT using reversed phase and ion exchange HPLC, Western blot analysis and real-time PCR were adapted or developed. HPLC methods are also compared with Hg saturation and microscaled Hg saturation methods, for this purpose, samples obtained from the waterborne experiment described in chapter 2 were used. These samples provided an excellent opportunity for comparisons between the different methods described in the present chapter.

Reversed-Phase and Ion Exchange High Performance Liquid Chromatography

Material and Methods

Solvents and chemicals

For HPLC analysis, all solvents were HPLC grade or better. Ultra pure water (< 18 MΩ) was used to make all buffers and mobile phase was filtered and degassed by filtering through a 0.22 μm filter (Millipore Corporation, Bedford, MA).

Sample preparation for HPLC detection

Liver tissue was homogenized using a Polytron homogenizer with a Teflon pestle (Brinkmann Instruments, Westbury, NY) in 10 mM NaH_2PO_4 buffer pH 7.0, containing 5 mM 2-mercaptoethanol to protect the sulfhydryl groups in MT from oxidation. The use

of dithiothreitol, as a protective agent is not recommended when metals bound to MT are to be analyzed because dithiothreitol can chelate metals with low binding affinities, including Zn. 2-mercaptoethanol reduces intermolecular disulphide bonds but does not destabilize bound metals (Suzuki, 1991).

The homogenate was heat treated at 95°C for 5 min and immediately cooled on ice for 4 min to remove heat-unstable high molecular weight proteins. For HPLC analysis it is necessary to assure that the MT protein completely separates from non-heat stable high molecular weight proteins present in the homogenate. Two centrifugation protocols were examined: 10,000 x g for 5 min and 20,000 x g for 90 min.

Recovery of MT-1 from a tissue matrix was examined by spiking a liver sample with MT-1 standard (0.5 mg/ml). The spike was added prior to homogenization to account for any possible losses during sample preparation. Three different filters were tested to account for the possibility that the filter material could retain the MT protein. Filter tubes (Millipore Corp., Bedford, MA) with pore sizes of 0.45 and 0.22 μm; 10,000 and 30,000 MW centrifugal filter devices (Millipore Corp., Bedford, MA) and 0.45 and 0.22 μm syringe filters (Millipore Corp., Bedford MA) were tested.

HPLC system

MT was analyzed by injecting the supernatant directly into a Gilson HPLC system (Middleton, WI) consisting of a Gilson 322 dual pump (Gilson, Middleton, WI), a 235p autoinjector (Gilson, Middleton, WI) and a Hewlett Packard 170 diode array detector (Hewlett Packard, Palo Alto, CA) to monitor UV absorbance at 214, 220 and 240 nm.

Reversed-phase column and gradient elution

Metallothionein was separated using a Waters (Millford, MA) 8 mm x 100 mm μBondapak C_{18} preparative column with a particle size of 10 μm and a 125 Å pore size, housed in a RCM 8 x 10 radial compression device. The column was maintained at room temperature (19 to 22°C) and eluted at a flow rate of 3.0 ml/min.

The mobile phase used to elute MT isoforms from the reversed-phase column consisted of two buffers, buffer A (10 mM NaH_2PO_4, pH 7.0) used as an equilibrator and buffer B (buffer A plus 60% acetonitrile) as the elution buffer. Metallothionein was eluted using a two-step linear gradient: 0-10% B from 0 to 5 min followed by 10-25% B from 5 to 20 min. The column was then purged with 100% B for 5 min and finally equilibrated with 100% A for 10 min prior to the next injection.

Ion exchange column and elution gradient

Anion exchange separation of metallothionein was performed with a TSK gel DEAE-5PW column (7.5 mm ID x 7.5 cm) from Tosoh Bioscience, Japan. A guard column packed with the same material protected the column from contamination. This particular column allows for the high injection volume of 500 μl used in these experiments.

Gradient elution was performed using 20 mM Tris-HCl, pH 7.4 (Buffer A) and 200 mM Tris-HCl, pH 7.4 (Buffer B). MT was eluted with a linear gradient from 0 to 60% Buffer B in 18 min at a flow rate of 1 ml/min. The column was regenerated with 100% Buffer A for 25 min. UV absorption was monitored at 224, 254 and 280 nm.

Metallothionein standards

Purified rabbit MT-1 and MT-2 (Sigma Chemical Co., St. Louis, MO) were used with the reversed-phase protocol to create separate standard curves for each isoform. Serial dilutions in Buffer A (10 mM NaH_2PO_4, pH 7.0) were done to obtain the following concentrations: 1 mg/ml; 0.5 mg/ml; 0.25 mg/ml; 0.125 mg/ml; 0.06125 mg/ml and 0.03125 mg/ml. Standard curves were constructed by plotting the concentration against the integrated peak area for UV absorbance at 214 nm.

For the ion exchange protocol, total rabbit MT was used as a standard (Sigma Chemical Co., St. Louis, MO), because MT-1 and MT-2 were no longer available. Total MT standards were dissolved in ultra pure water and diluted to obtain the following concentrations; 1, 0.5, 0.25, 0.125, 0.06125 and 0.03125 mg/ml.

Microscaled mercury saturation assay

Lake trout liver samples obtained from the waterborne EE2 experiment described in chapter 2 were analyzed using a microscaled mercury saturation assay (Shaw-Allen et al., 2003). Briefly, liver samples were homogenized 10% TCA and centrifuged for 10 min at 16,000 x g. The supernatant was saturated with 7 ppm Hg in 10% TCA and incubated for 10 min at 40°C. Free Hg was then scavenged from the mixture by adding 5% haemoglobin (Sigma-Aldrich, St. Louis MO) in saline. Precipitated haemoglobin was pelleted by centrifugation for 10 min at 16, 000 x g. The Hg saturated sample was then incubated at 70°C with BrCl overnight. After cooling, MT preparations were brought to a final volume of 1.6 ml with DDW, Hg was analyzed by cold vapour atomic fluorescence spectrometry. Metallothionein concentrations were calculated from

measured Hg concentrations using the stoichiometric relationship of 7 nmole Hg/ nmole MT.

Data analysis

A one way ANOVA, followed by Dunnet's or Tukeys multiple comparison tests were used to compare MT concentrations determined by HPLC, mercury saturation and microscaled saturation methods. Statistical significance was accepted at $p<0.05$.

Results and Discussion

Standard curves

Excellent correlation was observed between the standard concentrations of MT-1 ($r^2 = 0.997$) and MT-2 ($r^2= 0.997$) and their integrated peak areas using reversed-phase HPLC (Fig 3.1 and Fig 3.2 respectively). The linear relationship between the quantity of MT-1 injected onto the column and the integrated peak area for UV absorbance at 214 nm is shown.

Metallothionein standards analyzed by the ion exchange yielded only one peak, corresponding to MT-1. The regression analysis of the standard curve (Fig 3.3) showed a good correlation between peak area and concentration ($r^2 = 0.993$).

Analysis of liver samples from waterborne EE2 experiment using reversed-phase HPLC

Liver samples from the waterborne experiment described in chapter 2 were extracted and analyzed as described above. While the results were not statistically significant, there was a trend toward lower concentrations of MT were found in all

Figure 3.1 Metallothionein 1 standard curve by reversed phase HPLC showing the linear relationship between the quantity of MT-1 injected onto the column and the integrated peak area for UV absorbance at 214 nm (r^2=0.99).

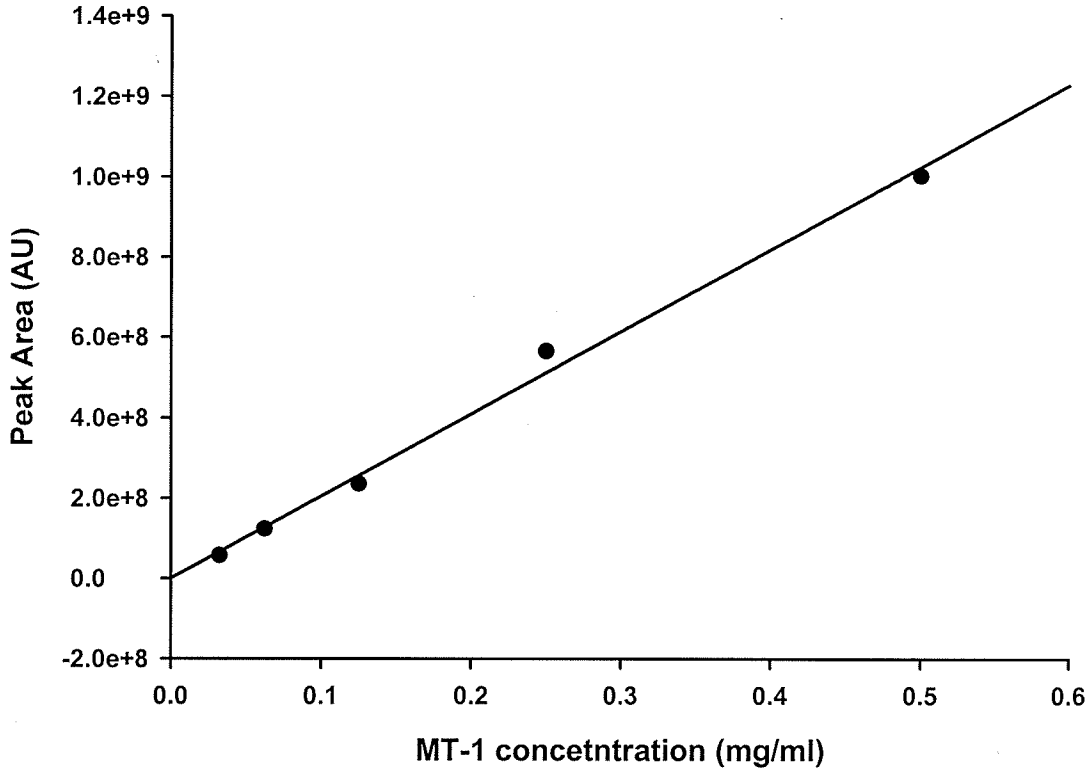

1.4e+9
1.2e+9
1.0e+9
8.0e+8
6.0e+8
4.0e+8
2.0e+8
0.0
-2.0e+8
Peak Area (AU)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
MT-1 concetntration (mg/ml)

Figure 3.2 Metallothionein 2 standard curve by reversed phase HPLC showing the linear relationship between the quantity of MT-2 injected onto the column and the integrated peak area for UV absorbance at 214 nm (r^2=0.99).

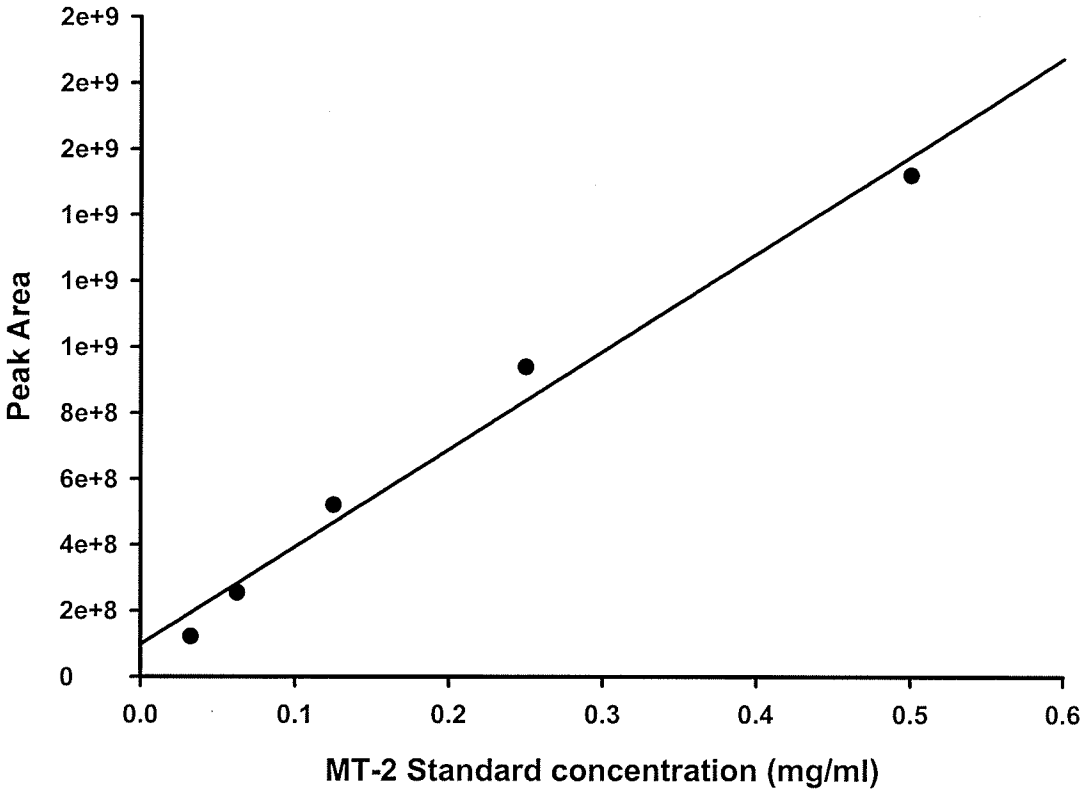
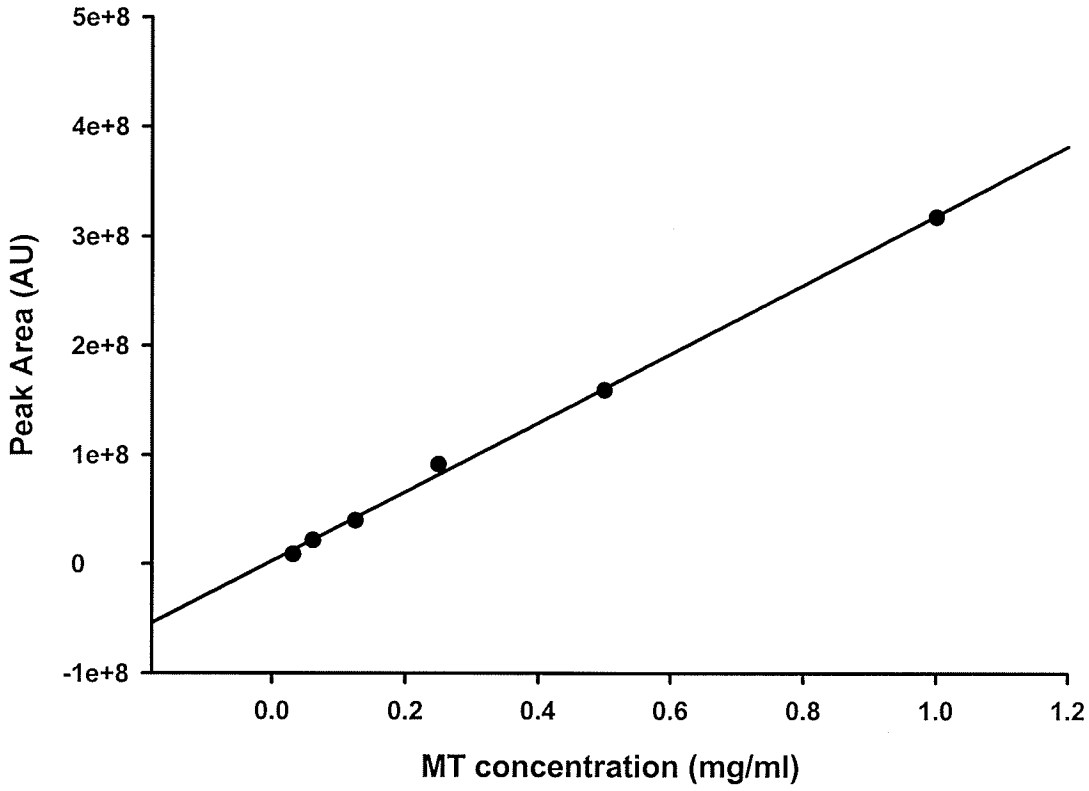

2e+9
2e+9
2e+9
1e+9
1e+9
1e+9
8e+8
6e+8
4e+8
2e+8
0
Peak Area
0.0
0.1
0.2
0.3
0.4
0.5
0.6
MT-2 Standard concentration (mg/ml)

Figure 3.3 Metallothionein standard curve by ion exchange HPLC showing the linear relationship between the quantity of MT injected onto the column and the integrated peak area for UV absorbance at 254 nm (r^2=0.996).


5e+8
4e+8
3e+8
2e+8
1e+8
0
-1e+8
Peak Area (AU)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
MT concentration (mg/ml)

groups exposed to EE2 compared to the control group. The group exposed to the highest dose of EE2 contained the least amount of MT per gram of liver tissue (Fig 3.4). When Hg saturation was used to analyze the same liver tissues as described in chapter 2, the same trend was observed, but differences between the two highest dose groups and the reference group were significant. The same trend was obtained when the samples were analyzed using the microscaled mercury saturation assay (Fig 3.5).

It is important to note that the differences in the amount of MT quantified using the three methods are orders of magnitude different. Because of the significant differences in the results obtained with the HPLC method, and the possibility that the recovery of MT with this method was poor, liver samples were added a spike of MT standard to evaluate the recovery of the spike and the sample after the extraction procedure.

Typical chromatograms for a 1 mg/ml MT standard and a liver sample are shown in figure 3.6, panels A and B. There were no significant differences between the filtration devices or the pore size used and the amount of MT recovered. Furthermore, the peak areas obtained from spiked samples did not correlate with the amount of MT standard added (Fig 3.7 A). To verify that the MT protein was not binding to the filters a standard was filtered with the different filters and analyzed with the HPLC. Recovery of the MT standard was 100% (Fig 3.7 B) suggesting that MT was being retained in the pellet following centrifugation.

Next, the speed and time of the centrifugation were increased from 10,000 x g for 5 min to 20,000 x g for 90 min as suggested in the literature (Suzuki, 1991;

Figure 3.4 MT-1 concentration in liver of lake trout exposed to waterborne EE2 (4, 40 and 400 ng/L) determined by reversed-phase HPLC.

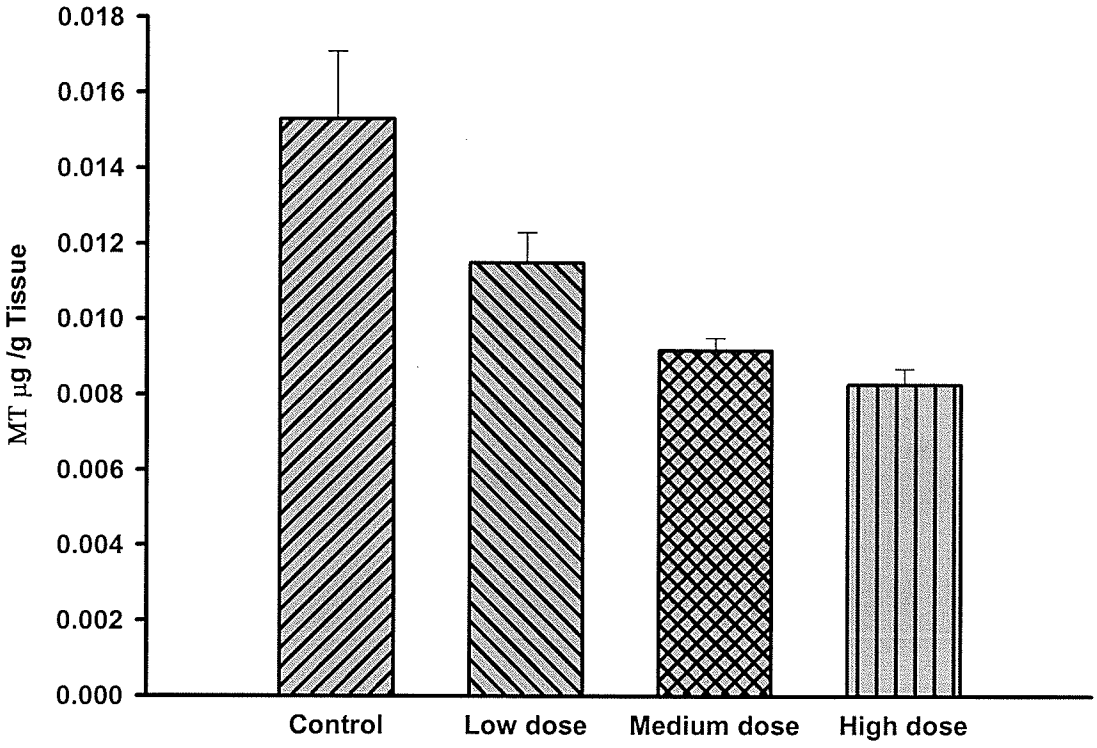

0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
MT µg /g Tissue
Control
Low dose
Medium dose
High dose

Figure 3.5 Comparison of MT-1 concentrations in livers of lake trout exposed to waterborne EE2 (4, 40 and 400 ng/L) determined by reversed-phase HPLC, microscale mercury saturation and mercury saturation analysis.

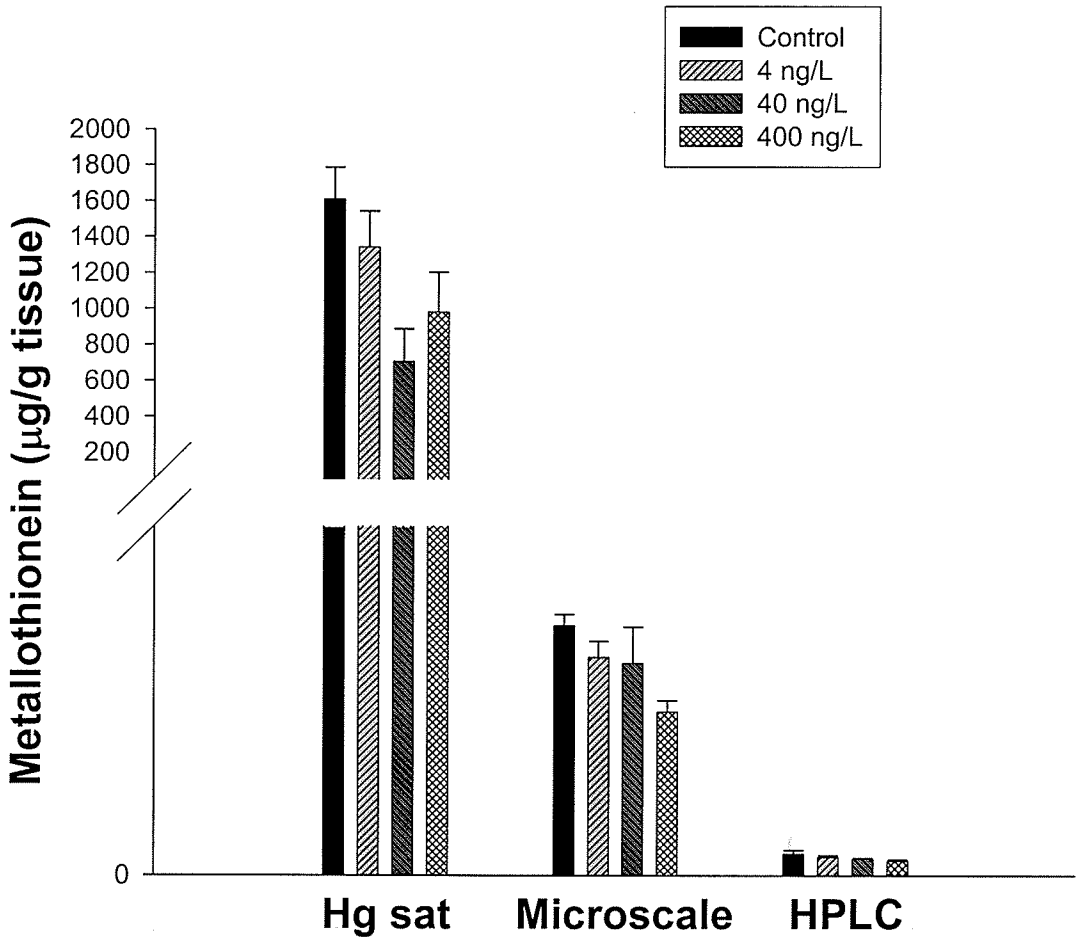
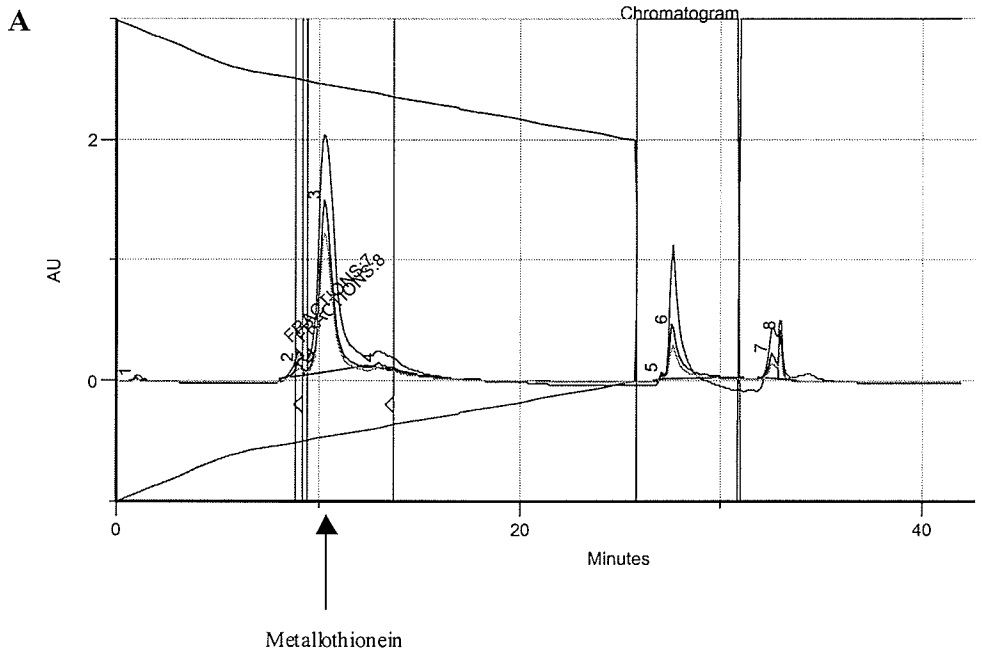

Control
4 ng/L
40 ng/L
400 ng/L
Metallothionein (µg/g tissue)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Hg sat
Microscale
HPLC

Figure 3.6 Typical chromatograms of MT-1 standard (panel A) and a liver sample (panel B) using reversed-phase HPLC. The arrow indicates the retention time where the standard and the sample were eluted from the column.


A
Chromatogram
AU
2
0
FRACTIONS:3
FRACTIONS:8
FRACTIONS:7
1
2
3
4
5
6
7
8
0
20
40
Minutes
Metallothionein

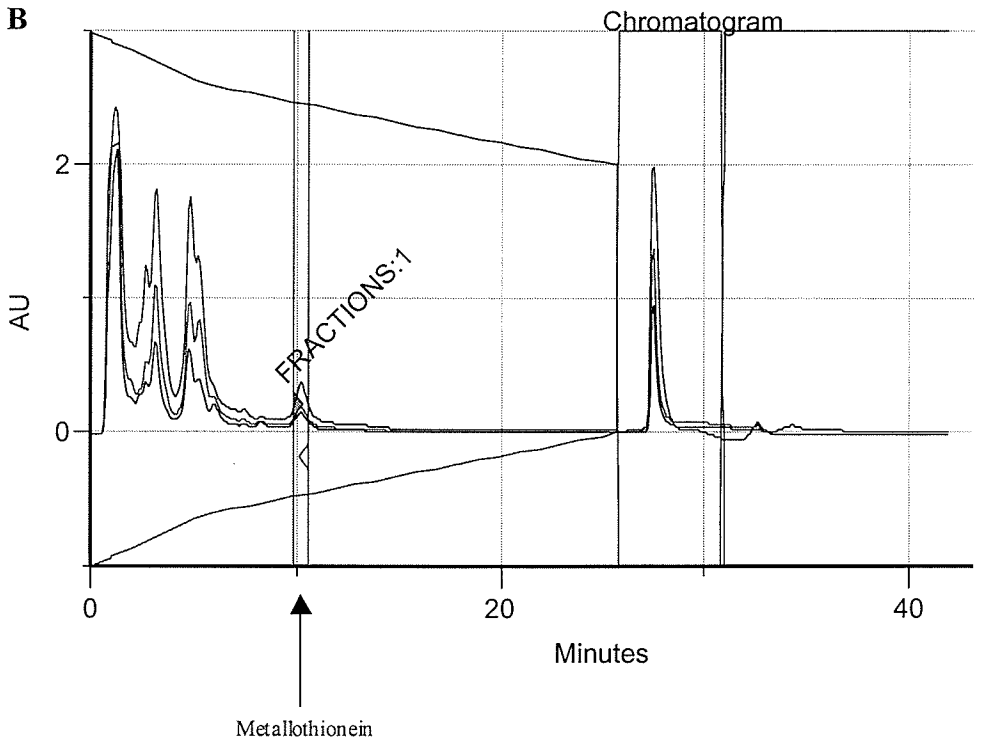
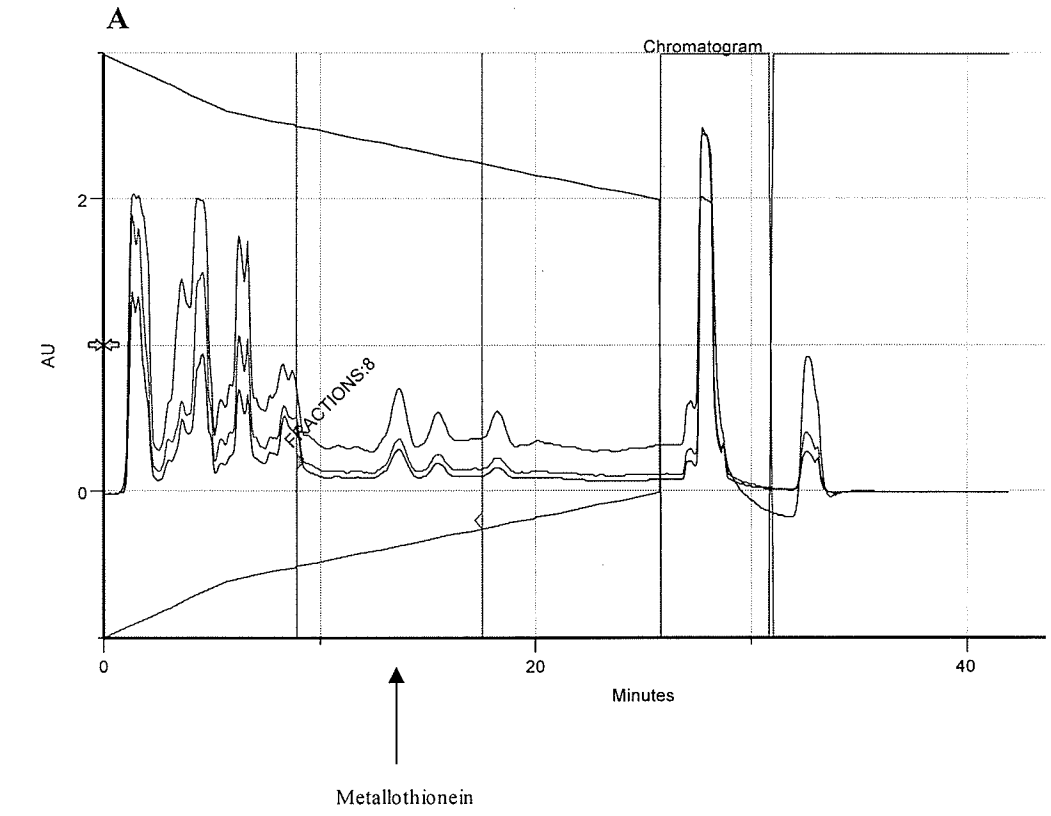
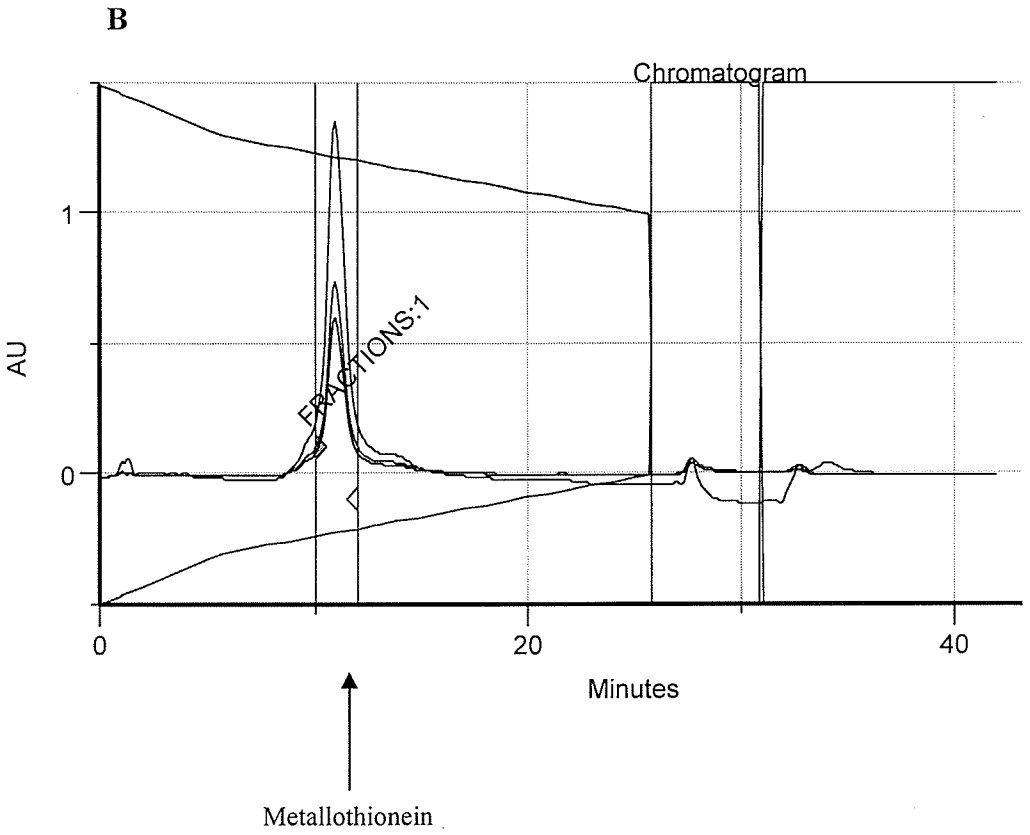

B
Chromatogram
AU
2
0
FRACTIONS:1
0
20
40
Minutes
Metallothionein

Figure 3.7 Chromatograms of MT-1 standard (panel A) and MT-1 recovered from the supernatant of a liver sample that was spiked with 0.5 mg MT-1 / ml (panel B). Both the standard and supernatant were filtered using a 0.22 μm filter prior to HPLC injection. The arrow indicates the retention time of the sample after elution from the column.


A
Chromatogram
2
AU
0
FRACTIONS:8
0
20
40
Minutes
Metallothionein


B
Chromatogram
1
AU
0
FRACTIONS:1
0
20
40
Minutes
Metallothionein

Mosleh et al., 2004). Using this approach the recovery of the spike significantly increased, reaching a 100% recovery of the spike. Once the methodology was adjusted, the final step in the quantification and identification of the MT fraction is to do a spectral analysis of the sample and compare it with the spectral analysis of the standards. It is important to note that each compound has a distinct absorption spectrum plot; and this plot measure the changes in absorbance at different wavelengths. It was found that the absorption spectrum plot for the standards is different from the absorption spectrum plot of the samples; this suggests that the peak obtained from the samples is different from the peak obtained for the standard (data not shown).

It has been suggested that HPLC is a useful tool to analyze metallothionein in tissues (Richards, 1991; Morcillo and Santamaria, 1993; Wu et al., 1999; Nöstelbacher, 2000; Mosleh et al., 2004). In this study we were not able to successfully separate metallothionein from fish tissues. One of the problems encountered was the different spectrum between the rabbit MT standards and the fish samples. Metallothionein is a highly conserved protein (Kägi, 1993; Gedamu et al., 1993) therefore; the use of a mammalian standard commercially available could be of use. On the other hand, it is known that small changes in the amino acid composition (1 - 15 amino acids) of a protein modifies the isoelectric point and the overall net negative charge of the protein, making HPLC a great method to separate protein isoforms that will have small changes in their amino acid composition (Lehman and Klaassen, 1986; Chassigen, 1998; Prange and Schaumloffel, 2002). With this in mind, when two different absorption spectrum plots were obtained from the standard and the fish samples it was assumed that different proteins were being separated.

Analysis of the protein sequences of MT isoforms from rabbit and rainbow trout showed that the proteins were significantly different. This may explain the different spectral plots (Fig 3.8). Furthermore, comparing rabbit MT-1 and MT-2 it is evident that there are three sets of amino acid sequences that are highly conserved and these sequences are separated by two other sequences that are not conserved. The differences between the two isoforms would facilitate their separation by HPLC. However, rainbow trout MT-1 and MT-2 amino acid sequences are highly conserved one amino acid inserted in MT-1 compared to MT-2 (Fig 3.8). this would make separation of fish MT-1 and MT-2 using HPLC more difficult than for the rabbit isoforms.

Protein Blast (www.ncbi.nlm.nih.gov) analysis was performed for rabbit and rainbow trout metallothionein. Few similarities were identified between fish and mammals. There is a displacement of one cysteine when the fish and mammalian MTs are compared, and there are fewer lysines in the fish protein sequence (Scudiero et al., 1997; Vergani et al., 2005). These differences in the amino acid sequences of Mt form the two organisms are strong enough to account for the different spectral plots. When rainbow trout MT was compared to mussel (*Mytilus galloprivincialis*) MT, Vergani (2005) showed differences in the UV absorption spectra from both proteins at 245 nm and 228 nm, where the mussel MT lacked a negative band (245 nm) and a positive band (228 nm). It was shown that the red-shifted spectra observed in mussel MT corresponded to an atypical secondary conformation.

Figure 3.8 Amino acid sequences of MT-1 (A) and MT-2 (B) in rabbit and rainbow trout. Underlined sequences are those that are different between the two isoforms in the same species. A= alanine; C=cysteine; D=aspartate; E=glutamate; G=glycine; I=isoleucine; K=lysine; M=methionine; N=asparagines; P=praline; Q=glutamine; S=serine; T=threonine and V=valine.

Amino acid sequences of MT-1 and MT-2 in Rabbit and Rainbow Trout

A

Rabbit MT-1
MDPNCSCA**TG NSCTCASSCK CKECK**CTSCK KSCCSCC**PAGCT**KCAQGCIC KGASDKCSCC A

Rainbow Trout Mt-1
MDPCECSKTG SCNCGGSCKC SNCACTSCKK **A**SCCDCCPSG CSKCASGCVC KGKTCDTSCC Q

B

Rabbit MT-2
MDPNCSCA**AA GDSCTCANSC TCKA**CKCTSC KKSCCSCCP**P GCA**KCAQGCI CKGASDKSCSC CA

Rainbow trout MT-2
MDPCECSKTG SCNCGGSCKC SNCACTSCKK SCCPCCPSDC SKCASGCVCK GKTCDTSCCQ

This shift results from a difference in the primary sequence of the proteins, specifically in the α-domain.

HPLC has proven to be an important tool in the separation and identification of metallothionein isoforms (Lehman and Klaassen, 1986; Micallef et al., 1992; Mocillo, 1993; Wan et al., 1993; Chassaigne and Lobinski, 1998a; Lobinski et al., 1998; Wu et al., 1999; Farrarello, 2000; Nöstelbacher, 2000; Prange and Schaumloffel, 2002; Mosleh et al., 2004). Differences in the amino acid sequence of mammalian and fish species has to be taken into account when separating these proteins if mammalian standards are to be used to identify the protein. Results could be strengthened in the future if fish are treated to over express metallothionein, for example by Cd treatment, followed by purification and separation of fish metallothionein isoforms to use as true standards. Also the coupling of HPLC to element- or molecule specific detectors would be a valuable advantage as the eluate is analyzed for its metal content giving a better understanding of the role of MT in physiological conditions. A promising step in the appropriate identification of the proteins separated by HPLC could be the analysis of the eluate by Western blot analysis. Western blot analysis using anti-cod metallothionein antibody is discussed in the following section.

Western Blot Analysis

Material and Methods

Sample preparation

Liver samples were prepared for western blot according to the method described previously. Briefly, liver tissue was homogenized in 10 mM NaH_2PO_4 buffer, pH 7.4

with 5 mM 2-mercaptoethanol. The homogenate was heat treated at 95°C for 5 min and cooled on ice for 4 min, followed by centrifugation at 20,000 x g for 90 min. The supernatant was collected and kept at -90°C until analyzed.

Electrophoresis

Protein content was measured using the Bio-Rad protein assay following manufacturer's recommendations (Bio-rad, Hercules, CA). Polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli's procedure (Laemmli, 1970) with some modifications to allow for the separation of low molecular weight proteins. Briefly, 16.5% Tris-Tricine resolving gels with 4% stacking gels (Bio-rad, Hercules, CA) were used to separate MT. This type of gel is recommended when small proteins or peptides are separated. The sample buffer consisted of 200 mM Tris-HCl, pH 6.8; 2% SDS; 40% glycerol and 0.04% Coomassie Blue G-250 (Bio-rad, Hercules, CA). The electrophoresis buffer was 100 mM Tris, pH 8.3; 100 mM Tricine and 0.1% SDS (Bio-rad, Hercules, CA).

The samples (100 μg protein per well) were heated in a boiling water bath for 5 minutes, cooled on ice and mixed with the sample buffer. The samples were run in a mini protean apparatus (Bio-rad, Hercules, CA) at a constant current (100 mA) until the dye front reached the bottom of the gel. The proteins were then transferred onto 0.22 μm pore size PVDF membrane (Millipore Corp, Bedford, MA) for 1 hour at a constant voltage (100V). Prior to transfer, the gels and membranes were equilibrated in cold transfer buffer until the membrane was completely wet, about 3 minutes. The transfer

buffer was 48 mM Tris, 39 mM glycine containing 0.04% SDS and 20% methanol at pH 8.3.

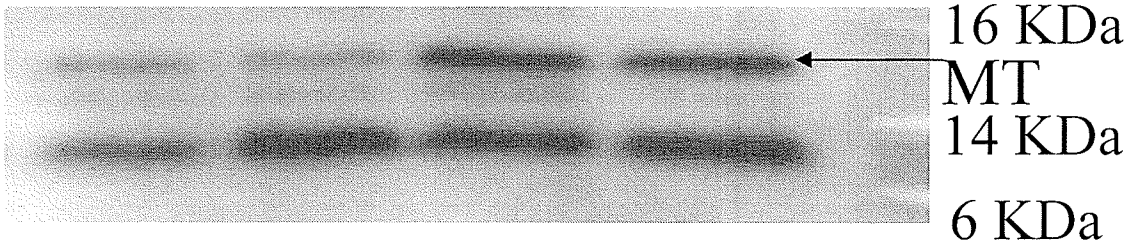
Metallothionein blotting

The membranes were blocked with 3% (w/v) bovine serum albumin (BSA) in Tris buffered saline/Tween 20 (TBST) [100 mM Tris-HCl, pH 7.5; 0.9% (w/v) NaCl; 0.1% (w/v) polyoxyethylenesorbitan monolaurate (Tween 20)] for 1 hour at room temperature. The membranes were incubated for 1 hour with a rabbit anti-Cod metallothionein polyclonal antibody (Biosense Laboratories, Bergen, Norway) at a 1:100 dilution in 1% BSA-TBST. The membranes were then washed with TBST with gentle agitation to remove unbound antibody, and then incubated with goat anti-rabbit IgG HRP (1:3,000) for 1 hour at room temperature. The membranes were once again washed in TBST and exposed to developer solution (Supers signal West Pico chemiluminescent substrate, Pierce Biotech, Rockford IL) for 10 min. The membranes were finally exposed to film to reveal the results.

Results and Discussion

Western blotting using the anti-Cod polyclonal antibody generated a band that was 14 KDa in size that corresponded to the MT protein (Fig 3.9). Because the antibody was not specific for lake trout, it was important to find the appropriate dilution to generate positive results. A 1:100 dilution was diemed suitable.

Figure 3.9 Western blot analysis of MT using anti-Cod anti Rabbit antibody.


16 KDa
MT
14 KDa
6 KDa
Spring
Summer
Fall
Winter
MW

Anti-Cod antibody is the only commercially available fish metallothionein antibody. It has been reported that this antibody cross-reacts well with a variety of different species, including whiting (*Merlangius merlangus*), yellow gurnard (*Trigla lucerna*), flounder (*Platichthys flesus*), as well as with members of the cypriniform, siluriform, salmoniform, perciform and rajiform families (Hylland et al., 1995). The degree of cross-reactivity differs between species; with respect to the salmoniforms, this antibody has a weak cross-reactivity with the anti-Cod antibody (Hylland et al., 1995). It is also important to note the blocking agent that is used. The use of 5% non-fat dry milk produces no binding of the MT antibody to the membrane or to the proteins embedded in it. On the other hand, the use of BSA as the blocking agent and to dilute the antibody to its working concentration works well.

Real-Time Polymerase Chain Reaction

Material and Methods

Primer development

In rainbow trout there are two known isoforms of metallothionein, MT-1 and MT-2. Two different genes, termed MT-I and MT-II encode these two isoforms. The complete mRNA coding sequence of MT-I spans 372 nucleotides in length, while that of MT-II spans 369 nucleotides. It is possible to align the sequences of these two mRNA's in order to compare the similarity between the sequences. The Blast 2 sequences program available from GenBank (www.ncbi.nlm.nih.gov) allows for the alignment of any two sequences. The program requires the accession numbers (M18103 for MT-I and M18104 for MT-II) in order to allow the user to search for specific proteins and

organisms using the nucleotide menu of the search engine. The blast tool allows sequences to be aligned to determine the homology between the sequences of interest.

After comparing the sequences of rainbow trout MT-I and MT-II using the blast tool, it was determined that the mRNA sequences for the two proteins have 85% homology. The major difference between the two sequences appears in the 5'UTR where no alignment is observed (Fig 3.10). There are a few other gaps in the sequence that are different, but because the regions in the 5'UTR are not conserved, primers developed to recognize this region will allow specific amplification of each isoform.

The program Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) was used to design the Taqman probes for later use in the real-time PCR assays. The mRNA coding sequences derived from GenBank were used in the Primer3 program. Parameters used to calculate the primers were retained as reported by the program with the exception of the melting temperatures (Tm) for the primers and the hybridization probe. The Tm of the probe needs to be 10°C higher than the Tm value for the primers, so the necessary adjustments need to be made to avoid overlapping of these temperatures. The melting temperatures were set as follows: Minimum temperature, 57.0; Optimum temperature, 60.0; Maximum temperature, 63.0. For the hybridization probes: Minimum temperature, 67.0; Optimum temperature 70.0; Maximum temperature, 73.0.

Another specific requirement when designing fluorogenic probes is that there should not be a guanosine (G) at the 5' end of the probe. Having a guanosine adjacent to the reporter dye quenches reporter fluorescence even after cleavage. Once the conditions are set, Primer3 generates a series of possible primers and probes indicating the start

Figure 3.10 MT-I and MT-II nucleotide sequence alignment after the blast 2 sequence analysis. Note that the major differences are in the 5'UTR.

Metallothionein I and II aligned using the Blast tool.

5'UTR

position, the length of the oligonucleotide, the melting temperature, the sequence of the oligonucleotides and the sequence size.

Besides designing primers and probes for MT-I and MT-II, monitoring a "housekeeping" geneis also necessary. The ribosomal 18s subunit and β-actin genes were both considered.

The first set of primers designed targeted rainbow trout MT-I (Fig 3.11) and 18s rRNA (forward primer: aaacggctaccacatccaag; reverse primer: cctccaatggatcctcgtta; probe: TETgcaggcgcgcaaattacccaBHQ^{TM}-1). The probe for MT-1 was labeled with FAM at the 5'end and BHQ^{TM}-1 at the 3'end. The primers were dissolved in Diethyl Pyrocarbonate (DEPC) water to a concentration of 100 μM; the probes were also dissolved in DEPC water to a concentration of 10 μM.

A second set of primers was designed for both MT-I and MT-II but also for β-actin. These changes were necessary to address the presence of two peaks in the melt curve analysis of the MT-I primer set, the high fluorescence signal obtained with the 18s ribosomal primers and the difficulty to create MT-II primers targeting the 5'UTR region. A more detailed analysis of both MT-I and MT-II sequences showed that there are parts in the coding sequence that show some differences when both, MT-I and MT-II are aligned (Fig. 3.12). These gaps gave a new area with which two sets of primers could be designed, giving the possibility of differentiating between the two gene products. The primers designed with the new target sequence were:

Figure 3.11 MT-I forward and reverse primer set with the location of the Taqman probe design using Primer3. Oligonucleotides in orange represent the forward primers, the oligonucleotides in red represent the reverse primers and the oligonucleotides in blue represent the Taqman probe.

CAACACACCA CTGACACCCA GACAAACTAC TACGATCCAT
TCGGATAAAG AAGGTAGCTC AAAAACTGGA AAATGGATCC
TTGTGAATGC TCCAAAACTG GATCTTGCAA CTGCGGTGGA
TCCTGCAAGT GCTCCAACTG CGCATGCACC AGTTGTAAGA
AAGCAAGTTG CTGCGACTGC TGTCCCTCCG GCTGCAGCAA
GTGTGCCTCA GGCTGCGTGT GCAAGGGCAA GACTTGTGAT
ACCAGCTGTT GTCAGTGAGG CCTGTGTATG ACATCACAAT
GCAGTCCATT CCCTATGACT ATGAAGTTGT ACCATCTTGA
GCATAGCTTT TGTACCTTGT CAAATGTAAG GAAATAAATT
GCATGTAACT T

GTTGTGTGGT GACTGTGGGT CTGTTTGATG ATGCTAGGTA
AGCCTATTTC TTCCATCGAG TTTTTGACCT TTTACCTAGG
AACACTTACG AGGTTTTGAC CTAGAACGTT GACGCCACCT
AGGACGTTCA CGAGGTTGAC GCGTACGTGG TCAACATTCT
TTCGTTCAAC GACGCTGACG ACAGGGAGGC CGACGTCGTT
CACACGGAGT CCGACGCACA CGTTCCCGTT CTGAACACTA
TGGTCGACAA CAGTCACTCC GGACACATAC TGTAGTGTTA
CGTCAGGTAA GGGATACTGA TACTTCAACA TGGTAGAACT
CGTATCGAAA ACATGGAACA GTTTACATTC CTTTATTTAA
CGTACATTGA A

Figure 3.12 MT-I and MT-II primer selection based on the differences in the two sequences, not including the 5"UTR. The nucleotides in pink denote those who are different between the two sequences. The oligonucleotide sequences in the yellow box correspond to the primers for MT-I and the oligonucleotide sequences in the green box correspond to the primers for MT-II. The oligonucleotides in the blue box correspond to the Taqman probe for MT-I and the oligonucleotides in the grey box correspond to the Taqman probe for MT-II.

MT-II

taaagaagcgcgatcaaaaactgaaaaatggatccttgtgaatgctctaaaactggctct

taaagaaggtagctcaaaaactggaaaatggatccttgtgaatgctccaaaactggatct

MT-I

tgcaactgcggtggatcctgcaagtgctcaaactgcgcatgcaccagttgtaagaa a

tgcaactgcggtggatcctgcaagtgctccaactgcgcatgcaccagttgtaagaaagca

agttgctgccctgctgrccttccgactgcagtaaatgtgcttcaggctgtgtgtgcaag

agttgctgcgactgctgtccctccggctgcagcaagtgtgcctcaggctgcgtgtgcaag

ggcaagacctgcgataccagctgttgtcagtgaggcctggtgcattaccgtgacaatgca

ggcaagacttgtgataccagctgttgtcagtgaggcct gtgtatga catcacaatgca

gt cattccctacgaaattg gttgtaccatcttgagcctagacttagtactttgtctg

gtccattccctatgactatgaagttgtaccatcttgagcatagctttgtaccttgtc

ttaaatgtaagaaaataaattccattaaactt

aaatgtaaggaaataaattgcatgtaactt

MT-I forward 5'tagctcaaaaactggaaaatggat3'; reverse 5'atcacaagtcttgcccttgc3'; probe FAMtccctccggctgcagcagcaagtBHQ-1.

MT-II forward 5'cgcgatcaaaaactgaaaaat3'; reverse 5'gcattgtcacggtaatgcac3'; probe TXRctgcccctgctgtccttccgBHQ-2.

Actin forward 5'agagctacgagctgcctgac3'; reverse 5'agcactgtgttggcgtacag3'; probe TETtcggcaacgagaggttccgcBHQ-1.

Sample preparation

RNA isolation was performed using the TRIzol (Invitrogen Corp, Carlsbad CA) extraction method. Liver tissue (100 mg) was homogenized with TRIzol reagent using a polytron homogenizer; the homogenate was incubated at room temperature for 5 min, followed by the addition of chloroform (0.2 ml / 1 ml TRIzol). The tubes were shaken vigorously for 15 seconds and incubated at room temperature for another 3 min. This mixture was centrifuged at 12,000 x g for 15 min at 4°C, and the chloroform layer transferred to new tubes. Isopropanol (0.5 ml / 1 ml TRIzol) was added followed by a 10 min incubation at room temperature. The tubes were then centrifuged once again at 12,000 x g for 10 min at 4°C. The isopropanol was discarded and 75% ethanol (1 ml / 1 ml TRIzol) was added to the pellet, followed by 1 hour incubation at -20°C. The tubes were then centrifuged at 7,500 x g for 5 min, the ethanol discarded, and the tubes let to dry. Once dried, the pellet was resuspended in 100 µl of DEPC water and kept at -70°C until analyzed.

To calculate the purity and concentration of the extracted mRNA the optical density at 260/280 and 260 nm was measured. The RNA was diluted to a concentration

of 1 μg/μl in deionized distilled water (DDW). Reverse transcription of RNA was performed using the Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) kit from Invitrogen (Carlsbad, CA) following the manufacturers recommendations. Briefly, 1μg RNA was added to chilled microcentrifuge tubes, to these a master mix containing 200 U/μl MMLV-RT, 5X Reverse transcriptase buffer (250 mM Tris HCl, pH 8.3; 375 mM KCl; 15 mM $MgCl_2$), 40 U/μl RNase out, 0.1M DTT, 0.1% BSA, 250 ng RT primer (5'gactcgagtcaacatcgattt$(ttt)^5$tt), 5 mM dNTP was aliquoted. The tubes were incubated at 37°C for 2 hours and kept at -20°C until used.

Real-time PCR conditions

The cDNA obtained by the reverse transcription of the lake trout liver mRNA was used with the primers designed as described above to run a real-time PCR reaction. OmniMix master mix (Cepheid; Sunnyvale, CA) was used on a Cepheid Smart Cycler (Sunnyvale, CA) following manufacturer's recommendations. Briefly, OmniMix master mix (3 U TaKaRa hot star Taq polymerase; 200 μM dNTP; 4mM MgCl; 25 mM HEPES buffer pH 8.0) was mixed with the primers and probes (100 μM and 10 μM respectively) or with the fluorescent dye SYBR green (Sigma Chemical Co., St. Louis, MO). The addition of cDNA to the tubes containing the master mix was followed by a brief centrifugation of the tubes to bring the contents to the bottom and then placed into the Smart Cycler.

Cycling conditions

After an initial denaturation cycle of 95°C for 150 sec, the polymerase reaction was stimulated over 30 thermal cycles; each consisting of 15 sec denaturation at 95°C, 30 sec annealing at 60°C and 30 sec extension at 72°C. When SYBR green was used, a further melt curve analysis was done with a 0.2 Deg/sec change in the temperature, starting at 60°C and ending at 95°C.

Results and Discussion

Ribosomal 18s RNA was assayed as a housekeeping gene in the real time PCR system to correct for sample to sample variations. High fluoresce indicative of high levels of transcript were found, the fluorescent signal was so high that it overwhelmed the smart cycler's ability to quantify it. Therefore, the cDNA was diluted 10 times to give a quantifiable signal.

When choosing a housekeeping gene, it is important that the sample content of this gene is similar to the content of the unknown gene, allowing for an accurate correction. The need to dilute the 18s sample compare to the MT-I sample to obtain similar amplicon signals suggested that 18s might not be the best housekeeping gene to be used with this system.

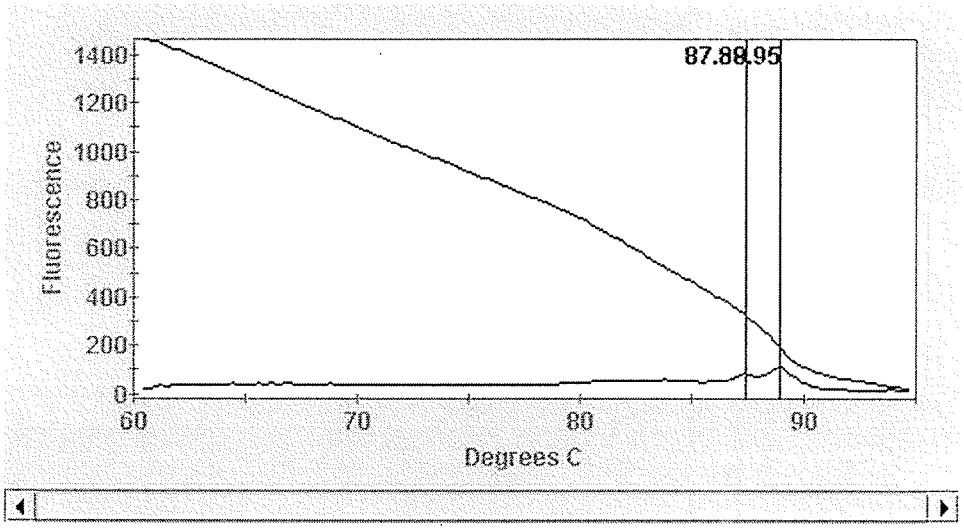
The inclusion of a housekeeping gene in real-time PCR allows to correct for sample to sample variation. The amplification of a cellular RNA that serves as an internal reference is suggested, this reference should be expressed at similar levels at any stage of the cell cycle. The comparison of changes in the unknown gene and the housekeeping gene allows for measurements due to cell activity. This becomes relevant

when the samples are obtained from different individuals and could lead to misinterpretation of the expression profiles of the target genes (Karge et al., 1998; Bustin, 2000).

The ideal housekeeping gene should be expressed at a constant level among different tissues within the organism and at all stages of development and should not be affected by the experimental treatment. There are several housekeeping genes that are commonly used, including β-actin, gyceradehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal RNA. Of these three genes, GAPDH has been shown to change with a variety of stimuli, like oxidative stress (Ito et al., 1996), Insulin (Barroso et al., 1999), growth hormone (Freyschuss et al., 1994), hypoxia (Zhong and Simons, 1999), etc. It is well known that MT is involved in the oxidative stress response in fish species (Schlenk et al, 1997; Kling and Olsson, 2000) which also suggests that GAPDH might not be the appropriate choice of housekeeping gene. Beta-actin was chosen as the housekeeping gene and the results obtained showed that this gene is present at a slightly higher concentration in the samples analyzed but was still in the same range as MT-I and MT-II.

The melt curve analysis of both MT-I and β-actin showed that the first sets of primers used were amplifying more than one product (Fig. 3.13). When designing MT-II primers that targeted the 5'UTR, the large AT content in that specific region precluded development of a primer. A high AT content in a sequence usually renders primers with

Figure 3.13 Melt curve analysis of the first set of MT-I primers. Note the appearance of two peaks in the fluorescence curve. The vertical lines define the melting temperature of the amplicons generated with this set of primers.


87.88,95
1400
1200
1000
800
600
400
200
0
Fluorescence
60
70
80
90
Degrees C

a low melting temperature. Primers with low melting temperatures are not useful in real-time PCR as this method takes advantage of an enzyme capable of transcribing cDNA at high temperatures to catalyze the extension of annealed oligonucleotide primers in a sequence specific manner.

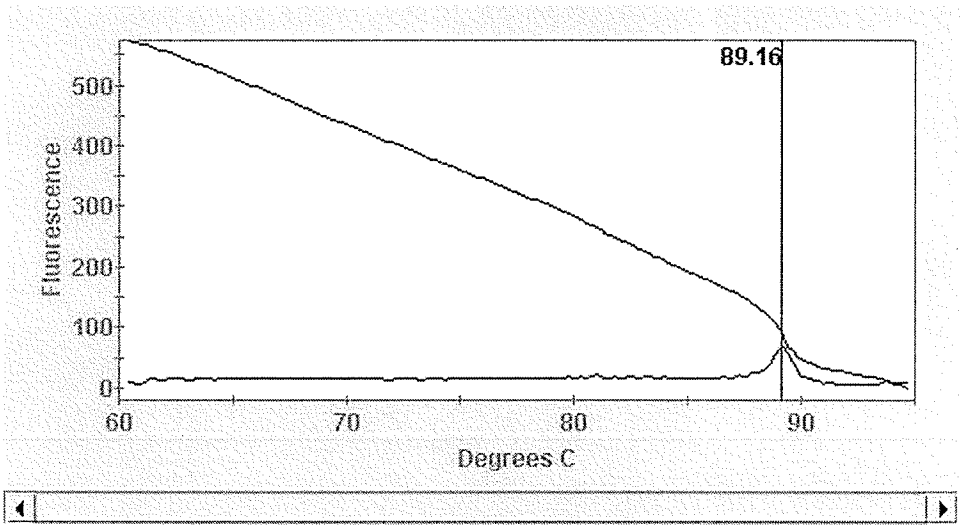
The melt curve analysis of the second set of primers gave only one product being amplified per primer set (Fig. 3.14). The second set of actin primers and probe were used at the same cDNA concentration as the concentration used for the quantification of MT-I and MT-II, making β-actin a better housekeeping gene for the purposes of this study.

The results obtained show that the set of primers targeting the differences in the sequence of the coding region of the rainbow trout gene are appropriate to quantify changes in MT-I and MT-II in the lake trout, *Salvelinus namaycush*. Furthermore, it has also been shown that the use of the β-actin gene as the housekeeping gene is a suitable choice, as its expression is similar to the expression of the genes in question.

Summary and Conclusions

Three different methodologies were developed to aid in the quantification of metallothionein. High performance liquid chromatography has been used to separate MT isoforms; two different approaches were used in this study, reversed-phase HPLC and ion exchange HPLC. Both techniques showed that separation of MT from commercially available standards was possible, but when the spectral analysis of the standards and the fish samples were compared differences were found. After careful analysis of the protein sequences of MT from both species it became obvious that the use of mammalian MT

Figure 3.14 Melt curve analysis of the second set of MT-I primers. The vertical line shows the melting temperature for the amplicon generated by this set of primers.


89.16
500
400
300
200
100
0
Fluorescence
60
70
80
90
Degrees C

protein as a standard for fish work is not appropriate, given the dissimilar nature of their sequences.

Western blot analysis was then chosen as a way to quantify metallothionein in fish tissues. A commercially available anti-Cod metallothionein antibody showing good cross-reaction with lake trout liver protein worked extremely well, once the appropriate blocking agent was utilized. As mentioned above, 5% non-fat dry milk commonly used in western blots is not useful when MT antibodies are to be used, but the use of 3% BSA worked very well. Another important thing to consider is that even though an antibody directed to fish species is commercially available, the concentration at which it is used varies among the target species. These differences are mainly due to the cross-reactivity of the antibody with the different organisms is targeting (Hylland et al., 1995), but good results were obtained in this experiment.

The third technique developed for the quantification of lake trout MT was real-time PCR. This technique allows for the quantification of mRNA when reverse transcription is performed prior to PCR. Problems inherent with this methodology are the difficulties encountered when looking for gene sequences of non modeled species. When analyzing samples from this kind of organisms the strategies followed to find suitable species whose sequences are known and that are quite close to the group in question become important. In the case of the lake trout, the sequences of rainbow trout were used based on the assumption that the gene sequence of this highly conserved protein is not that different between organisms of the same family (Hylland et al., 1995). When dealing with proteins that are not as conserved as metallothionein, different approaches need to be used, like comparing the sequence of closely related species and looking for

the most conserved part of those sequences in order to target that area when designing primers. In the case of MT this was not necessary and the primers used in these experiments worked well.

Finally, the use of western blot and real-time PCR allows for a rapid and accurate quantification of MT protein and mRNA, respectively. The technical aspects of these two techniques, once they are optimized, are easy to follow and highly reproducible. The data obtained with these techniques is of great value due to the possible use of MT as a bioindicator of contamination. On the other hand, HPLC is a more technically challenged technique but could provide useful information about the correlation between the 2 isoforms found in fish as well as its metal content. This could be useful when describing the physiological functions of this protein.

Chapter 4

Expression of metallothionein during a reproductive cycle in wild lake trout (*Salvelinus namaycush*) and the effects of cadmium and ethynylestradiol

Introduction

Over the past two decades the use of biochemical endpoints has become an increasingly important tool for evaluating the effects of exposure to chemicals in wild organisms. Bioindicators allow rapid assessments of organism health but also relate dose- or time- dependent exposures to the degree of dysfunction that a contaminant has produced (Huggett et al., 1992). Specific indicators include enzyme activities or proteins that respond to classes of chemicals. Initially, their relevance to higher level effects may not be completely understood or established, but because toxicological responses to a chemical are mediated by the interaction between the chemical and a receptor, biochemical responses are presumed to occur before effects at higher levels of organization. Therefore subcellular bioindicators have the greatest potential as early warning indicators of contaminant specific effects (Roberts and Oris, 2004). Before bioindicators can be effectively applied, some knowledge of their natural variability is required (Livingstone, 1993). Seasonal changes arising from biotic and abiotic factors such as metabolism, feeding status, sexual cycle, temperature, and salinity can all potentially affect basal levels of many bioindicators and alter their responses to pollutants (Olsson et al., 1987; Ronisz et al., 1999; Gorbi et al., 2005).

Metallothionein (MT) is a low molecular weight protein that has been proposed for use as a bioindicator of metal exposure in many animals. MT is expressed in a dose dependent manner in aquatic organisms exposed to class IB and IIB metals (Couillard et al., 1993, 1995, 1997; Roeva et al., 1999; Wu et al., 1999; Van Campenhout et al., 2004; Xie and Klerks, 2004). However MT has also been shown to respond to other stressors such as infection, oxidative stress, glucocorticoids, starvation, inflammation, X-

irradiation, etc (Olsson et al., 1987; Ronisz et al., 1999; Wu et al., 1999; Gorbi et al., 2005). Furthermore, while MT concentrations increase after initial metal exposure (McClain et al., 2003; Heerden et al., 2004; Roberts and Oris, 2004), longer-term exposures have produced mixed results. For example, MT increased in white prawns (*Litopenaeus vannamei*) exposed to waterborne Cd (0.2 mg $CdSO_4$/L) after one month, declined after two months and after three months of exposure there was an overall decrease in MT concentrations (Wu and Chen, 2005).

The effects of long term exposure to environmentally realistic concentrations of Cd were investigated in a four year whole lake addition experiment at the Experimental Lakes Area (ELA) in northwestern Ontario, Canada (Malley et al., 1991). The main objective of this study was to evaluate the effectiveness of the current Canadian Water Quality Guideline for Cd (0.2 μg/L for water hardness less than 60 mg/L calcium carbonate) for protecting aquatic life. A secondary objective was to evaluate the usefulness of MT as an early warning bioindicator of disruption at higher levels of organization. A well-defined population of wild lake trout (*Salvelinus namaycush*) was resident in the study lake, providing an opportunity to examine the effects of long term Cd exposure on MT and its expression over seasons, throughout reproductive cycle.

Fifteen years after Cd additions were terminated, the concentrations of Cd remained elevated in tissues of lake trout from the treated lake relative to the nearby reference lake (V. Palace, DFO, Winnipeg, personal com.). Since the time of this experiment, additional information regarding natural variability of MT in fish has become available. For example, it has been suggested that metallothionein levels change during the reproductive cycle of fish in response to hormone changes and physiological

requirements for the essential metal Zn (Olsson et al., 1987). It is not known if the changes in MT concentration observed under normal physiological conditions also occur when fish are exposed to heavy metal concentrations that could potentially induce MT chronically.

More recently, hormone active compounds have been identified in receiving waters (Belfroid et al., 1999; Ternes et al., 1999; Arukwe et al., 2000). How these impact Zn mobility and MT concentrations is also not known. Similar to the Cd whole lake experiment, a recent whole-lake addition of ethynylestradiol (EE2), the active component of the birth control pill, offered an opportunity to examine variability in the MT response (Palace et al., 2006). Ethynylestradiol is found in receiving waters downstream from sewage treatment plants (Harries et al., 1996, 1997; Belfroid et al., 1999; Solè et al., 2000). MT concentrations were examined in lake trout from the lakes treated with Cd and EE2 to examine the effect of prolonged exposures to these compounds on MT expression. Given that the fish are often exposed to hormone active compounds and metals in complex effluents, the information gained from these experiments is relevant to understanding variability and for the future application of MT as a biomarker.

Material and Methods

Cadmium additon

Starting in June 1987, Cd was added to the epilimnion (the upper most layer of the water column) of Lake 382 continuously during the ice-free season. Target concentrations were 0.10, 0.12 and 0.16 μg/L for each of the years from 1987 to 1990,

respectively. Over the four years, a total of 3.8 Kg of Cd were added raising the Cd water concentration in the epilimnion to 0.16 μg/L (Malley et al., 1991).

Ethynylestradiol addition

17α-ethynylestradiol was added biweekly to the epilimnion of Lake 260 during the ice-free season as described by Palace et al. (2006). Mean concentrations of EE2 in epilimnetic waters ranged between 4.5 and 8.1 ng/L during the three years (2001-2003) (Palace et al., 2006).

Fish collections and sampling

Mature female lake trout were collected from Lakes 468 (reference), 382 (Cd addition), 442 (reference) and 260 (EE2 addition) during the fall, winter, spring and summer of 2000-2001 (lakes 382 and 468) and 2001-2002 (lakes 260 and 442) sampling periods. Adult lake trout were collected with gill nets retrieved at 30 min intervals. Upon retrieval, fish were anaesthetised for 5 min in buffered (pH 7.0) tricaine methanesulphonate (MS222; 250 mg/L). Weight and length were measured and condition factor ($K=Wt/L^3 \times 100$) determined. Liver and gonads were dissected and weighed to obtain liver (LSI=[Liver Wt/Wt-Liver Wt] x 100) and gonadal (GSI=[Gonad Wt/Wt-Gonad Wt] x 100) somatic indices. After weighing, livers were immediately frozen on dry ice and transported to the Freshwater Institute where they were kept at -90°C until analysed.

Western blot analysis of metallothionein protein

Liver tissue was homogenized in 10 mM NaH_2PO_4 pH 7.0; 5mM 2-mercaptoethanol, heat treated at 95°C for 5 min, cooled for 4 min and centrifuged at 20,000 x g for 90 min. The supernatant was used to separate and identify MT using western blots.

SDS/PAGE was performed as described by Laemmli (1970), using a 16.5% Tris-Tricine precast gel system (Bio-rad, Hercules, CA). Electrophoresis was carried out at 100 mA (constant current). The separated proteins were then transferred onto 0.2 μm Immobilon P^{sq} PVDF membranes (Millipore Corp, Bedford, MA) for 1 hr at 100 V (constant voltage). The membranes were blocked with 3% BSA at room temperature followed by 1 hr incubation with the primary antibody (anti Cod-MT). After washing the excess primary antibody, the membranes were incubated with secondary antibody (horseradish peroxidase goat anti rabbit antibody) for 1 hr and again washed. The membranes were then exposed to developer solution (Super signal west Pico chemiluminescent substrate, Pierce Biotech, Rockford IL) and exposed to film for 8 min to reveal the results. The density of the bands obtained was analysed using a FluorChem IS-8900 imaging system (Alpha Innotech, San Leandro, CA).

RNA extraction

Total RNA was obtained from liver tissue of fish from each of the experimental Lakes using the Trizol extraction protocol as described in Chapter 3. Briefly, 100 mg of liver tissue were homogenized in Trizol reagent (Invitrogen, Corp., Carlsbad CA) using a polytron homogenizer. The homogenate was incubated at room temperature for 5 min,

followed by the addition of chloroform. The tubes were shaken vigorously for 15 seconds, incubated at room temperature for 3 min and centrifuged at 12,000 x g for 15 min at 4°C. The top layer was transferred to new tubes and isopropanol was added followed by a 10 min incubation at room temperature. The tubes were centrifuged at 12,000 x g for 10 min at 4°C, the isopropanol discarded and 75% ethanol was added to the pellet and incubated for 1 hour at -20°C. The tubes were centrifuged at 7,500 x g for 5 min, the ethanol discarded, and the tubes let to dry. Once dried, the pellet was resuspended in 100 µl of DEPC water and kept in the -70°C freezer until analyzed.

Reverse transcription of the extracted mRNA was done using the Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) kit from Invitrogen (Carlsbad, CA) following manufacturers recommendations. Briefly, 1µg total RNA was added to chilled microcentrifuge tubes followed by the addition of 200 U/µl MMLV-RT, 5X Reverse transcriptase buffer (250 mM Tris HCl, pH 8.3; 375 mM KCl; 15 mM $MgCl_2$), 40 U/µl RNase out, 0.1M DTT, 0.1% BSA, 250 ng RT primer (5'gactcgagtcaacatcgattt$(ttt)^5$tt) and 5 mM dNTP. The tubes were incubated at 37°C for 2 hours and kept at -20°C until analysed.

Northern blot probe development and preparation

A probe for the MT-I gene of rainbow trout (*Oncorhynchus mykiss*) was developed using the program *GeneFisher* - Software Support for the Detection of Postulated Genes (www.techfak.uni-bielefeld.de/ags/pi/GeneFisher/). The sequence of the probe targeted the 5'UTR region of the MT-I gene. The primers generated by this program were as follows: MTA forward caccactgacacccag and MTA reverse

tgtcatacacaggcctc. These primers were used to amplify the MT-I cDNA obtained from liver samples. In order to optimize the reaction 1 or 3 μg of cDNA and 30, 35 or 40 PCR thermal cycles were used to amplify the product. The PCR reaction was performed in a Techne cyclogene thermocycler (Techne, Cambridge, UK). Briefly, 1 or 3 μg of cDNA were added to pre-chilled PCR reaction tubes followed by addition of a master mix containing 5 u/μl Taq DNA polymerase (Invitrogen, Corp., Carlsbad, CA), 50 μM $MgCl_2$, 50 μM MTA forward, 50 μM MTA reverse and 10 mM dNTP. The tubes were placed in the thermocycler programmed to denature the cDNA at 94°C for 1 min, anneal the primers at 52°C for 1 minute and finally to extend the product at 72°C for 1 min. The reaction was allowed to cycle 40 times and samples were taken at 30, 35 and 40 cycles. The PCR products obtained were kept at -20°C until further analysis.

Once amplified the PCR products were run on a 1% Agarose gel in TBE (89 mM Tris, 89 mM Boric Acid, 2 mM Ethylenediaminetetraacetic acid or EDTA, pH 8.4) and 10 mg/ml Ethidium Bromide (EtBr) to separate the product for further purification.

Probe purification and labelling

Once the PCR products were run, the agarose gel was exposed to ultraviolet light to visualize the band or bands obtained after the PCR. A single band confirmed the specificity of the primer. The band was cut out of the gel and cleaned using the Perfctprep gel cleanup kit (Eppendorf AG, Hamburg Germany) following manufacturer recommendations. Briefly, the band cut out of the gel was placed in a microcentrifuge tube and 3 μl of binding buffer was added per 1 mg of gel cut out, followed by a 10 minute incubation at 50°C vortexing the solution every 2 – 3 min. One volume of

isopropanol / mg of band was added and mixed by inversion. Up to 800 µl of sample were added to a spin column in the collection tube provided. The sample was then centrifuged at 10,000 x g for 1 min and the filtrate discarded. The pellet was washed with 750µl of wash buffer, centrifuged at 10,000 x g for 1 min and the filtrate discarded. The purified DNA was the eluted from the column with 30 µl of elution buffer by centrifugation at 10,000 x g for 1 min. The purified DNA was stored at -20°C until it was labelled.

The probe was labelled using Rediprime II random prime labelling system (Amersham Pharmacia, Pittsburgh PA). 25 ng of DNA were diluted in 45µl TE (10 mM Tris HCl pH 8.0, 1 mM EDTA) buffer. The DNA was the denatured by heating to 100°C for 5 min and snap cooled on ice for 5 min after denaturation. The denatured DNA was mixed with 5 µl Redivue [^{32}P] dCTP, incubated at 37°C for 10 min and the reaction stopped by the addition of 0.2 M EDTA.

Once the probe was labelled, it was separated from any unbound radioactive isotope using a sephadex G50 in $T_{10}E_1$ (10 mM Tris; 1 mM EDTA; pH 7.5) column. The fraction obtained was mixed with pre-hybridization buffer (50% formamide, 5X Denhardt's solution [1% Ficoll 400, 1% polyvinylpyrrolidone, 1% BSA fraction V], 5X SSPE [3 M Sodium Choride, 0.2 M Sodium phosphate, 0.02 M EDTA], 0.1% Sodium dodecylsuphate [SDS]; 10µg/ml denatured salmon sperm DNA) boiled for 5 min, cooled on a mixture of ethanol plus dry ice and added to the membrane for hybridization.

Northern blot analysis

Electrophoresis and transfer

RNA obtained from the liver samples of fish from the different lakes was separated in 1% Agarose, 2.2 M Formaldehyde (37%) and 10 mg/ml EtBr. Before loading the sample onto the gel, 40 µg of RNA in formamide-formaldehyde were placed in a 65°C water bath for 15 min, cooled on ice water and mixed with gel loading solution type I (Sigma-Aldrich, Inc. St Louis, MO). The gel was run at 100 V for approximately 4 hours. Following separation, the mRNA was transferred onto a 0.45 µm Optitran supported nitrocellulose membrane (Schleicher & Schuell BioScience, Inc. Keene NH) by capillary blotting. Briefly, a glass plate was placed in a glass dish containing 20X SSC (3.0 M Sodium chloride; 0.3 M Sodium citrate). Two pieces of Whatman 3M paper covered the glass plate and extended into the buffer. The gel was placed onto the Whatman paper ensuring that no air bubbles were trapped between them. Plastic wrap was placed over the edges of the gel to prevent any loss of buffer to the sides of the glass plate. The membrane, previously wetted was placed on top of the gel, again excluding air bubbles. Two more pieces of Whatman paper and ~ 6cm of paper towels were held down with a weight on top of the membrane and left overnight. After the capillary blotting was complete, the membrane was baked at 85°C for 2 hrs in a vacuum oven and kept at -80°C until hybridized (< 2 weeks).

Northern blot hybridization

The membrane was incubated with pre-hybridization solution for 2 hrs in a hybridization oven set at 42°C and rotating at 5 rpm. The membrane was then incubated

with the probe in the hybridization oven for 18 hrs at the same conditions. Following hybridization the membrane was washed in 2 X SSC plus 0.1% SDS to remove unbound probe, a second wash (0.1X SSC, 0.1% SDS) was performed to ensure that only the probe tightly bound to the membrane was left. The membrane was exposed to photographic film, kept in the -80°C freezer overnight and developed to reveal the results. The bands obtained were analyzed by densitometry using a FluorChem IS-8900 imaging system (Alpha Innotech, San Leandro, CA).

Real time PCR

The program Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) was used to design Taqman probes and primers for MT-I (M18103) and MT-II (M18104), as well as for the housekeeping gene β-actin to use in the real-time PCR assays (as described in chapter 3). For the real-time PCR experiments all the data was expressed as changes in the inverse of the relative ΔCt (MT isoform-Actin). This way of expressing the data was chosen to more easily compare the data obtained with the real-time PCR and the data obtained with the northern blot analysis. This became necessary because a lower value of ΔCt signifies an increase in induction of the gene in question. Conversely, in northern blot an increase in density of the band means an increase in the levels of mRNA present in the sample.

Data analysis

For western and northern blot analysis, a one way ANOVA followed by Dunnet's or Tukey's multiple comparison tests were used to analyze the differences between the reference lakes versus the treated lakes. Statistical significance was accepted at $p<0.05$.

For real-time PCR a pair wise fixed reallocation randomisation test was performed. This test is based on the probability of the observed effect occurring under the hypothesis of no treatment effect. If this hypothesis is true, the values in one treatment group were just as likely to have occurred in the other group. The randomization test repeatedly and randomly reallocates the observed values to the two groups, and notes the apparent effect each time (Pfaffl, 2001; Pfaffl et al., 2002).

Results and Discussion

Somatic parameters

Somatic parameters for fish from reference and treated lakes are shown in Table 4.1. Significant differences were found in length among fish from different lakes in each season. There were differences in the length of fish captured throughout the year only in Lake 382, where fish from the fall and winter were significantly longer than fish sampled during summer and spring. Fish from Lake 382 were consistently (30%) lighter that fish from the other lakes. There were no significant differences in weight of fish within any of the lakes throughout the year. The differences in length and weight observed in Lake 382 could be attributed to their chronic Cd exposure A decrease in growth rate in fish chronically exposed to Cd has been associated with a shift in energy

Table 4.1 Mean somatic parameters of female lake trout from reference (Lake 468 and Lake 442 and Cd added (Lake 382) and EE2 added (Lake 260).

Season	Lake	n	Length (cm)	Weight (g)	GSI	LSI	CF
Fall							
	Lake 468	4	44.2±6.7[1]	1021±199[1]	12.52±2.20[1,a,b,c]	0.44±0.09[a,b,c]	1.15±0.06[2]
	Lake 442	4	39.8±0.5	740±28	11.59±0.78[1,a,b,c]	0.50±0.04[1,a,b,c]	1.17±0.08[1]
	Lake 382	4	37.2±0.4[1,2,a]	552±23[1]	ND	0.41±0.03[a,b,c]	1.07±0.02
	Lake 260	4	42.5±0.6[2]	766±36	3.27±2.56[1]	0.31±0.02[1,a,b]	0.99±0.11[1,2]
Winter							
	Lake 468	3	39.2±0.9[1]	688±77	1.14±0.36[a]	1.22±0.17[a]	1.13±0.11
	Lake 442	3	42.3±1.7[2]	808±148	1.11±0.09[a]	0.99±0.13[a]	1.04±0.09
	Lake 382	3	37.8±1.0[1,2,b]	451±27	0.71±0.08[a]	0.77±0.08[a]	1.07±0.06
Spring							
	Lake 468	4	47.9±5.7[1]	1305±494[1]	1.48±0.39[1,b]	0.98±0.03[1,b]	1.06±0.04
	Lake 442	4	38.92±1.09	648±49[2,4]	1.44±0.43[1,b]	0.99±0.02[2,b]	1.03±0.03
	Lake 382	3	36.8±0.65[1,2,b]	490±6[1,2,3,]	0.99±0.32[b]	0.73±0.01[1,2,b]	0.98±0.08
	Lake 260	4	44.7±1.18[2]	851±50[3,4]	0.83±0.38	0.77±0.08[b]	0.99±0.08
Summer							
	Lake 468	4	39.47±1.58	700±77	4.40±1.51[c]	1.24±0.08[1,3,c]	1.12±0.02
	Lake 442	3	38.87±1.96	693±112	3.05±1.40[c]	1.26±0.13[2,4,c]	1.16±0.04[1]
	Lake 382	4	36.35±0.81[1,a]	515±47	3.21±0.40[a,b]	0.89±0.09[1,2,c]	1.06±0.06
	Lake 260	3	42.96±1.79[1]	836±115	2.537±1.18	0.79±0.05[3,4,c]	1.04±0.05[1]

Means labelled with the same number are significantly different within the same season (ANOVA followed by Tukeys or ANOVA on ranks followed by Dunn's test). Means labelled with same superscript letter are significantly different within the same lake (ANOVA followed by Bonferroni or ANOVA on ranks followed y Dunn's test). Values are expressed as mean ± SD; $p<0.05$.

metabolism from growth to tissue repair, detoxification and elimination of this toxicant (Reynders et al., 2006; Szczerbik et al., 2006).

Condition factor was lower in Lake 260 during the fall and summer compared to the reference sites. No other significant differences in condition were found in fish from any of the lakes.

As expected, gonad size and the calculated GSI increased in the fall before spawning and dropped significantly after spawning. Adequate numbers of pre-spawning fish could not be obtained from Lakes 382 and 260 (Table 4.1).

Liver size and the calculated LSI varied seasonally with high values in the spring and summer, lower values in the winter and the lowest weight in the fall. The decrease during the fall is likely due to a depletion of hepatic lipids that are being transferred into the gonads (Jerez et al., 2006). Similarly, the increase in LSI observed in spring and summer in all groups is likely due to an increase in the synthesis of the yolk precursor lipophosphoglycoprotein VTG, in the liver (Carlson and Williams, 1999; Gerpe et al., 2000; Norris et al., 2000).

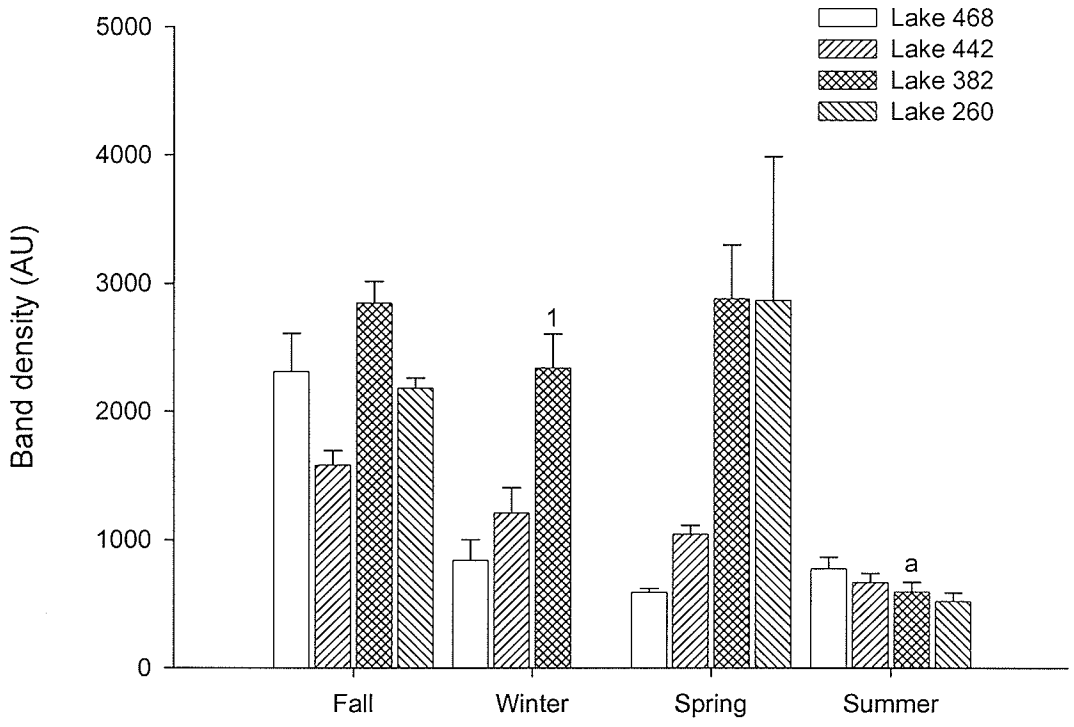
Lake trout from Lake 382 had significantly lower LSI compared to both reference lakes during the spring and summer. Similar findings were obtained in brown trout (*Salmo trutta*) sampled from Eagle river, a tributary of the Colorado River that contains 1.2 μg/L Cd. In those fish, a significant decrease in LSI compared to fish from uncontaminated part of the same river was observed (Norris et al., 2000). In juvenile carp exposed to 1.6 μg/l waterborne Cd for 14 days, a decrease in LSI was also observed when the exposed fish were compared to non-exposed fish (de la Torre et al., 2000).

LSI in fish from Lake 260 was significantly lower compared to the reference lakes during the fall and summer. These findings are contrary to what has been observed in fish treated with estradiol (Van Bohemen et al., 1982; Hogstrand et al., 1996; Olsson et al., 1995) or ethynylestradiol (Werner et al., 2003) where an increase in LSI was observed as a result of the treatment. It is possible that these fish are not spending enough time in the epiliminion, where the EE2 was added. Lake trout spend most of their time in the deepest layer of the watercolumn (hypolimnion) and only move to the epilimnion to feed, therefore the time that they effectively are exposed to this compound is minimal compared to for example, pearl dace or fathead minnows (Palace et al., 2002; 2006). The concentration of EE2 is also a factor that could determine the differences observed in this study. In chapter 2 it was shown that lake trout respond to high concentrations of EE2 (40 -400 ng/L), but at low concentrations like the ones achieved in the whole lake addition, lake trout only showed induction of VTG without any changes in MT concentrations (Werner et al., 2003).

Western blot analysis

Western blot analysis was used to evaluate hepatic MT protein in fish from lakes 382 (Cd added) and 260 (EE2 added) as well as the two reference lakes (Fig. 4.1). In the reference lakes, an increase in MT protein was observed during the fall, followed by a decrease during winter, spring and summer, where the lowest levels in MT protein content were found. The decrease in MT content during the summer compared to the other seasons suggests a correlation between the start of production of vitellogenin and low MT content. The increase in VTG related metabolism would be expected to coincide

Figure 4.1 Western blot analysis of liver metallothionein protein from fish exposed to Cd (Lake 382), EE2 (Lake 260) and fish from the reference lakes (Lakes 468 and 442) during the 2000-2001 season. The data are presented as the density of the bands obtained from western blots. [1]Significantly different from the reference lakes during the same season. [a]Significantly different from the fall, winter and spring. $P<0.05$, data are expressed as mean ± SEM (n=4).


Lake 468
Lake 442
Lake 382
Lake 260
Band density (AU)
5000
4000
3000
2000
1000
0
1
a
Fall
Winter
Spring
Summer

with the concomitant use of the essential metals Cu and Zn as cofactors for enzyme activity and a decrease in MT content (Olsson et al., 1987; Roesijadi, 1992, 1994; Olsson et al., 1996; Olsson et al., 1996b; Gorbi et al., 2005). The increase of MT concentrations in the fall coincides with the end of vitellogenesis and the beginning of spawning. The Zn that had been incorporated into organelles for the production of VTG would become free, subsequently inducing an increase in MT. It has been shown that during the winter months, food might not be readily available in northern climates (Morgan et al., 2000; Riddell et al., 2005; Lapointe et al., 2006). This would explain a decrease in MT during the winter months.

As in fish from the other lakes, fish from Lake 382 had the lowest MT protein content in summer. However, whereas fish from reference lakes had relatively low MT content in winter and spring, concentrations remained high in fish from Lake 382 at these times. MT was significantly higher in Lake 382 relative to the other lakes in the winter. The elevated levels of MT protein found in lake trout from Lake 382 suggest that the Cd added 15 years ago is still present in the water column. As a result MT protein is still upregulated in these fish.

It was anticipated that EE2 exposure might down-regulate MT concentrations as a secondary result of chronic induction of vitellogenin production (Palace 2006; Olsson et al., 1989). However, Figure 4.1 shows that MT concentrations in fish from Lake 260 were similar to those in reference lakes at each of the sample seasons. The concentrations of EE2 added to lake 260 were sufficient to greatly alter VTG production in resident fathead minnows, *Pimephales promelas* (ie. 10,000 fold induction of VTG) (Palace et al., 2002), pearl dace, *Margariscus margarita* (ie. 1500 – 3000 fold induction),

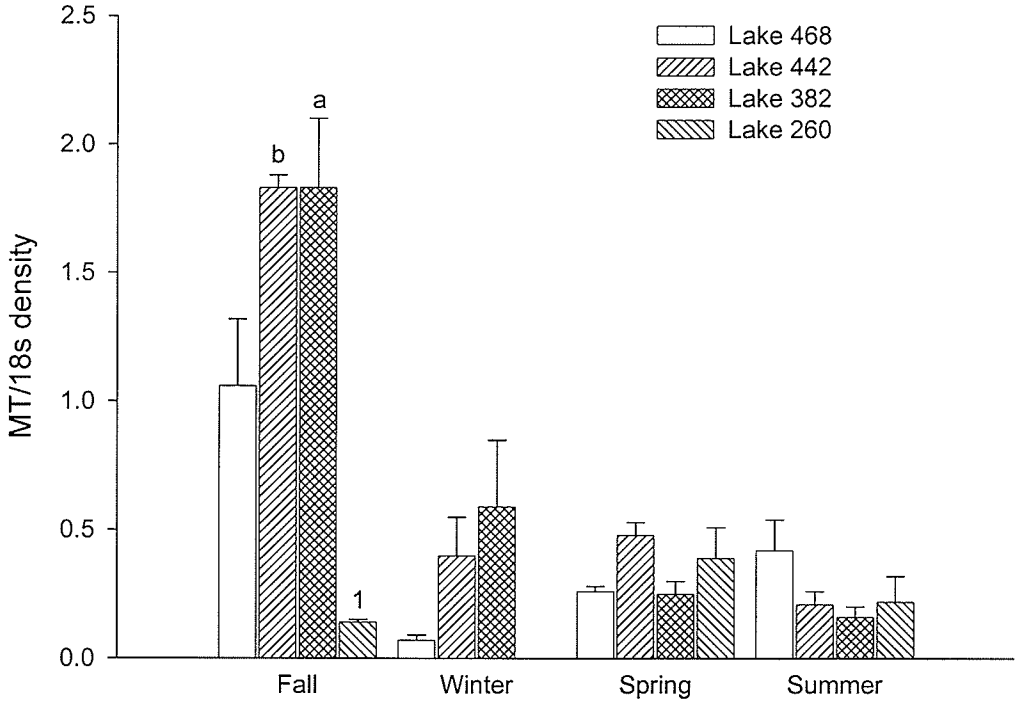
(Palace et al., 2006) and lake trout (V. Palace, unpublished Observ.). But the induction of VTG was far lower in lake trout than in pearl dace and fathead minnows collected from the same lake. Lake trout are residents of the deepest portions of the lake (hypolimnion) where EE2 concentrations were significantly lower (e.g. 1-2 ng/L) (Palace et al., 2002) compared to the concentration in the epilimnion where the fathead minnows and pearl dace reside. The low exposure dose of EE2 for lake trout in this study may explain why MT concentrations were not significantly altered relative to fish from reference systems. This is consistent with the results from chapter 2 where lower exposure doses of EE2 (4ng/L) were insufficient to alter hepatic MT concentrations.

Northern blot analysis

Results from northern blot analysis of MT mRNA in fish from lakes 382 (Cd), 260 (EE2) and both reference lakes are shown in Fig. 4.2. Similar to the results of protein analysis, MT-mRNA was generally highest in the fall relative to the other seasons. MT mRNA was down regulated in fish from lakes 260 and 382 during the fall, relative to the other lakes. There were no statistical differences between any of the lakes during the other three seasons.

The increase observed in MT mRNA during the fall in all lakes with the exception of Lake 260, is in agreement with what has been observed in other fall spawning species. At the end of the period of vitellogenesis, a redistribution of the Zn liberated from the enzymes involved in this process effectively induces the transcription of the MT gene (Olsson et al., 1987, Roesijadi, 1992; Olsson et al., 1996b). Once the gene is translated the newly synthesized protein can bind the excess Zn from the cytosol and in this way

Figure 4.2 Northern blot analysis of metallothionein mRNA in lakes 468, 442 (reference), 382 (Cd addition) and 260 (EE2 addition) during the 2000-2001) season. [1]Significantly different from lakes 468, 442 and 382. [a]Significantly different from the summer. [b]Significantly different from winter, spring and summer ($P<0.05$). Data are expressed as mean ± SEM (n=4).


2.5
2.0
1.5
1.0
0.5
0.0
MT/18s density
Lake 468
Lake 442
Lake 382
Lake 260
a
b
1
Fall
Winter
Spring
Summer

prevent the toxic effects of this metal. The newly synthesized protein also provides a pool of available Zn for the next round of vitellogenesis or any other metabolic function that requires this metal as a cofactor.

Prolonged Cd accumulation in fish from Lake 382 was expected to induce increased levels of protein as well as MT mRNA. Contrary to what was found with the protein content, northern blot analysis of MT mRNA did not show an increased induction in the fish from Lake 382 when compared to the reference lakes. The differences between protein and mRNA induction have been previously shown in other systems, suggesting that MT expression may be regulated transcriptionally as well as translationally (Bargelloni et al., 1999; Vasconcelos et al., 2002). It is also possible that the fish from Lake 382 being constantly exposed to Cd, and having elevated protein levels are not faced with the need of *de novo* synthesis of the protein to successfully handle the Cd present in their system and the Zn used during normal metabolism.

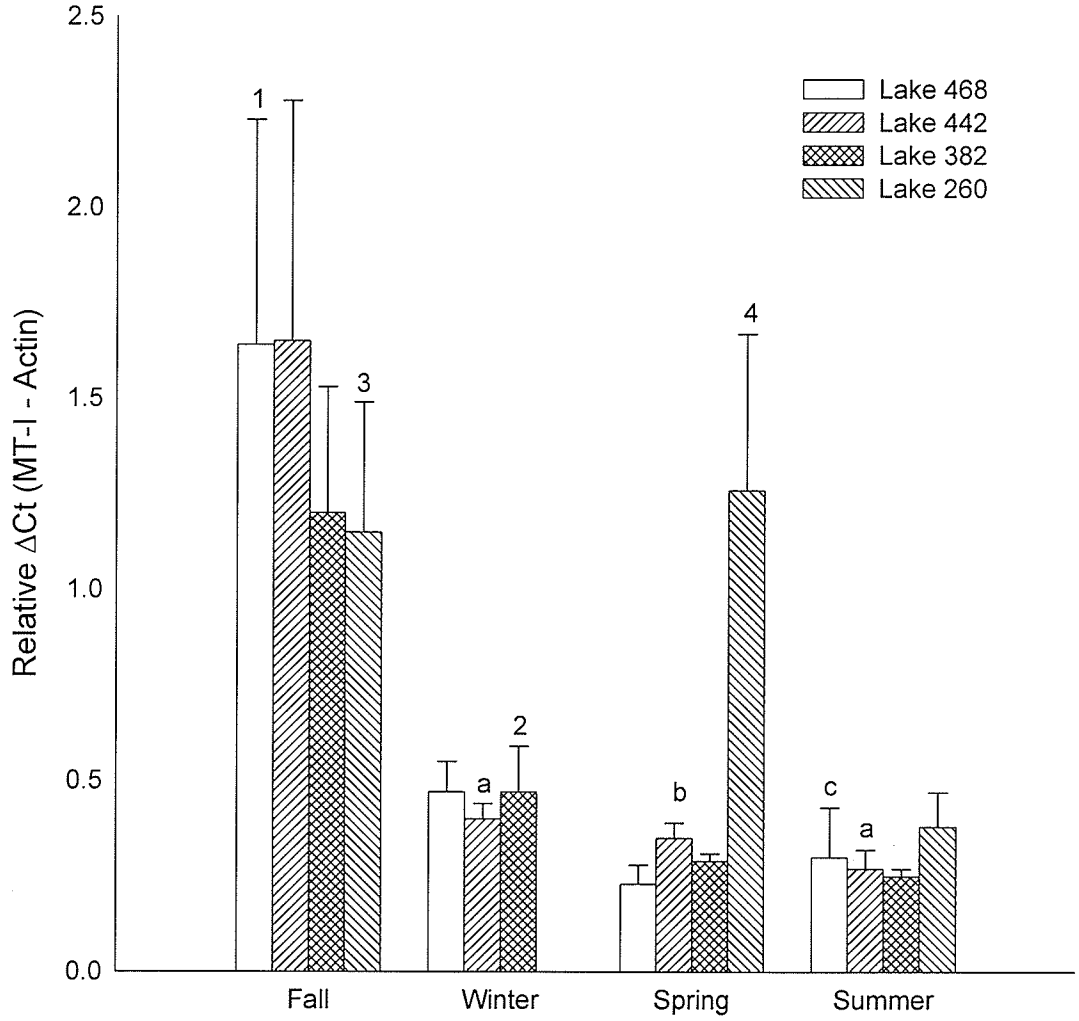
Female lake trout from Lake 260 had no significant differences in MT mRNA content in any of the seasons studied. Contrary to what was observed with the other lakes studied, fish from Lake 260 did not show an increase in MT mRNA during the fall. In fact, compared to the other lakes, MT mRNA was down-regulated in this season. It has been reported that when estrogen levels are high enough to induce changes in MT expression, a down-regulation of MT mRNA as well as a decrease in MT is observed (Olsson et al., 1987). In this study a decrease in MT mRNA during the fall was observed, but a decrease in protein during this season was not. It is possible that these changes reflect the increased sensitivity of MT mRNA as an indicator of EE2 exposure. While this idea requires further investigation, mRNA expression has been used as an early

indicator of EE2 exposure in fathead minnows, where VTG mRNA induction has been observed earlier than the changes in protein (Lattier et al., 2002; Scholz et al., 2004). During spring and summer MT mRNA followed a similar pattern to what was observed in the other lakes. This suggest that the EE2 concentration present in the lake was either not sufficiently high to induce further changes (Werner et al., 2003), or that the lake trout are not spending sufficient time in the epilimnion to be affected by the EE2.

Real time PCR

Real time PCR analysis was performed to examine the specific seasonal differences between mRNA of the two-metallothionein isoforms in lake trout from the study lakes (Fig. 4.3). When fish from each lake were analyzed separately during their respective reproductive cycles, both reference lakes showed an increase in MT-I mRNA content during the fall followed by a decrease in winter, spring and summer. The changes were significantly different in the fall and winter for Lake 468, but not for Lake 442. In Lake 382 there was a slight increase in MT-I mRNA during the fall that decreased during the winter and spring ($P<0.05$), reaching its lowest point during the summer. The changes were different in Lake 260 where the highest levels of MT mRNAwere found during the spring ($p<0.05$), while summer and fall were lower. The changes in MT-I mRNA induction during the fall in all lakes are in agreement with the idea of increased induction at the end of the period of exogenous vitellogenesis. During gonad development an increase in metabolic activity resulting in an increase in the utilization of Zn, would keep the levels of MT low (winter spring and summer). At the end of vitellogenesis, a release of Zn from the enzymes involved in this process

Figure 4.3 MT-I mRNA content in livers of female lake trout exposed to Cd in lake 382, to EE2 in lake 260 and from the reference lakes (468 and 442) as determined by real time PCR. [1]Significantly different from the winter. [2]Significantly different from fall and spring. [3]Significantly different from spring and summer. [4]Significantly different from summer. [a]Significantly different from lake 382. [b]Significantly different from lake 260. [c]Significantly different from lake 260. $P<0.05$, data are expressed as mean ± SEM (n=4).

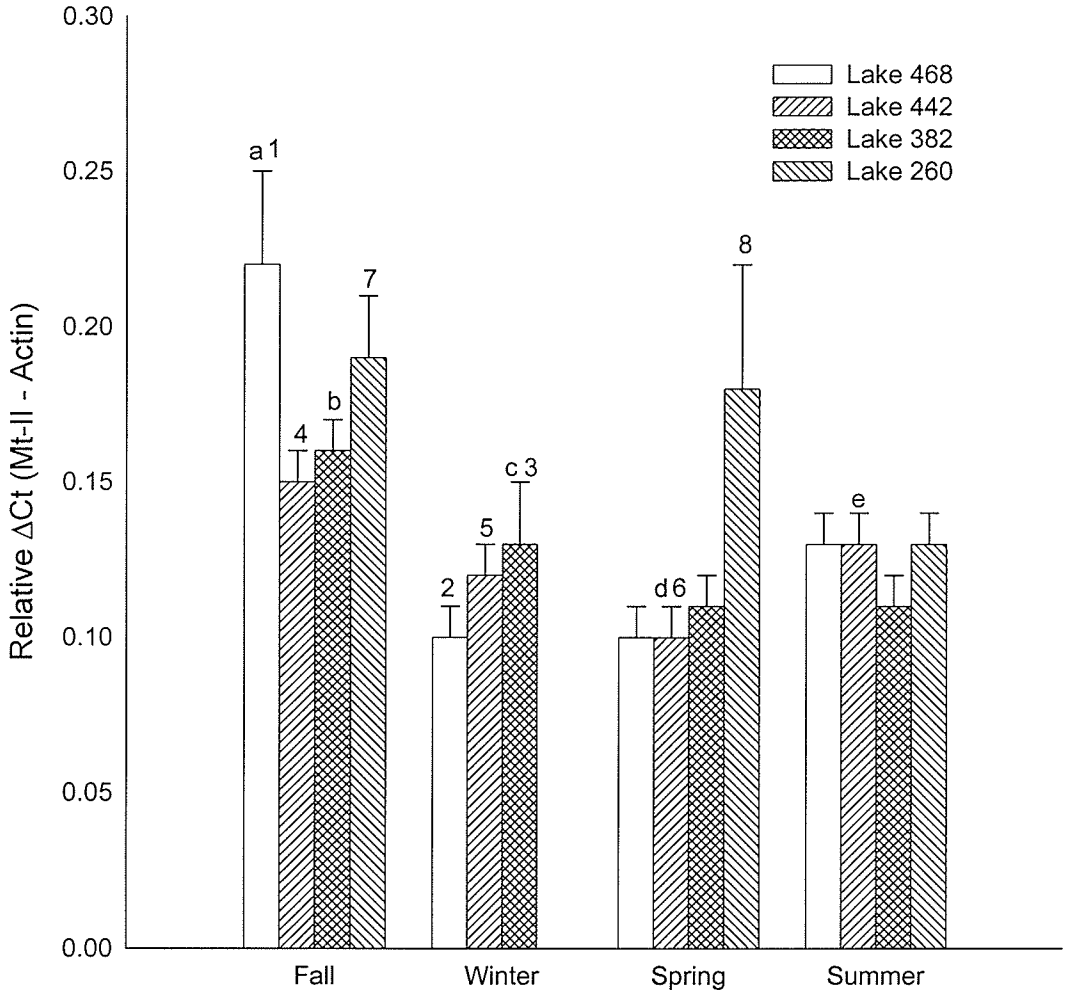

2.5
2.0
1.5
1.0
0.5
0.0
Relative ΔCt (MT-I - Actin)
Lake 468
Lake 442
Lake 382
Lake 260
1
2
3
4
a
b
c
a
Fall
Winter
Spring
Summer

induces the transcription of the MT-I gene and the production of MT protein (Olsson et al., 1987; Roesijadi, 1994; Hogstrand et al.,, 1995; Olsson et al., 1996b; Banks et al., 1999; Van Cleef et al., 2000; Noaksson et al., 2004; Gorbi et al., 2005). The specificity in the response of this isoform is the result of the differential binding of metals to each of the two isoforms. In salmonid fish, MT-I preferentially binds Zn while MT-II preferentially binds Cu (Zafarullah et al., 1990). Although a decrease during the fall was also observed using real-time PCR, it was not as significant as the results obtained when northern blot was used to analyze the same samples. The down-regulation of MT-I mRNA during the fall could be an early indication of EE2 exposure. The concentrations of EE2 encountered by lake trout in this experiment were not high enough to elicit a response at the level of protein, but might be enough to induce some changes in mRNA content. Additional studies are required.

MT-II mRNA (Fig. 4.4) concentrations were also analysed using real-time PCR. As with the protein and MT-I mRNA, expression was highest in fish captured in fall from each of the lakes. MT-II mRNA expression was similar to the pattern of MT-I mRNA expression. This was surprising because MT-II preferentially binds Cu (Zafarullah et al., 1989; 1989b; 1990) and during a reproductive cycle, the levels of Cu do not change (Olsson et al., 1987). While MT-I and MT-II isoforms preferentially bind Zn and Cu respectively, both can bind Cu and Zn (Zafarullah et al., 1989). Therefore it is possible that the changes observed in MT-II are a reflection of MT-II binding Zn when that element is liberated from enzyme binding sites.

MT-I expression was approximately three times higher than MT-II. It has been shown that MT-I has three pairs of MRE, but there is only one cluster so far identified in

Figure 4.4 Real time PCR content in MT-II mRNA content in livers from female lake trout exposed to Cd (lake 382), EE2 (lake 260) and females from the reference lakes (468 and 442). [1]Significantly different from winter and summer. [2]Significantly different from spring and summer. [3]Significantly different from fall and spring. [4]Significantly different from winter and summer. [5]Significantly different from spring and summer. [6]Significantly different from summer. [7]Significantly different from spring and summer. [8]Significantly different from summer. [a]Significantly different from lakes 382 and 260. [b]Significantly different from lake 468. [c]Significantly different from lakes 468 and 442. [d]Significantly different from lakes 382 and 260. [e]Significantly different from lakes 468 and 382. Data are expressed as mean ± SEM, $P<0.05$ (n=4).


0.30
0.25
0.20
0.15
0.10
0.05
0.00
Relative ΔCt (Mt-II - Actin)
Lake 468
Lake 442
Lake 382
Lake 260
a 1
4
b
7
2
5
c 3
d 6
8
e
Fall
Winter
Spring
Summer

MT-II, this difference might explain the higher induction of MT-I (Olsson et al., 1995b; Carginale et al., 1998). Furthermore, in a rainbow trout gonadal cell line it has been shown that Zn and Cd are able to induce MT-I to a higher extent compared to Cu, but Cu shows higher inducibility for MT-II (Kling and Olsson et al., 1995).

Summary and Conclusions

The developed real time PCR methodology can now be used to differentially quantify the two MT isoforms. This methodology also demonstrated that there is a difference in the basal levels of induction between the two MT isoforms with MT-I production being higher than MT-II. The persistence of Cd after the end of the additions (15 years ago) and the increase in MT protein show the utility of MT as bioindicator of chronic exposure to heavy metals. Results from this study also show that alterations to MT by estrogen or estrogenic compounds require higher concentrations than those that are typically encountered in the environment.

Two different methodologies were used to compare MT mRNA levels in this study. Northern blot and real time PCR yielded similar results. This important finding validates primers and methods that were developed for real time PCR.

Chapter 5

Laboratory study
Metallothionein expression in lake trout (*Salvelinus namaycush*) during a reproductive cycle induced by photoperiod manipulation

Introduction

Field experimentation provides an excellent opportunity to study the effects of complex mixtures of contaminants in aquatic ecosystems, but control over many biotic and abiotic factors is lacking. For example, when studying wild fish there is variability associated with different ages of the fish, nutritional status and overall health. These factors could potentially affect the way the fish responds to toxic contaminants.

Laboratory experiments offer the advantage of controlling many factors that can influence experimental results in fish exposed to contaminants. However, changes in the environmental factors like water temperature, light cycle changes, the effects of weather and the interactions between the different species present in a natural aquatic environment cannot be exactly reproduced. Despite this limitation results from laboratory experiments can often be extrapolated for population modelling (Hutchinson et al., 2006).

Toxicant effects can easily be studied in a laboratory setting. Mechanism or mode of action of stressor chemicals, factors that modulate toxicant response, dose-response relationships over a wide range of concentrations, differences in exposure route (waterborne, diet), long term versus short term exposure effects, etc., are some examples (Ankley and Johnson, 2004; Hinton et al., 2005). Laboratory trials are especially useful for validating and quantifying bioindicator responses and for demonstrating how the responses might relate to fish exposed in the wild (Codi et al., 2004).

Because bioindicators of metal exposure can be greatly affected by water chemistry, the laboratory is an excellent place to study and validate their use. Having demonstrated that concentrations of the metal binding protein metallothionein can vary

during a reproductive cycle in wild lake trout (Chapter four), the same parameters were examined in a laboratory setting where parameters like water temperature, light cycles and nutrition could be controlled and a better understanding of the dynamics of MT protein and mRNA in fish could be achieved. As in chapter 4, western blotting and real time PCR were used to evaluate MT expression.

Materials and Methods

Fish

Lake trout (*Salvelinus namaycush*) (946 ± 16 g) were randomly distributed into two 800 L fibreglass tanks (20 fish per tank), and acclimated for 14 days. Each tank received 1 L of aerated and de-chlorinated Winnipeg City tap water per minute. Temperature was maintained between 11.5 and 13.1 °C and dissolved oxygen was at least 90% saturation at all times. The fish were fed commercial dry pellet feed (Martin Mills, Inc., Elmira Ontario Canada) at a ration of 1% body weight per day (Monday to Saturday). During the acclimation period the fish were kept on a 12 hour light : 12 hour dark light schedule.

Light cycle

After the 14 day acclimation period, the light cycle was adjusted every two weeks to simulate natural seasonal changes. Lighting was provided above each tank with 40 W full spectrum fluorescent lamps. The intensity of light at the surface of the water in each tank was approximately 200 lux. Lights were regulated with an automatic timer that was adjusted every week according to sunrise and sunset data provided by Environment

Canada (http://weatheroffice.ec.gc.ca/canada_e.html). In addition to the fluorescent lamps above each tank, room lights were also turned on or off 30 minutes before or after the fluorescent light timers to simulate dawn and dusk.

Fish sampling

Female fish were selectively sampled from each tank once every season starting in the winter of 2000. Sample times coincided with the week after fish were collected from the field locations detailed in chapter 4. Fish were anaesthetized in pH buffered tricaine methanesulphonate (MS222) (0.8 g/L, pH 7.0). When all fin movements had ceased (<0.3 min), fish were blotted dry, weighed and measured. Condition factor (K= [Total Wt / L^3] x 100) was calculated to evaluate the overall health of the fish. Liver and gonad tissues were dissected and weighed to obtained liver somatic index (LSI = [Liver Wt / Body Wt] x 100) and gonadal somatic index (GSI = [Gonad Wt / Body Wt] x 100). Liver samples were frozen in sterile plastic bags on dry ice before storage at -90°C until they could be analysed.

Western blot analysis of metallothionein protein

To determine MT protein content, liver tissue was homogenized in buffer (10 mM NaH_2PO_4 pH 7.0; 5mM 2-mercaptoethanol), heat treated at 95°C for 5 min, cooled for 4 min and then centrifuged at 20,000 x g for 90 min. The supernatant was collected and used to separate and identify MT using western blot analysis.

SDS/PAGE was performed as described by Laemmli (1970), using a 16.5% Tris-Tricine precast gel system (Bio-rad, Hercules, CA). Electrophoresis was carried out at

100 mA (constant current). The electrophoresis was terminated when the dye front reached the bottom of the glass plates. The separated proteins were then transferred onto 0.2 μm Immobilon P^{sq} PVDF membranes (Millipore Corp, Bedford, MA) for 1 hr at 100 V (constant voltage). The membranes were blocked with 3% BSA at room temperature followed by 1 hr incubation with the primary antibody (anti Cod-MT). Anti cod-MT andibodies cannot differentiate between the tow isoforms, therefore total MT was detected. After washing the excess primary antibody, the membranes were incubated with secondary antibody (horseradish peroxidase goat anti rabbit antibody) for 1 hr followed by washing of the secondary antibody. The membranes were then exposed to developer solution (Super signal west Pico chemiluminescent substrate, Pierce Biotech, Rockford IL) and exposed to film for 8 min to reveal the results. The density of the bands obtained was analysed using a FluorChem IS-8900 imaging system (Alpha Innotech, San Leandro, CA).

RNA extraction

Total RNA was obtained from liver tissue of fish at each of the sample times using the Trizol extraction protocol as described in chapter 3. Briefly, 100 mg of liver tissue were homogenized in Trizol reagent (Invitrogen, Corp., Carlsbad CA). The homogenate was then incubated at room temperature for 5 min, followed by the addition of chloroform. The tubes were shaken vigorously for 15 seconds and incubated at room temperature for another 3 min. This mixture was centrifuged at 12,000 x g for 15 min at 4°C, and the top layer transferred to new tubes. Isopropanol was added followed by a 10 min incubation at room temperature. The tubes were then centrifuged at 12,000 x g for

10 min at 4°C. The isopropanol was discarded and 75% ethanol was added to the pellet, followed by 1 hour incubation at -20°C. The tubes were then centrifuged at 7,500 x g for 5 min, the ethanol discarded, and the tubes let to dry. Once dried, the pellet was resuspended in 100 µl of DEPC water and kept in the -70°C freezer until analyzed.

The purity and concentration of the extracted mRNA was measured by the optical density at 260/280 (purity) and 260 (concentration) nm. Reverse transcription was performed using the Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) kit from Invitrogen (Carlsbad, CA) following manufacturers recommendations. Briefly, 1µg mRNA was added to chilled microcentrifuge tubes followed by the addition of 200 U/µl MMLV-RT, 5X Reverse transcriptase buffer (250 mM Tris HCl, pH 8.3; 375 mM KCl; 15 mM $MgCl_2$), 40 U/µl RNase out, 0.1M DTT, 0.1% BSA, 250 ng RT primer (5'gactcgagtcaacatcgattt$(ttt)^5$tt and 5 mM dNTP. The tubes were incubated at 37°C for 2 hours and kept at -20°C until used.

Real time PCR

As described in chapter 3 the program Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) was used to design Taqman probes and primers for MT-I (M18103) and MT-II (M18104), as well as for the housekeeping gene, β-actin to use in the real-time PCR assays. The primers and probes obtained using Primer3 were:

MT-I forward TAGCTCAAAAACTGGAAAATGGAT

MT-I reverse ATCACAAGTCTTGCCCTTGC

Probe [FAM]TCCCTCCGGCTGCAGCAGCAAGT[BHQ-1]

MT-II forward CGCGATCAAAAACTGAAAAAT

MT-II reverse GCATTGTCACGGTAATGCAC

Probe [TxR]CTGCCCCTGCTGTCCTTCCG[BH-2]

Actin forward AGAGCTACGAGCTGCCTGAC

Actin reverse GTGTTGGCGTACAGGTCCTT

Probe [TET]TCGGCAACGAGAGGTTCCGC[BH1]

Primers were dissolved in DEPC water to a concentration of 100 μm; while probes were also dissolved in DEPC water to a concentration of 10 μm. Once resuspended in DEPC water they were aliquoted and stored at -20°C.

Real-time PCR conditions

The cDNA obtained by the reverse transcription of the lake trout liver mRNA was used with the primers designed as described above to run a real-time PCR reaction. OmniMix master mix (Cepheid; Sunnyvale, CA) was used on a Cepheid Smart Cycler (Sunnyvale, CA) following manufacturers recommendations. Briefly, OmniMix master mix (3 U TaKaRa hot star Taq polymerase; 200 μM dNTP; 4mM MgCl; 25 mM HEPES buffer pH 8.0) was mixed with the primers and probes (100 μM and 10 μM respectively) or SYBR green, followed by addition of the cDNA, briefly centrifuged to bring the contents to the bottom of the tube and placed in the Smart Cycler.

Cycling Conditions

After an initial denaturation cycle of 95°C for 150 sec, 30 cycles were performed; each consisting of 15 sec denaturation at 95°C, 30 sec annealing at 60°C and 30 sec extension at 72°C.

Data analysis

For the data obtained from western blots, a one way ANOVA followed by Dunnet's or Tukey's multiple comparison tests were used to analyze the differences between MT in fish in the different seasons. Statistical significance was accepted at $p<0.05$.

For real-time PCR a pair wise fixed reallocation randomisation test was performed. This test is based on the probability of the observed effect occurring under the hypothesis of no treatment effect. If this hypothesis is true, the values in one treatment group would be expected to be just as likely to have occurred in the other group. The randomization test repeatedly and randomly reallocates the observed values to the two groups, and notes the apparent effect each time (Pfaffl, 2000; Pfaffl, 2001).

Results and Discussion

Somatic parameters

Somatic parameters from the female fish sampled during the laboratory experiment are shown in table 5.1. There were no significant differences in length, weight, condition factor or gonad somatic index among the fish at the different light induced seasons. While GSI appeared to vary considerably among seasons, considerable variability within each season was observed. For example, during the fall only one female had mature gonads. As a result, mean gonad weight and GSI were elevated in that fish at that time even while the rest of the females had smaller gonads. Similarly, in summer, four females had large gonads and elevated GSI and three females had smaller gonads with lower GSI values. This high variability among fish gonad sizes within the

Table 5.1 Mean somatic parameters in female fish exposed to different light cycles to reflect seasonal changes.

Season	n	Weight (g)	Length (cm)	K	GSI	LSI
Winter	3	881±107	43.4±1.2	1.05±0.04	0.80±0.16	1.23±0.12*
Spring	4	685±25	42.2±0.3	0.90±0.02	0.38±0.07	0.94±0.05
Summer	7	1010±45	46.1±0.7	0.98±0.02	8.54±1.22	0.75±0.02*
Fall	4	1123±48	48.2±0.8	1.00±0.03	2.75±1.13	0.96±0.04

*Significantly different from each other based on ANOVA followed by Bonferroni. Data are expressed as mean ± SE, p=0.05.

same season resulted in there being no significant differences between the overall mean gonad sizes among seasons.

Liver somatic index was significantly different between fish sampled in the winter and the summer (Table 5.1). The highest LSIs were found in summer and the lowest during the fall in the wild female lake trout described in chapter 4. In contrast, the highest LSI was observed during the winter in the laboratory fish from this chapter's experiments. This would suggest that gonad development in fish held in the lab was one full season behind the wild fish. It should be expected that if MT induction peaks in the season following vitellogenesis when liver size is elevated, that maximum MT would be found during the spring in fish from the lab. It would appear then, that altering the light cycle to reflect ambient conditions did not elicit synchronized physiological responses of the lake trout in our laboratory.

In the aquaculture industry, fish are selected to achieve an appropriate size at a given time of the year. This is routinely done by manipulating the light regime. Fish are usually moved to light controlled chambers after their last spawning event. Buss (1980) showed for rainbow trout that spawned in late October and November, that it was possible to induce them to spawn by the following June, if the fish were placed in the light control facility during the last week of November. It was reported that when lights were installed outside the light room, fish failed to ripen at the same time, an effect observed when fish are kept at constant light.

The changes in the light regime in the present experiment were not as stringent as the regimes suggested in the study by Buss (1980). Although the room lights were completely extinguished during "dark" times, some light may have been able to "leak"

into the room from the adjacent corridor. This light may have been enough to prevent all fish from developing synchronously. As a result, high variability in gonad and liver weights as well as gonad development was observed. Had the fish been subjected to a second year under the light control conditions, there would likely have been a decrease in the number of early spawners (Corson, 1955). The abrupt change from a 12L:12D cycle to the manipulated light cycle may also have contributed to the high variability (Corson, 1955).

It has also been suggested that besides changes in light, fish in the Northern Hemisphere are cued by water temperature. In our experiments the water temperature was maintained constant. The lack of changes in water temperature naturally occurring in nature could account for the difference between the results obtained in the laboratory compared to the differences obtained in the field experiment (Chapter 4). In the laboratory, fish spawned during the summer and continued during the fall, in contrast, in the field experiment fish only spawned during the fall.

Western blot analysis

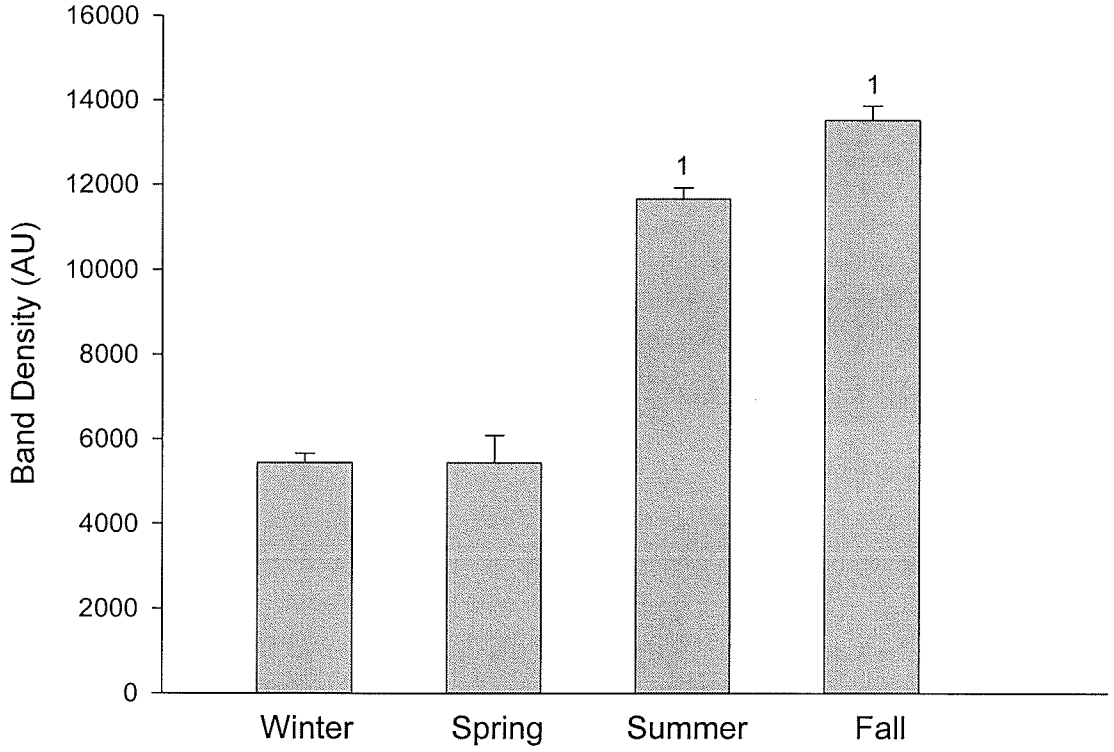
Metallothionein protein was quantified in liver by western blot analysis using a rabbit anti-cod metallothionein antibody. This antibody has good cross-reactivity with lake trout samples as outlined in chapter 3, allowing for the relative quantification of MT protein in liver samples from females exposed to changes in light to induce changes related to their reproductive cycle. It has been suggested that changes in free available hepatic Zn result from changes in metabolic activity in female fish during the latter stages of gonadal development (Olsson et al., 1987; Roesijadi, 1992; Coyle et al., 2002). These

changes are especially important during the process of endogenous vitellogenesis when the essential metals Cu and Zn are used as cofactors for enzymatic activity associated with the production and export of the lipophosphoglycoprotein vitellogenin (Olsson et al., 1987; Roesijadi, 1994; Olsson et al., 1996b). MT concentrations are regulated by hepatic concentrations of essential metals, including Cu and Zn, and so MT may decline as a result of the decline in intracellular free metal concentrations during vitellogenesis (Olsson et al., 1987; Banks et al., 1999; Gorbi et al., 2005).

Hepatic MT protein concentrations in lake trout exposed to an artificially manipulated light cycle are shown in Figure 5.1. There was a significantly higher MT protein concentration during summer and fall compared to winter and spring. This corresponds with the early gonad development of these fish. Fish exposed to the change in light regime started gonad maturation during the summer and continued through the fall. This was reflected in the GSI and LSI measures. MT protein increased following gonad maturation, and this would be expected if Zn were being released from cellular enzymes after vitellogenesis ceases. A decrease in MT protein concentrations coincides with the onset of the process of vitellogenesis that induces an increase in metabolic activity and the utilization of Zn as an enzymatic cofactor. This period also coincides with a resulting increase in LSI (Olsson et al., 1987; Roesijadi, 1994; Olsson et al., 1996b).

Lake trout typically spawn in the fall and the peak of vitellogenesis takes place during the summer months. Vitellogenesis usually starts in late spring - early summer, and during this time levels of the natural estrogen 17β-estradiol increase, resulting in

Figure 5.1 Hepatic MT protein in lake trout exposed to light cycle changes determined by western blot. [1]Significantly different from winter and spring. Data are expressed as mean ± SEM, $P<0.05$ (n=4).


16000
14000
12000
10000
8000
6000
4000
2000
0
Band Density (AU)
1
1
Winter
Spring
Summer
Fall

increased production of VTG. Conversely, at the end of vitellogenesis a redistribution of Zn from the microsomal and mitochondrial fractions to the cytosolic fractions has been observed. The increase of Zn in the cytosolic fraction has been associated with an increase in MT protein (Olsson et al., 1987, Olsson et al., 1989; Banks et al., 1999). Apart from a shift of one season compared to the wild fish, the relative changes in GSI, LSI and MT protein in the lab fish are in agreement with those described in chapter 4.

Real time PCR – MT mRNA

Salmonid fish express at least two MT isoforms, MT-1 and MT-2. These isoforms are expressed by two different genes, also denoted MT-I and MT-II. Apparently each isoform is preferentially induced by metals that preferentially bind to the expressed protein (Vasconcelos et al., 1996; Kawata et al., 2006). For example, MT-1 preferentially binds Zn and Cd, while MT-2 binds Cu in salmonid fish (Zafarullah et al., 1990). MT mRNA levels were examined in female lake trout exposed to altered light regimes as a means to examine the changes in expression of each individual isoform. There was a significant increase in MT-I mRNA during the fall compared to the rest of the seasons (Fig. 5.2).

Moreover, gonadal maturation was active in the summer in some of the fish and the fall in other fish. MT protein was elevated during the summer and fall but MT-I mRNA expression was only elevated during the fall. MT-II expression did not show the same pattern as MT-I. Specifically, MT-II mRNA decreased in the fall and winter and was elevated in spring and summer (Fig. 5.3). While MT-II mRNA is more responsive to

Figure 5.2 MT-I mRNA expression determined by real-time PCR in female lake trout exposed to light cycle changes. [1]Significantly different from winter, spring and summer. Data are expressed as mean ± SEM, $P<0.05$ (n=4).

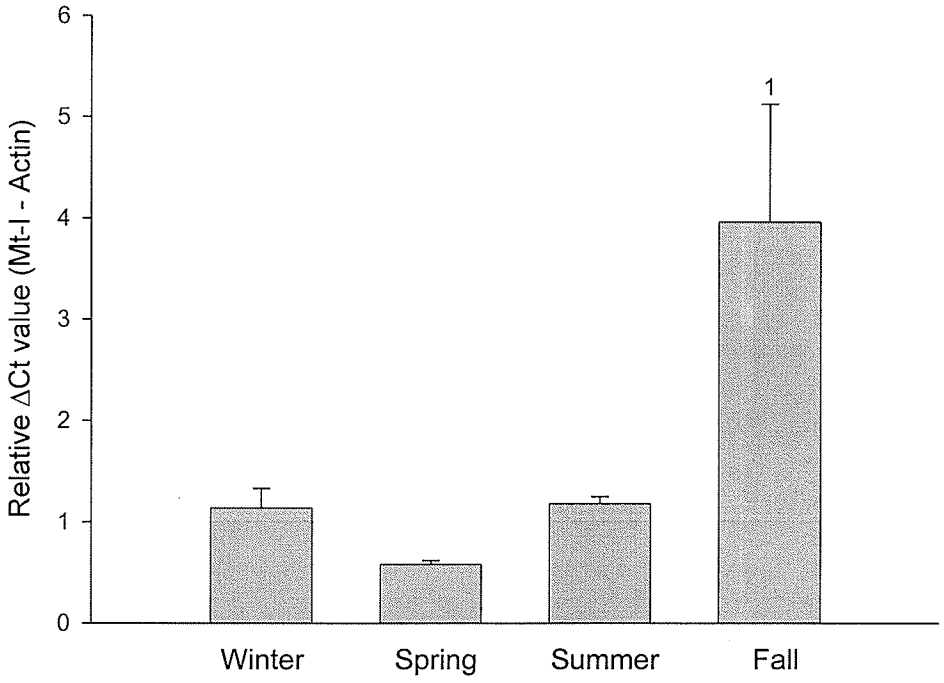
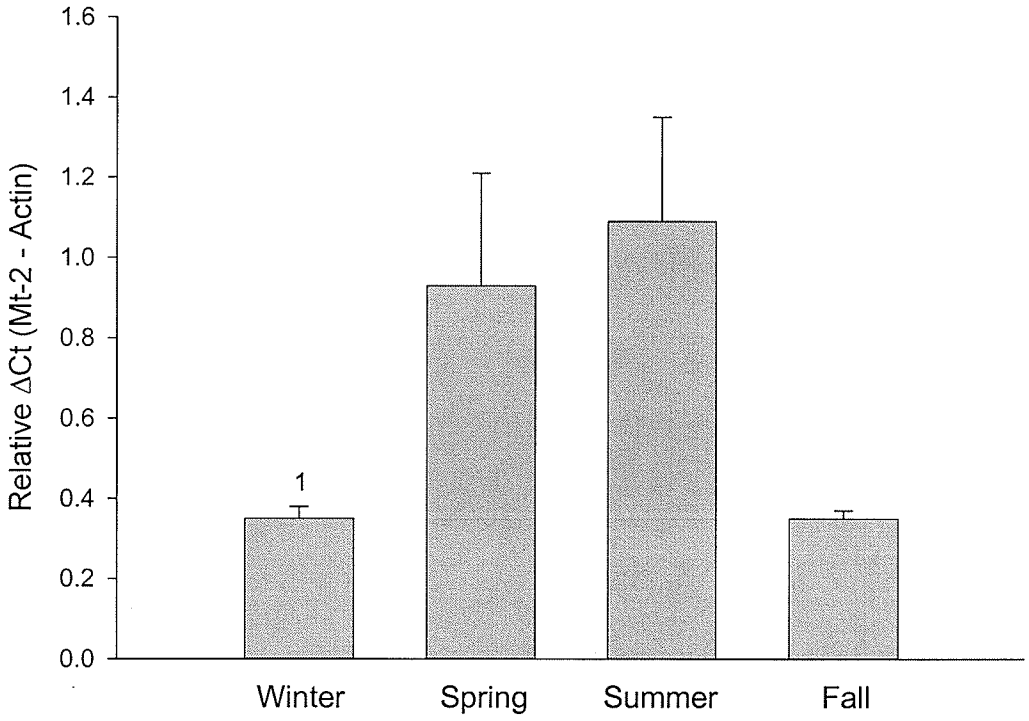

Relative ΔCt value (Mt-I - Actin)
6
5
4
3
2
1
0
1
Winter
Spring
Summer
Fall

Figure 5.3 MT-II mRNA expression determined by real-time PCR in female lake trout exposed to light cycle changes. [1]Significantly different from spring. Data are expressed as mean ± SEM, $P<0.05$.


Relative ΔCt (Mt-2 - Actin)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
Winter
Spring
Summer
Fall

Cu, a clear relationship between this metal and the reproductive cycle has not been established.

Protein concentrations observed in the different seasons of this study did not necessarily correlate with mRNA expression. Specifically, MT protein concentration wasincreased during the summer compared to the winter and spring, but when MT-I mRNA is analyzed the increased in mRNA expression is just slightly higher compared to winter and spring. Previous studies have also identified that mRNA and protein concentrations do not correlate (Scudiero et al., 1997; Carginale et al., 1998; Gygi et al., 1999; Vasconcelos et al., 2002). One suggestion is that MT expression may be regulated transcriptionally as well as translationally (i.e. mRNA may be generated but it may not necessarily be translated into protein). This has previously been observed in rats (Vasconcelos et al., 2002) where injection of Cd (8.9 μmol/Kg) significantly increased renal MT-1 and MT-2 mRNA levels but protein was unaffected. When the rats were injected with Cu (8.7 μmol/Kg) mRNA levels of both isoforms decreased but MT protein increased. It is possible that the discrepancies observed between protein and mRNA expression are a reflection of separate regulatory mechanisms (Bargelloni et al., 1999; Vasconcelos et al., 2002).

The mRNAs for the two isoforms have been analysed with respect to their appearance in polyribosomes in rats injected with either Cd or Cu (Vasconcelos et al., 1996). The ratio of MT-I mRNA abundance in polyribosomes to that in total mRNA remained unchanged but the ratio for MT-II mRNA was reduced, showing that MT-I mRNA is being recruited by the polyribosomes to make MT-1 protein but not MT-II

(Vasconcelos et al., 1996). These results were consistent when either of the metals were injected. This data show that there is a translational control of the two MT isoforms.

As we observed in the field experiments of chapter 4, there were differences in the expression between the two MT isoforms. The levels of MT-I mRNA isoform seems to be up to 4 times higher compared to the MT-II isoforms in this study. Although these results are not conclusive they give an insight into the difference in metal regulation as a result of a reproductive cycle. Differential expression of the two isoforms has also been reported in carp (*Cyprinus carpio*) where basal levels, in brain, muscle, liver and kidney of the MT-I gene product were higher compared to the MT-II gene product. Moreover, in the heart of these fish lower levels of MT-I compared to MT-II were observed (Hermesz et al., 2001). In rats it was shown that Cu induces MT-II mRNA to a greater extent that MT-I mRNA, but when Cd is injected more MT-I mRNA is induced compared to MT-II mRNA (Vasconcelos et al., 1996). It has been shown that in salmonid fish Cu is preferentially bound to MT-2, whereas MT-1 preferentially binds Zn and Cd (Zafarullah et al., 1990).

In the present study MT-I mRNA seems to be expressed to a greater extent compared to MT-II mRNA, suggesting that MT-I is regulating Zn, as an increase of Zn as a result of the completion of vitellogenesis coincides with an increase in MT content in liver cells (Valencia et al., 1998; Banks et al., 1999). It is important to remember that vitellogenesis is triggered by an increase in 17β-estradiol that also induces an increase in LSI through elevated metabolic activity and VTG production (Thompson et al., 2001). An increase in metabolic activity requires the utilization of Zn as a cofactor for various enzymes resulting in a decrease in MT mRNA. At the end of vitellogenesis, Zn is

released into the cytosolic fraction inducing the increase in MT-I mRNA and protein observed in the lab and wild fish from this study. On the other hand, MT-II mRNA does not change in a similar manner to MT-I suggesting that this isoform is not involved in Zn homeostasis. Copper concentrations are not altered as a result of seasonal 17β-estradiol alterations and so it makes sense that MT-II would be less seasonally variable (Olsson et al., 1987).

Summary and Conclusions

Manipulating light to induce fish to undergo the changes that take place during a reproductive cycle is a useful approach to control variability that might appear in wild populations. Here we have used the approach to examine changes in a bioindicator, MT, in a controlled environment. Despite the variability encountered in the fish used in this study, light changes did induce reproductive stages, which were reflected in changes to the MT protein concentrations as well as changes in the mRNA expression. MT protein increased during the summer and fall followed by a decrease during the winter and spring. Similar changes were also observed for MT-I mRNA. The alterations of GSI, LSI and MT observed in this study are closely reflected in the reproductive cycle of the fish. Specifically, a high level of MT coincides with the end of vitellogenesis during the fall, followed by a decrease in MT during the following months. Changes observed in MT-II mRNA did not follow the same pattern as MT-I, likely as a result of different controlling mechanisms.

General Summary and Conclusions

The complete physiological roles of the metal binding protein metallothionein are not known but it is widely thought that MT is involved in the maintenance of homeostatic concentrations of the essential metals Cu and Zn, in the detoxification of heavy metals and the scavenging of free radical species. MT expression appears to be mediated by the displacement of Zn from cellular ligands or other MT proteinsby other metals. This displaced Zn is then translocated into the nucleus where it binds to specific sequences in the MT gene called metal transcription inhibitors (MTI) releasing them from the metal transcription factors (MTF). The binding of the MTF to metal response elements (MRE) in the MT gene allows transcription initiation that result in the synthesis of newly formed MT protein. Zn can be displaced from its cellular ligands by other metals including Cd, Ag, Hg, Co, Ni, etc. By allowing Zn to be displaced and by binding these other metals, metallothionein induction provides a mechanism to sequester these toxic metals for long term storage. Clearly, Zn plays an important role in controlling MT concentrations.

Because of the central role of Zn in MT regulation, other factors that influence intracellular Zn concentrations can secondarily affect MT concentrations. For example, recent evidence has focused on the ability of hormones (e.g. estrogen) to down regulate metallothionein secondarily through the induced alteration to Zn homeostasis. Specifically, in mature female fish, Zn is mobilized to liver organelles under the control of estrogen during the production of vitellogenin. Low concentrations of MT are found during this period. However, at the end of vitellogenesis, when estrogen levels decline and free Zn concentrations rise, an induction of MT has been observed. What was not understood at the time that this thesis work began was whether exposure to

environmentally relevant concentrations of an estrogen mimic could chronically induce VTG synthesis and secondarily down regulate MT synthesis. Additionally, it was not known if chronic exposure to a heavy metal could chemically up regulate MT and inhibit the natural cycle of vitellogenesis by disturbing the intracellular availability of Zn for the support of organelle formation required for that process.

In addition to understanding other factors that can potentially affect a bioindicator, methodological considerations need to be taken into account. When developing a bioindicator, it must be reproducible, cost effective and easy to apply. Four methods to measure MT protein and mRNA were developed. For protein quantification both HPLC and western blot were evaluated. HPLC has been shown to be a useful tool to separate compounds from complex mixtures. It was initially thought that HPLC could be applied to the analysis of metallothionein and its two isoforms MT-1 and MT-2. These differ at neutral pH by a single negative charge, making the separation of the two MT isoforms found in Salmonid fish excellent candidates for separation through HPLC.

However, the ability of HPLC to discern between isoforms is currently limited by the availability of specific standards for the species in question. Metallothionein is a highly conserved protein among many taxa. Fish MT differs from mammalian MTs by the displacement of one cysteine and by having fewer lysine residues compared to mammals. When commercially available rabbit MT standards were used to compare to lake trout MT a difference in spectral analysis between the two proteins was found suggesting fundamental differences in the proteins. When the amino acid sequence for both proteins was compared significant differences were confirmed, outlining the need for species appropriate standards in MT analysis using HPLC.

Western blot is another technique used to evaluate changes in MT protein content. This method has the advantage of the availability of a fish antibody that has good cross-reactivity with various species. The anti-Cod anti-rabbit antibody is commercially available and cross-reacts well with the lake trout protein. This antibody generates a band of 14 KDa in size that corresponds to metallothionein. The technique is straightforward, and allows MT proteins from several fish species to be analyzed in the same assay. The major disadvantage of this method is the inability to discriminate between the two isoforms of MT.

Isoform specific determinations of MT can be achieved by examining gene products. For the measurement of mRNA, northern blot and real time PCR were used. Both methods report the relative content of mRNA in the tissues. Development of these methods necessitated gene sequencing. For the purposes of this study, the sequence for the rainbow trout MT-I and MT-II genes were used as surrogates for lake trout. This was done under the assumption that the MT gene sequences are highly conserved among Salmonid fish. Northern blot is an excellent technique that besides the identification of the RNA in question can also give information about mRNA size, alternative splicing and the integrity of the RNA samples.

Real time RT-PCR has also been used to determine changes in mRNA content in aquatic organisms. This technique allows for the differentiation of the two isoforms by designing specific primers targeting the differences between the two isoforms. Once the primers are tested and deemed appropriate there are two approaches to analyze the tissue samples. Specific probes can be design at the same time as the primers or the addition of a DNA binding dye can be used. The idea behind both approaches is similar, to quantify

the increase in fluorescence as new DNA strands are being generated. Real time PCR is an easy to use technique, but reagents can be costly.

The methods described above were used to examine MT protein and mRNA concentrations in lake trout from laboratory and field experiments. These included basic examinations of MT concentrations at different times during a reproductive cycle, both in the wild and in fish held in the lab that were exposed to an artificial light regime. Results from these experiments support the hypothesis that MT is up regulated following gonad maturation. This is consistent with the prevailing hypothesis in the literature that Zn is remobilized after vitellogenesis is complete and secondarily enhances the signalling of MT mRNA resulting in higher MT protein concentrations (Olsson et al., 1987).

The effects of waterborne exposure to EE2 throughout a reproductive cycle in lake trout were also investigated using the techniques developed in this thesis. This was examined in a laboratory setting as well as a field study. Both experiments showed that at environmentally relevant concentrations of EE2 (ie. those to which fish would be expected to encounter in receiving environments) MT protein and mRNA concentrations were not affected relative to unexposed fish. At concentrations above 40 ng/L EE2, however, there was a decrease in MT protein in the livers of lake trout exposed to EE2 and an increase in kidney MT in the same fish.

Finally, the effects of chronic exposure to Cd on MT were explored in wild lake trout exposed in a whole lake where historical Cd addition had taken place. Cadmium addition took place in Lake 382 at ELA beginning in 1987. Fifteen years after the addition, lake trout were once again analyzed for MT content to evaluate the recovery of this aquatic ecosystem. Cadmium is still present at elevated concentrations in the

sediment and overlying water column. Higher concentrations of MT protein were observed in fish from Lake 382, but mRNA concentrations were not concurrently increased. These differences might be attributed to transcriptional and translational regulation of the MT mRNA.

This thesis has provided useful data for interpreting concentrations of MT protein or mRNA as a bioindicators in fish. Specifically, useful techniques for quantifying different isoforms of the protein and mRNA have been developed and used to demonstrate seasonal variability. The effects of environmentally relevant contaminants (Cd and EE2) have also been explored. However, there remain gaps in our knowledge of MT that must be addressed to allow MT to gain widespread use as a bioindicator.

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