

# Killifish metallothionein messenger RNA expression following temperature perturbation and cadmium exposure

Kathleen A. Van Cleef-Toedt,<sup>1</sup> Lisa A. E. Kaplan,<sup>1,2</sup> and Joseph F. Crivello<sup>1</sup>

<sup>1</sup>Department of Physiology and Neurobiology, University of Connecticut, 3107 Horsebarn Hill Road, U-156, Storrs, CT 06269, USA

<sup>2</sup>School of Arts and Sciences, Teikyo Post University, Country Club Road, Waterbury, CT 06723, USA

**Abstract** Metallothionein (MT), a cysteine-rich metal binding protein, is considered to play an essential role in the regulation of intracellular metals. Induction of MT in mammalian and nonmammalian tissues following heavy metal exposure may serve as a defense mechanism and a biomarker of environmental exposure to chemical stressors such as toxic metals. In this study, MT messenger RNA (mRNA) expression was characterized in male and female non-spawning and spawning killifish (*Fundulus heteroclitus*) following an 8-day exposure to specific sublethal stressors, which included temperature perturbation (26°C or 10°C) and/or 6 ppb of waterborne cadmium chloride (CdCl<sub>2</sub>). Hepatic, gill, and intestinal MT mRNA, expressed as copy number per microgram of total RNA, was assessed by reverse transcriptase–polymerase chain reaction and electrochemiluminescence using winter flounder (*Pleuronectes americanus*) MT complementary DNA primers. Liver, gill, and intestine MT mRNA expression was significantly ( $P < 0.05$ ) increased in nonspawning killifish exposed to 26°C compared with those exposed to 19°C (control). In addition, a significant ( $P < 0.05$ ) increase in gill MT mRNA induction was observed in nonspawning killifish exposed to 6 ppb of waterborne CdCl<sub>2</sub> compared with controls. The results of this study demonstrate significant MT mRNA induction in nonspawning killifish following short-term exposure to physiological and chemical stressors. Thus, further research may be necessary before the use of killifish MT mRNA induction as a biomarker of environmental chemical stress exposure alone.

## INTRODUCTION

Metallothioneins (MTs) are a family of low-molecular-weight cytosolic proteins that contain highly conserved cysteinyl residues. These residues allow MT to bind, transport, and store various transition metals via thiolate bonding (Hamer 1986; Dunn et al 1987). MT genes have been cloned and sequenced in several species of teleosts, including rainbow trout (*Oncorhynchus mykiss*), plaice (*Pleuronectes platessa*), winter flounder (*Pleuronectes americanus*), and stone loach (*Noemacheilus barbatulus*) (Bonham et al 1987; Chan et al 1989; Zafarullah et al 1989; Kille et al 1991). Although the specific physiological role of MT remains unclear (Palmiter 1998), MT has been proposed to have a significant role in regulating the intracellular

availability of essential metals, such as zinc (Zn) and copper (Cu), and detoxification of poisonous metals such as cadmium (Cd) and mercury (Hg) (George et al 1990). MT is not only capable of donating Cu and Zn to metallo-enzymes (Karin 1985; Brouwer and Brouwer-Hoexum 1991), but also donating metals, such as Zn, to transcription factors (Zeng et al 1991). The existence of specific metal-activated transcription factors for MT gene expression supports the hypothesis that MT induction by metals may be a specific cellular response to cellular metal concentrations (Thiele 1992). Metal sequestration may be a mechanism whereby MT confers organismal protection against toxicity from metals such as Cd (Klaassen et al 1999). Therefore, increased cellular resistance to metal toxicity may be conferred by processes that result in an increased capacity for MT synthesis (Roesijadi 1994, 1996).

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Correspondence to: Joseph F. Crivello, Tel: 860 486-5415; Fax: 860 486-3303; E-mail: crivello@predator.pnb.uconn.edu.

Inductions of MT and/or MT mRNA in aquatic organisms by heavy metals have been considered potential biomarkers for environmental metal exposure (Olafson and Thompson 1974; Pruell and Engelhardt 1980; Stegeman et al 1992; Linde et al 1999; Cajaraville et al 2000). In addition, field studies demonstrate that MT concentrations in fish and shellfish correlate well with environmental levels of exposure to Cd, Zn, and Cu (Roch et al 1982; Weidow et al 1982; Engel and Brouwer 1984, 1987; Roch and McCarter 1984; Brown et al 1987; Hogstrand et al 1991; Hamza-Chaffai et al 2000; Wong et al 2000).

Because MT gene transcription may also be induced by factors such as glucocorticoids, inflammatory and cytotoxic agents, and general stress-producing conditions (Kagi 1991; Palmiter 1998), MT may be involved in generalized cellular stress responses (Overnell et al 1987; Jacob et al 1999). Very little is known about the stress-induced MT gene expression in the killifish (*Fundulus heteroclitus*). Knowledge concerning MT gene expression in killifish is particularly important given the recent use of these fish in laboratory studies to assess the toxic effects of environmental pollutants (Weis 1984; Kaplan et al 1995).

The killifish is a small, bottom-feeding teleost that is common in estuaries along the US Atlantic coast from the Gulf of St Lawrence to Florida (Crawford and Powers 1992). It adapts well to laboratory conditions and is found in both contaminated and pristine habitats (Weis and Weis 1989). Killifish can tolerate relatively high temperature extremes (up to 34°C) and a wide range of salinities. Killifish spawn between April and August and are widely used as experimental animals because of their hardiness (Atz 1986). Effects of salinity and temperature on Cd toxicity and metal tolerance in killifish have been addressed previously (Eisler 1971; Weis and Weis 1989). Although considered to be a relatively metal-tolerant species, killifish may still potentially serve as sentinel species in many estuaries contaminated with xenobiotics due to their ubiquitous distribution along the Atlantic coast of North America.

MT mRNA levels, quantitated by Northern blot (Kaplan et al 1995), reverse transcriptase-polymerase chain reaction (RT-PCR) (Schlenk et al 1997, 2000), or RT-PCR and electrochemiluminescence (ECL) (Jessen-Eller et al 1994; Jessen and Crivello 1998; Van Cleef et al 2000; Van Cleef-Toedt et al 2000) have been useful in mechanistic studies on gene regulation of the stress response in several teleosts (Ryan and Hightower 1996). In this study, RT-PCR with ECL was used to characterize liver, gill, and intestine MT mRNA expression in spawning and nonspawning killifish exposed to temperature perturbation and in nonspawning killifish exposed to both temperature perturbation and waterborne Cd. The results of these studies may contribute to an overall understanding of the com-

plex biochemical mechanisms that occur following exposure to physiological and toxic heavy metal stressors in aquatic organisms.

## MATERIALS AND METHODS

### Animals

Adult male and female killifish were collected with a box trap from the Great South Bay (Nassau County, Long Island, NY, USA) during the 1995–1996 nonspawning and 1996 spawning seasons. Nonspawning animals were collected in September and spawning animals were collected in May. After collection, approximately 200 killifish were housed in 200-L, oval, plastic holding tanks equipped with constant aeration and carbon filtration (Magnum filters, Marineland, Moorpark, CA, USA). Fish were maintained in clean water for 4 weeks. Filtered tap water (pH 7.5–8.0) was treated with 0.1% sodium thiosulfate (50 mL, 10 L<sup>-1</sup>) and Instant Ocean (Aquarium Systems Inc, Mentor, OH, USA) (28 ppt) to ensure proper salinity. Animals were fed ad libitum with Tetra Marine (Tetra, Blacksburg, VA, USA) fish flakes during the 4-week period. All tanks were kept in temperature-controlled rooms at 19°C with light-dark cycles as follows: 14:10 hour (May) and 13:11 hour (September). The temperature and light-dark cycles resembled natural environmental conditions during the trapping periods.

### Experimental design

#### Temperature challenge experiments

Following the 4-week holding period, subset groups of killifish were removed from the oval tanks, separated by sex (as determined by coloration and external markings), and placed in standard 20-L glass tanks (maximum of 10 fish per tank) for an 8-day period. Nonspawning ( $n = 24$ ) and spawning ( $n = 24$ ) control animals were maintained at 19°C. For both the nonspawning and spawning seasons, temperature stress included 10°C ( $n = 50$ , nonspawning;  $n = 40$ , spawning) or 26°C ( $n = 24$ , nonspawning;  $n = 24$ , spawning). Approximately equal numbers of male and female animals were used for all temperature experiments. During each 8-day exposure period, animals were maintained in static water with constant water aeration. Fish were fed once daily with fish flakes and debris subsequently siphoned. Salinity and light-dark cycle conditions were maintained as described in the above section.

#### Temperature challenge with waterborne CdCl<sub>2</sub> experiments

During the nonspawning season, temperature challenge in addition to waterborne CdCl<sub>2</sub> exposures were per-

formed. Following the 4-week holding period, subset groups of killifish were removed from the oval tanks, separated by sex (as determined by coloration and external markings), and placed in standard 20-L glass tanks (maximum of 10 fish per tank). Killifish were then exposed to 6 ppb of waterborne CdCl<sub>2</sub> (Sigma, St Louis, MO, USA) while maintained at 19°C (control; *n* = 22), 10°C (*n* = 40), or 26°C (*n* = 20) for an 8-day period. Approximately equal numbers of male and female animals were used for each exposure. During the 8-day exposure period, animals were maintained in static water with constant aeration. Fish were fed once daily with fish flakes and debris subsequently siphoned. Salinity and light-dark cycle conditions were maintained as described in the above section.

Following all exposure regimens, all fish were pithed. Total length (in centimeters), weight (in grams), sex, spawning condition, and gross pathological condition (fin rot, ichthyophthiriasis, dropsy) were noted. Liver, gill, and intestine tissues were removed from each animal, snap frozen in liquid nitrogen, and stored at -70°C for subsequent analysis.

#### RNA analysis using quantitative RT-PCR

MT mRNA was quantified with RT-PCR and the PerkinElmer (Norwalk, CT, USA) QPCR 5000. This method, detailed by Jessen-Eller et al (1994) and Van Cleef (1998), is briefly described below. Tissues stored at -70°C were thawed on ice and homogenized with a variable-speed tissue tearer in RNAzol reagent (guanidium-thiocyanate and phenol). Total RNA was extracted as described by Creamer et al (1991). Total RNA concentration was determined by absorbance at 260 nm, and purity was determined by the 260/280-nm absorption ratio. Approximately 1 µg of total RNA was used in RT reactions with thermostable rTth enzyme (PerkinElmer) to generate complementary DNA (cDNA).

An aliquot of each cDNA sample was amplified via PCR with winter flounder MT-specific primers (Jessen-Eller et al 1994). These primers were previously shown to target MT in killifish cDNA templates (Van Cleef 1998). Biotin was attached to the 5' end of one primer, whereas a trivalent oxidized form of ruthenium (tributyl ruthenium) was attached to the 3' end of the other primer. Optimal PCR conditions to yield the desired 75-bp product were as follows: 94°C (30 seconds), 56°C (30 seconds), and 72°C (45 seconds) using 2.5 mM MgCl<sub>2</sub>, 0.3 µM primers, and 1 U Taq polymerase in a final reaction volume of 100 µL.

At time zero (cycle 0) and between cycles 16 and 30, 5-µL aliquots of each 100-µL reaction were sampled. The product in each aliquot was captured by streptavidin beads and analyzed via the QPCR 5000 (PerkinElmer) for

chemiluminescence (DiCesare et al 1993). The PCR product was expressed as ECL units. Each unit is equivalent to  $7.4 \times 10^{-13}$  moles MT mRNA (Jessen-Eller et al 1994).

cDNA samples derived from liver, gill, and intestine were randomized within each 96-well PCR plate. MT mRNA was standardized to a plasmid MT cDNA for winter flounder. Each PCR plate included serial dilutions of this plasmid MT cDNA (data used to construct a standard curve), 1-5 replicates of each sample, and at least 5 control (blank) wells. Percentage of recovery of product was determined by spiking at least one replicate with the plasmid MT cDNA. MT mRNA (normalized to the plasmid MT cDNA) was expressed as starting copy number of MT per microgram of total RNA.

To confirm that the cDNA PCR product obtained using winter flounder primers (WF<sup>Ru</sup>: 5'-TGC ACC ACC TGC AAC AAG AGC-3'; bases 73-93 and WF<sup>2</sup>: 5'-GCA CAC GCA GCC AGA GG-3'; bases 131-147) during quantitative RT-PCR was killifish MT, the PCR product was cloned using the TA Cloning System (Invitrogen, Groningen, The Netherlands), and sequenced using the Cy5 AutoRead Sequencing System (Pharmacia, Peapack, NJ, USA) with the ALF DNA Sequencer (Pharmacia) (Van Cleef 1998).

#### Metal analysis

Water samples were taken from the exposure tanks both before and after each experiment for metal analysis (Cd). Metal analysis (Cd) was also conducted on water taken from the Great South Bay (Nassau County, NY, USA) near the location of the killifish collection site. These samples were acid treated, prepared according to US Environmental Protection Agency (EPA) method 7131A, and analyzed with a graphite furnace (GFAA).

Fish food flakes were prepared for metal analysis according to a scaled-down version (10 mL total volume) of the nitric acid/hydrogen peroxide EPA method 3050. Arsenic (As), Cd, and Cu were analyzed using GFAA (EPA methods 7060A, 7131A, and 7211, respectively), and Zn and nickel (Ni) were analyzed with an inductively coupled plasma spectrometer (EPA method SW 846-6010). The respective detection limits for all these metals were 1.0, 0.5, 1.0, 5.0, and 20.0 µg/L. Standard metal analysis quality controls were used to assess percentage of recovery and repeatability within and across experiments and were calibrated with Trace Metal AA Standard 9954.

#### Statistical analysis

Nonparametric data were analyzed across and within tissue groups using the Kruskal-Wallis Test (*P* < 0.05) and Tukey's Studentized Range Test (SAS System, Cary, NC,

USA). Statistical analysis was based on the ranking of each fish liver, gill, and intestine MT mRNA copy number values within a comparison group. Mortality was compared between exposure groups using  $\chi^2$  analysis ( $P < 0.05$ ).

## RESULTS

### MT cDNA

Sequencing of the killifish cDNA-nested PCR product yielded a 75-bp fragment (Van Cleef 1998) that was more than 90% similar to other teleost MT cDNA sequences such as winter flounder (Chan et al 1989), plaice (George et al 1990), stone loach, northern pike (*Esox lucius*), rainbow trout MT-A (Kille et al 1991), goldfish (*Carassius auratus*), and tilapia (*Tilapia mossambica*) (Chan 1994) between base positions 73–150. Based on the size of the cDNA PCR product, similarity with other teleost MT cDNA sequences, and high cysteine content, the cDNA PCR product obtained using the ruthenium (WF<sup>Ru</sup>) and biotin-labeled (WF<sup>2</sup>) heterologous primers during the quantitative RT-PCR procedure was assumed to be killifish MT (Van Cleef 1998).

For all tissue types, duplicate RT-PCR samples within the same PCR plate using the same master mix averaged a 10% error as demonstrated by quantitative PCR. This variability increased to approximately 15% when duplicate samples were compared between PCR plates where a different master mix was used.

### Animals

The average length and weight of all fish used in this study were 7.12 ( $\pm 1.23$ ) cm and 5.65 ( $\pm 0.78$ ) g, respectively. No significant differences in length and weight between nonspawning and spawning killifish were observed within any experimental group. Mature female and male spawning killifish maintained reproductive season colorations and markings throughout the experimental period. Both nonspawning and spawning control animals maintained at 19°C exhibited no mortality or morbidity during the 4-week holding period and during all 8-day experiments. For other experimental groups that exhibited mortality during the 8-day exposure period, MT mRNA expression was only characterized in those killifish that survived the 8-day period. No gross pathological condition was observed at the time of pithing. No significant differences in tissue MT mRNA expression between male and female killifish were observed within or between any control or exposure group (data not shown). Unless otherwise noted, male and female MT mRNA expression data were pooled for each category.

**TABLE 1** Mortality among spawning and nonspawning *Fundulus heteroclitus* during an 8-day simultaneous exposure to temperature perturbation and waterborne cadmium chloride (CdCl<sub>2</sub>)

Treatment group	Reproductive season <sup>a</sup>	Temperature (°C)	CdCl <sub>2</sub> (ppb)	Sample size	No. of deaths	Mortality (%)
1	NS	10	0	53	3	6
2	NS	10	6	52	12	23
3	NS	19	0	24	0	0
4	NS	19	6	26	4	15
5	NS	26	0	39	15	38 <sup>b</sup>
6	NS	26	6	55	35	64 <sup>c</sup>
7	S	10	0	44	4	9
8	S	19	0	24	0	0
9	S	26	0	60	36	60 <sup>d</sup>

<sup>a</sup> NS, nonspawning; S, spawning.

<sup>b</sup> Significantly different ( $P < 0.05$ ) from group 3.

<sup>c</sup> Significantly different ( $P < 0.05$ ) from groups 2, 4, and 5.

<sup>d</sup> Significantly different ( $P < 0.05$ ) from groups 5 and 8.

### Temperature experiments

Spawning killifish exposed to 26°C exhibited significantly higher ( $P < 0.05$ ) mortality following the 8-day exposure period compared with those maintained at 19°C (Table 1). Also, spawning animals exposed to 26°C demonstrated significantly higher ( $P < 0.05$ ) mortality compared with nonspawning killifish housed at the same temperature. In addition, nonspawning killifish exposed to 26°C exhibited significantly higher ( $P < 0.05$ ) mortality compared with those exposed to 19°C (control) (Table 1).

During the nonspawning season, killifish exposed to 26°C exhibited significantly higher ( $P < 0.05$ ) MT mRNA expression in all 3 tissues analyzed compared with killifish exposed to 19°C (control) (Table 2). There were, however, no significant differences in liver, gill, or intestine MT mRNA expression between killifish exposed to 19°C (control) and those exposed to 10°C during the nonspawning and spawning seasons.

### Temperature challenge with waterborne CdCl<sub>2</sub> experiments

Metal analysis of all 20-L exposure tanks confirmed the Cd exposure conditions to be 0 ppb of Cd (control tanks) and 6 ppb of Cd (waterborne CdCl<sub>2</sub> tanks) before the addition of animals. Cd concentration in each tank was determined immediately following removal of animals after 8-day exposure; control tanks contained 0 ppb of Cd, and exposure tanks (previously containing 6 ppb of Cd) were found to contain 4 ppb of Cd. No Cd was detected following metal analysis of water samples from the killifish capture site (Great South Bay, Nassau County, NY, USA) or from food source.

Nonspawning killifish simultaneously exposed to 6 ppb of Cd and 26°C exhibited significantly higher ( $P < 0.05$ ) mortality compared with those exposed to 26°C alone (Table 1). Also, nonspawning killifish exposed to

**TABLE 2** Spawning and nonspawning *Fundulus heteroclitus* metallothionein (MT) messenger RNA (mRNA) expression in liver, gill and intestine tissues among animals exposed to 8 days of temperature perturbation

Tissue	Reproductive season <sup>a</sup>	Sample size	Temperature (°C)	Median MT mRNA copy number (per µg RNA)	±SE
Liver	NS	50	10	3 × 10 <sup>7</sup>	2 × 10 <sup>2</sup>
	NS	24	19	2 × 10 <sup>5b</sup>	3 × 10 <sup>2</sup>
	NS	24	26	1 × 10 <sup>8b</sup>	2 × 10 <sup>1</sup>
	S	40	10	5 × 10 <sup>7</sup>	1 × 10 <sup>2</sup>
	S	24	19	3 × 10 <sup>7</sup>	2 × 10 <sup>2</sup>
	S	24	26	2 × 10 <sup>7</sup>	2 × 10 <sup>2</sup>
Gill	NS	50	10	3 × 10 <sup>4</sup>	3 × 10 <sup>2</sup>
	NS	24	19	5 × 10 <sup>2c</sup>	2 × 10 <sup>2</sup>
	NS	24	26	1 × 10 <sup>5c</sup>	3 × 10 <sup>2</sup>
	S	40	10	5 × 10 <sup>5</sup>	2 × 10 <sup>2</sup>
	S	24	19	1 × 10 <sup>5</sup>	3 × 10 <sup>2</sup>
	S	24	26	3 × 10 <sup>5</sup>	3 × 10 <sup>1</sup>
Intestine	NS	50	10	5 × 10 <sup>6</sup>	2 × 10 <sup>2</sup>
	NS	24	19	4 × 10 <sup>5d</sup>	3 × 10 <sup>2</sup>
	NS	24	26	2 × 10 <sup>8d</sup>	2 × 10 <sup>1</sup>
	S	40	10	3 × 10 <sup>6</sup>	2 × 10 <sup>2</sup>
	S	24	19	5 × 10 <sup>6</sup>	2 × 10 <sup>2</sup>
	S	24	26	9 × 10 <sup>6</sup>	2 × 10 <sup>2</sup>

<sup>a</sup> NS, nonspawning; S, spawning.

<sup>b</sup> Significantly different ( $P < 0.05$ ) from each other.

<sup>c</sup> Significantly different ( $P < 0.05$ ) from each other.

<sup>d</sup> Significantly different ( $P < 0.05$ ) from each other.

<sup>e</sup> See footnote a in Table 3.

26°C in addition to 6 ppb of Cd exhibited significantly higher ( $P < 0.05$ ) mortality compared with those exposed to 19°C with 6 ppb of Cd and to those exposed to 10°C with 6 ppb of Cd (Table 1).

When killifish were maintained at 19°C, there was a significant difference observed in gill (not liver or intestine) MT mRNA expression between killifish exposed to 0 ppb of Cd (control) and those exposed to 6 ppb of Cd (Tables 2 and 3).

There were no significant differences in liver, gill, or intestine MT mRNA expression between animals exposed to heat stress (26°C) and those exposed to 26°C in addition to 6 ppb of Cd during the 8-day period (Tables 2 and 3). There were also no significant differences observed in liver, gill, or intestine MT mRNA expression between nonspawning killifish exposed to 10°C (cold stress) and those exposed to 10°C in addition to 6 ppb of waterborne Cd (Tables 2 and 3).

## DISCUSSION

An understanding of the relationships between MT mRNA expression and exposure to exogenous stresses will undoubtedly contribute to improved strategies for use of MT in assessing aquatic organismal health. For this to be achieved, there needs to be a better understanding

**TABLE 3** Metallothionein (MT) messenger RNA (mRNA) expression in liver, gill, and intestine tissues in nonspawning *Fundulus heteroclitus* exposed to 8 days of temperature perturbation and 6 ppb of waterborne cadmium chloride (CdCl<sub>2</sub>)

Tissue	Sample size	Temperature (°C)	Median MT mRNA copy number (per µg RNA)	±SE
Liver	40	10	5 × 10 <sup>7</sup>	1 × 10 <sup>2</sup>
	22	19	3 × 10 <sup>7</sup>	2 × 10 <sup>2</sup>
	20	26	8 × 10 <sup>7</sup>	1 × 10 <sup>2</sup>
Gill	40	10	9 × 10 <sup>4</sup>	2 × 10 <sup>1</sup>
	22	19	1 × 10 <sup>6a</sup>	2 × 10 <sup>2</sup>
	20	26	1 × 10 <sup>7</sup>	4 × 10 <sup>2</sup>
Intestine	40	10	3 × 10 <sup>7</sup>	6 × 10 <sup>2</sup>
	22	19	6 × 10 <sup>6</sup>	1 × 10 <sup>2</sup>
	20	26	3 × 10 <sup>7</sup>	3 × 10 <sup>2</sup>

<sup>a</sup> Significantly different ( $P < 0.05$ ) from median gill MT mRNA copy number in nonspawning killifish exposed to 19°C without cadmium (see footnote e in Table 2).

of MT function and the dynamics of MT mRNA induction (Roesijadi 1992). The presence of MT protein has been previously demonstrated in killifish (Pruell and Engelhardt 1980; Weis 1984). To the authors' knowledge, this is the first study to demonstrate MT mRNA expression in liver, gill, and intestine tissues of killifish following acute exposure to a physiological stress such as temperature perturbation.

The killifish has been found to be successful in surviving and reproducing in metal-polluted estuaries (Weis 1984). However, previous studies by Mitton and Koehn (1976) demonstrated specific morphological divergence in north shore populations of Long Island killifish exposed to thermal stress alone (compared with controls). Although synthesis of MT in these feral animals may be a response mechanism to heavy metal exposure within the aquatic environment, this study demonstrated that exposure to thermal stress alone results in a significant elevation in liver, gill, and intestine MT mRNA expression in killifish compared with controls during the nonspawning season.

Mammalian in vitro studies by Bauman et al (1993) using normal rat hepatocytes demonstrated that exposure to short-term heat shock (15–60 minutes at 43°C–44°C) did not increase MT protein. Although early in vitro studies with embryonic Chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout hepatoma cells also demonstrated that heat shock did not induce MT or MT mRNA (Heikkila et al 1982; Misra et al 1989), recent studies with yeast (*Saccharomyces cerevisiae*) have shown that MT gene expression may be activated through the action of heat shock factor and heat shock elements located within the yeast MT gene promoter upstream regulatory region following exposure to both heat and oxidative stress

(Tamai et al 1994; Santoro et al 1998). Thus, MT biosynthesis is considered an important response to heat shock stress in yeast (Silar et al 1991). Bonneton et al (1996) recently demonstrated enhanced expression of 2 *MT* transgenes of *Drosophila melanogaster* (*Mtn* and *Mto*) after heat shock treatment. As suggested by Bauman et al (1993), there may be a relationship between conditions of heat shock and *MT* gene expression. The significant increase in *MT* mRNA expression observed in nonspawning killifish exposed to 8 days of heat stress (compared with controls) supports previous suggestions that *MT* may belong to the common group of heat shock or general stress proteins (Ryan and Hightower 1996). This proposed role of *MT* as a protein involved in the general heat shock response metabolic cascade is also supported by the observation that no difference in *MT* mRNA expression was observed in the same tissues when nonspawning killifish were exposed to cold stress (compared with controls).

The effect of heat stress was also demonstrated in this study by the significantly high mortality observed in nonspawning and spawning killifish exposed to heat stress compared with controls. The high mortality observed in spawning killifish exposed to heat stress compared with spawning controls may reflect various endogenous gonadal and hormonal fluctuations and adaptations occurring during teleost reproduction, such as sex steroid synthesis and increased plasma cortisol levels (Day and Taylor 1984). Previously, 100% mortality was observed in both nonspawning and spawning killifish exposed to 30°C for an 8-day period (Van Cleef 1998). These findings support past work that indicates that 26°C may be the highest temperature extreme for killifish maintained in aquaria (Hellner 1990).

An increase in murine *MT* mRNA has been shown to correlate well with a rise in physiological stress-induced serum corticosterone (Jacob et al 1999). Also, studies with largemouth bass (*Micropterus salmoides*) have confirmed that fish exposed to physiological stress display elevated serum cortisol, Cu, and Zn levels (Weber et al 1992). Following the observation that cortisol and corticosterone induced a 2-fold increase in *MT* levels of rainbow trout hepatocytes in primary culture, it was suggested that cortisol may be involved in the regulation of *MT* in rainbow trout (Hyllner et al 1989). In addition, Muto et al (1999) demonstrated that exposure to physical stress in crucian carp (*Carassius cuvieri*) may induce *MT* synthesis, in part, by the release of endogenous factors such as glucocorticoids. Although the *MT* gene has not been thoroughly characterized in killifish, recent structural and functional analysis of many teleost *MT* genes has demonstrated the presence of upstream metal responsive element (MRE) and glucocorticoid responsive element sequences that are similar to those observed for mammalian *MT* (Olsson et

al 1995; Olsson and Kille 1997). In addition to mediating Zn induction of *MT* synthesis, MREs may make important contributions to nonmetal-induced promoter activity (Samson and Gedamu 1998).

MRE-binding transcription factor 1 (MTF-1) has been shown to play a central role in the transcriptional activation of specific mammalian and teleost *MT* genes through MRE binding in response to metals such as Zn and oxidative stress (Samson and Gedamu 1998; Andrews 2000). Expression of the pufferfish (*Fugu rubripes*) cDNA in mammalian cells by Auf der Maur et al (1999) showed that pufferfish MTF-1 had the same DNA-binding specificity as its mammalian counterpart. In addition, Dalton et al (2000) showed that both zebrafish (*Danio rerio*) and rainbow trout express MRE-specific binding activities immunologically similar to mammalian MTF-1. Interestingly, Zn reversibly modulated 1 of the 2 isoforms of MTF-1 in rainbow trout in a temperature-dependent manner (Dalton et al 2000). Future studies are needed to examine the 5' upstream region(s) of the *MT* gene(s) in killifish to elucidate the events related to *MT* mRNA expression following exposure to physiological stress.

A significant increase in gill *MT* mRNA expression was observed in killifish exposed to Cd compared with controls. This result supports previous studies with teleosts that have demonstrated a significant increase in gill *MT* and *MT* mRNA expression following Cd exposure (Cosson 1994; George et al 1996). Interestingly, Cd, which has been shown to have little effect on the DNA-binding activity of mammalian and teleost MTF-1 in vivo or in vitro, is often considered a more potent inducer of *MT* gene expression than Zn (Chu et al 1999; Andrews 2000; Dalton et al 2000).

The effects of multiple environmental stressors on *MT* expression in fish have been unclear (Thomas and Wofford 1984; Olsson et al 1987; Overnell et al 1987). Previous studies have demonstrated a temperature dependence of hepatic *MT* synthesis in fish exposed to Cd, such that Cd sequestration by *MT* increased in fish maintained at 20°C compared with those reared at 10°C (Carpene et al 1992). Also, the accumulation of Cd has been shown to be a temperature-dependent process (Failla et al 1979). However, the results of the current study demonstrated that nonspawning killifish exposed to thermal stress alone exhibited significantly elevated liver, gill, and intestine *MT* mRNA expression compared with controls. Thus, exposure to a low dose of an additional stress (6 ppb of Cd) did not result in further significant *MT* mRNA expression when comparing nonspawning killifish exposed to heat stress. The results of the current study also showed no significant increase in liver, gill, or intestine *MT* mRNA expression in nonspawning killifish exposed to cold stress compared with those exposed to cold stress and Cd. As the temperature is lowered, the fluidity of plasma

membranes has been shown to decrease (Krasne et al 1971). This process could then affect ion channel permeability of Cd into tissues and subsequent MT mRNA induction at lower temperatures.

To identify relationships between physiological stressors and MT mRNA expression, characterization of a model system using a sentinel species must be performed with an evaluation of specific inducers. For utilization in potential environmental monitoring projects, RT-PCR has been shown to provide the ability to sensitively and accurately quantitate levels of MT mRNA expression in target tissues of killifish and various other aquatic species (Jessen-Eller et al 1994; Kaplan et al 1995; Schlenk et al 1997, 2000; Jessen-Eller and Crivello 1998; Van Cleef et al 2000; Van Cleef-Toedt et al 2000). Because the effects of heat stress on MT mRNA expression in this aquatic organism may possibly serve to confound the analysis of MT mRNA expression in killifish sampled from contaminated aquatic ecosystems, further analyses are required before the use of MT mRNA induction in killifish as a biomarker of heavy metal exposure alone.

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