

## Quantitation of metallothionein mRNA from the New Zealand common bully (*Gobiomorphus cotidianus*) and its implications for biomonitoring

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**Abstract** Metallothionein (MT) is a protein found in most eukaryotes where its primary role is the regulation of homeostasis of the essential metals copper and zinc. MTs have an additional protective role through their binding of toxic metals such as cadmium and mercury. Increased expression of MT in response to harmful levels of these metals has been demonstrated for several aquatic species. In this study, the expression of MT in the liver tissue of the New Zealand common bully *Gobiomorphus cotidianus* was measured by quantifying MT mRNA. A statistically significant increase in MT expression was observed in fish exposed to copper for 48 h, but not those exposed to zinc. Field sampling of common bullies showed a wide variation in individual hepatic MT mRNA levels which did not correlate with fish age, sex, or sampling location. A comparison of two populations of common bullies from a polluted and a control site showed a two-fold higher mean MT mRNA level in fish from the polluted site. Implications for using MT expression in the common bully as a bio-monitoring tool are discussed.

**Keywords** metallothionein; Taqman-PCR; mRNA; *Gobiomorphus cotidianus*; biomarker; sentinel species

### INTRODUCTION

Metallothioneins (MTs) are low molecular-weight cytoplasmic proteins that act as biological chelators of heavy metals through formation of metal-thiolate bonds with their numerous cysteine residues (Kagi & Schaffer 1988). These proteins play an important role in the transport and storage of the essential metal ions copper (Cu) and zinc (Zn), which are required as co-factors for many metabolic enzymes. MTs have an additional protective role through their binding and sequestration of toxic metal ions such as cadmium (Cd) and mercury (Hg), and protect against toxic levels of Cu and Zn (Roesijadi 1992). MTs also play an important cellular role in scavenging reactive oxygen species (ROS) such as peroxide and hydroxy radicals, which bind to the cysteine residues in MT (Ghoshal & Jacob 2001).

In many species studied (e.g., mammals, yeast, fish) heavy metals such as Cd, Zn, and Cu have been shown to be potent inducers of MT at the transcriptional level. These ions bind to metal responsive factors (MRF) which in turn bind to metal responsive elements (MRE) of the MT gene promoter, stimulating transcriptional output (reviewed in Samson & Gedamu (1998) and Ghoshal & Jacob (2001)). MT gene expression may also be activated by ROS since a number of MT promoters contain antioxidant-responsive elements (Dalton et al. 1994). However, even in the absence of elevated metal or ROS concentrations, the basal level of expression of MT is relatively high, because of its essential role in maintaining homeostasis of Zn and Cu.

The measurement of MT levels in fish has been touted as a viable approach to evaluating exposure of biota to heavy metals in aquatic environments. European monitoring programmes of marine and estuarine environments currently include measurement of MT levels in mussels and oysters, for which a clear correlation has been shown with ambient levels of metal contaminants (Geffard et al. 2002 and references therein). The correlation of fish MT levels with environmental metal concentrations has been more difficult to demonstrate, partly because of the

mobility of fish and therefore a greater uncertainty of their exposure. Controlled exposures of fish to either waterborne metals (Gerpe et al. 1998; Dang et al. 2000; Wu et al. 2000), or by intra-peritoneal (IP) injection (Gerpe et al. 2000), have demonstrated a dose-dependent induction of MT with metal concentration, as has exposure of fish cell cultures (e.g., rainbow trout (*Oncorhynchus mykiss*) hepatocytes) to elevated metal levels (Risso-de Faverney et al. 2000). Studies of wild fish have not shown such a clear correlation. Specific quantitation of MT isoforms in North Sea dab (*Limanda limanda*) showed MT levels were poorly correlated with IP exposure (post-capture) to Cd, and were primarily influenced by water temperature and onset of gonad development (Lacorn et al. 2001). In a New Zealand study, MT levels in two populations of yellowbelly flounder (*Rhombosolea leporina*), sampled near Auckland, correlated with body burdens of Zn and Cu but did not correlate with the assumed exposure to metals; fish from the polluted Manakau Harbour had lower MT levels than the control population (Evans et al. 2001). However, a study of brown trout (*Salmo trutta*) in Cu- and Cd/Zn-contaminated rivers in Norway showed that gill concentrations of Cu and Cd/Zn MT reflected the presence of these metals in the rivers (Olsvik et al. 2000). When trout from one river were caged in the other, and vice versa, the MT levels reflected metal concentrations in the new environment, supporting the utility of MT as an indicator of metal exposure in these fish.

Although New Zealand does not have a major heavy metals problem, in several parts of the country elevations of metal concentrations above background levels have occurred in waters, sediments, and biota, as a result of industrial discharges, geothermal inputs, mining activities, and urban stormwater discharges (Smith 1986; Rob 1988). Better practice in discharging industrial waste means this source is no longer a significant problem, although elevated heavy metal levels persist in sediments in many localities. Urban stormwater runoff may represent the most important source of Cd, Cu, Zn, and Pb since, with increasing urbanisation, such inputs into urban streams and rivers is certain to continue (Smith 1986).

The New Zealand common bully (*Gobiomorphus cotidianus* (McDowall 1975)) is a small (30–120 mm total length), endemic, eleotriid fish present in lakes, rivers, and streams throughout New Zealand (McDowall 1978). The wide distribution of the common bully and the diversity of aquatic habitats in which it prospers suggests it is tolerant to a wide

range of environmental parameters. Indeed, common bullies can tolerate a pH range of 6.1–10.6 (West et al. 1997) and dissolved oxygen levels as low as 3–4 mg litre<sup>-1</sup> (Dean & Richardson 1999), and are less sensitive to ammonia toxicity than salmonids (Richardson 1997). The common bully could be considered representative of fauna present in New Zealand aquatic environments since it is used in a protocol to assess the toxicity of effluents (NIWA 1998).

Despite the apparent ability of the common bully to prosper under adverse conditions, there is no way of monitoring the effect of insidious chronic pollutants that may result in sub-lethal effects and long-term impacts on populations. In this study we have examined whether water-borne exposure of common bullies to Cu or Zn induces MT expression in the liver, and whether this biomarker could be a reliable indicator of exposure of these fish to heavy metals. Normalised hepatic MT mRNA levels were determined by quantitative RT-PCR analysis using Taqman real-time PCR. Because induced MT levels represent a protective response to an environmental challenge, detection of such a response may be an early warning of compromised individual health, which can be detected before the onset of deleterious changes in fish population structure (Roesijadi 1992).

## METHODS

### Nucleotide sequencing of metallothionein cDNA from *G. cotidianus*

Common bully liver tissue was homogenised in Qiagen RLT buffer using an Ultra-turrex T8 homogeniser, then total RNA was extracted using a Qiagen RNeasy kit, incorporating an on-column DNase digestion step, and eluted in 40 µl RNase-free water. RNA was used as a template in a reverse transcription (RT) reaction using an oligo(dT) primer to generate cDNA (Thermoscript RT-PCR system, Invitrogen). The incubation conditions of the RT reaction were as described below. MT gene-specific primers designed based on a consensus sequence of MT genes from other fish species (oligo14, CTGACCCTGAAGTACCCC and oligo15, CATCTCCTGCTCGAAGTC) were used to amplify a 183-bp PCR product from the cDNA preparation. Cycling conditions were: 94°C, 2 min; 30 cycles of 94°C, 30 s/50°C, 30 s/72°C, 30 s; and 72°C, 10 min, in a Techne Cyclogene thermocycler. The nucleotide sequence of the amplified product was determined

using the ET-dye terminator system (Amersham) with the oligo14 and oligo15 primers by the Waikato DNA Sequencing Facility (Hamilton, New Zealand) (Genbank accession no. AY239389).

### Design and optimisation of Taqman PCR assay

Primer Express software (Applied Biosystems) was used to design a 114-nt amplicon using the derived cDNA sequence of the *G. cotidianus* MT gene, consisting of primers:

(18F, CACCTGTGGTGGATCATGCA; 123R, GCAGCCGGAAGCACTT)

and a Taqman probe:

(FAM-TGCTCTTGCTGTCCATCAGGTTGCA-TAMRA). Optimised primer concentrations were 300 nM of each primer, and 100 nM probe.

Quantitation of 18S rRNA in each sample was used to normalise MT levels, using a Ribosomal RNA Control (Applied Biosystems) which consists of an optimised primer set and VIC-labelled probe homologous to all eukaryotic 18S rRNA.

Real-time PCR analysis of both MT and 18S rRNA from *G. cotidianus* cDNA was performed in the same tube (multiplex PCR) using an Applied Biosystems SDS7700 thermal cycler using the default settings (50°C, 2 min; 95°C, 10 min; 40 cycles of: 95°C, 15 s; 60°C, 1 min). Reactions were 25 µl volumes using Platinum Quantitative PCR Supermix-UDG (Invitrogen).

(FAM, TAMRA, ROX, and VIC are abbreviations for fluorophore or quencher molecules monitored during the Taqman PCR analysis.)

### Routine MT Taqman analysis protocol

Total RNA was extracted from liver tissue samples preserved in RNA-later (stored at 4°C) as described above (including the DNase step), and eluted in 40 µl RNase-free water. cDNA was synthesised from 2.5 µl RNA using the Thermoscript RT-PCR system (Invitrogen) in 10 µl reaction volumes, using random hexamers as the primer. The primer/RNA/dNTP mix was incubated at 65°C for 5 min followed by 5 min on ice, after which the remaining reaction components were added and the reaction incubated as follows: 25°C, 10 min; 55°C, 30 min; 85°C, 5 min. RNaseH (0.5 µl) was added and the reaction mix incubated at 37°C for 20 min. cDNA samples were diluted 1:50 in water and 5 µl diluted cDNA was analysed in triplicate using the Taqman PCR assay. Each reaction contained 13 µl Platinum Quantitative PCR Supermix-UDG including ROX dye (Invitrogen), 1.25 µl 20×MT primer/probe mix, 1.25 µl 20×18S rRNA primer/probe mix, 4.5 µl water,

and 5 µl diluted cDNA. Real time PCR analysis was performed on an ABI Prism SDS7700 machine as described above.

### Field sampling of fish

Common bullies ranging from 35 to 65 mm total length were netted from rivers at four locations in the South Island, New Zealand (Rakaia River, Ashley River, Wairau River, Motueka River). Fish were immersed briefly in MS222 (3-aminobenzoic acid ethyl ester) (Finquel), then killed by severance of the spinal chord, and the liver removed and placed in 500 µl RNA-later solution (Qiagen). Preserved tissue was stored at ambient temperature for up to 48 h, thereafter at 4°C until processing (within 1 week).

### Controlled exposure of fish to metals

Live fish netted from back-waters within braided sections of the Ashley or Rakaia Rivers were transported to the laboratory and transferred to a large polythene tank containing 20 litres of tap water (Canterbury Agricultural and Science Centre (CASC) bore supply, hardness 28 ppm) amended with malachite green and aerated with an air stone. Fish were acclimatised for 4 days, during which time they were fed commercially available bloodworms, and half the water was replaced daily. Exposure tanks consisted of 2-litre beakers containing tap water aerated with an air stone, amended with either Cu(SO<sub>4</sub>) (Sigma) or Zn(SO<sub>4</sub>) (Sigma). Fish were randomly allocated to exposure tanks (4–5 fish per tank) where they were kept for 48 h without feeding, after which time fish were killed and the liver removed and preserved as described above. Approval from the Landcare Research Animal Ethics Committee was obtained before fish exposure experiments were performed.

### Analysis of results

MT mRNA levels were derived using the Comparative C<sub>T</sub> Method (User Bulletin No. 2. ABI Prism 7700 Sequence Detection System: Relative Quantitation of gene expression). This analysis does not utilise a standard curve for calculation of absolute MT mRNA template levels, but expresses MT mRNA levels relative to that of a calibrator sample (i.e., non-exposed control fish). Output from the Taqman analysis is as a threshold cycle (C<sub>T</sub>) value for each target, i.e., the cycle at which the amplification curve crosses a threshold value. For each liver cDNA sample two C<sub>T</sub> values were derived: for the 18S rRNA endogenous reference, and for the MT target. To normalise MT against 18S,

the 18S  $C_T$  is subtracted from the MT  $C_T$  to obtain a  $\Delta C_T$  value. To compare normalised MT mRNA levels between samples, the  $\Delta C_T$  values are compared with the reference  $\Delta C_T$  (i.e., that of the control sample) to derive a  $\Delta\Delta C_T$  value. The amount of MT target, normalised to the 18S rRNA endogenous reference and relative to a calibrator, is given by:  $2^{-\Delta\Delta C_T}$ . For example, if the  $\Delta\Delta C_T$  for a sample is  $-2$ , this sample has 4-fold more MT mRNA than the control sample. Experiments confirmed the validity of the multiplex PCR, and the reproducibility of  $\Delta C_T$  results from separate cDNA syntheses from an RNA preparation, and of separate RNA extractions from different portions of the same liver (data not shown). Statistical analysis of  $\Delta C_T$  and normalised MT mRNA levels (relative to non-exposed controls) was by single-factor ANOVA using a 95% confidence limit.

### Metal analysis

Water samples from the river sampling sites were analysed by Environmental Laboratory Services Ltd, Lower Hutt, New Zealand using ICP-MS. Limits of detection are Cu,  $\geq 1 \mu\text{g/litre}$ ; Zn,  $\geq 3 \mu\text{g/litre}$ ; Cd,  $\geq 0.3 \mu\text{g/litre}$ .

## RESULTS

### Nucleotide sequence of *G. cotidianus* MT cDNA

Based on the length of MT cDNA sequences from other fish species, the 183-bp MT product amplified by RT-PCR from RNA extracted from *G. cotidianus* liver represents the entire coding region of the MT gene. Comparison of this sequence with MT genes from other fish species revealed the *G. cotidianus* MT cDNA sequence was most similar to that of *Liza aurata* (golden grey mullet) (Genbank accession no. AAB51591) (86% nt, 93% aa), and showed c. 90% aa identity to MT from many other fish species, including 90% and 88% to MT-B from *Salmo salar* (Atlantic salmon) and *Oncorhynchus mykiss* (rainbow trout). Although some fish species are known to possess two isoforms of MT which differ by an amino acid insertion at position 31 (Bonham et al. 1987; Kille et al. 1991; Gerpe et al. 1998, 2000; Egg et al. 2000; Ren et al. 2001), *G. cotidianus* appears to have only a single expressed MT gene. Sequencing of the amplified MT cDNA indicated this was a pure PCR product since the sequence was unambiguous and not affected by frame shifts or mixed product artefacts.

### Concentration-response exposures

To determine whether a concentration-response relationship exists between water-borne metals and liver MT mRNA, four experiments were performed in which common bullies were exposed to a range of metal concentrations (either Cu or Zn) for 48 h. The upper limit for Cu exposure was 500  $\mu\text{g/litre}$  which is similar to the acute  $LC_{50}$  for the common bully (520  $\mu\text{g/litre}$ ) (C. Hickey pers. comm.). We were not aware of data for Zn toxicity to common bullies, but since it was assumed to be less toxic than Cu, the upper value for the exposures was 1000  $\mu\text{g/litre}$ . Table 1 shows mean values calculated from the fish exposed to each metal concentration, and the derived relative MT mRNA levels obtained by comparing these values with that of the control (non-exposed) fish (see Methods section for an explanation of  $\Delta C_T$  and  $\Delta\Delta C_T$  values).

The first Cu exposure experiment suggested MT mRNA was a sensitive indicator of exposure to high concentrations of metals (36-fold increase). Subsequent exposures to Cu and Zn showed a less dramatic increase in MT mRNA. The second Cu exposure showed significant induction at 300 and 500  $\mu\text{g/litre}$  Cu (2.89- and 2.26-fold), and although the Zn exposures showed elevated levels relative to the control samples, these increases were not significant because of the level of variation between individuals (Table 1).

For all experiments the highest metal concentrations resulted in an increase in MT mRNA relative to the mean level of the control fish, although only 500  $\mu\text{g/litre}$  Cu (CuSO<sub>4</sub> Experiment 1), and 300 and 500  $\mu\text{g/litre}$  Cu (CuSO<sub>4</sub> Experiment 2) showed a statistically significant increase. The range in relative MT mRNA levels (indicated in parentheses) for each concentration was usually quite high, indicating the high inter-fish variability in MT expression. There was no correlation between levels of MT mRNA and fish sex or size (data not shown).

### Field sampling

The high variation in MT mRNA levels obtained from the concentration-response experiments prompted us to assess the "natural" variation of MT mRNA in fish inhabiting streams and rivers in the South Island. Fish were captured from four rivers (Wairau River, Motueka River, Ashley River, Rakaia River) during early December 2002, and immediately killed and the liver tissue preserved, so that MT mRNA levels should reflect that of the fish in its natural habitat. Subsequent analysis of MT mRNA levels in the 28 samples showed a range of

$\Delta C_T$  values between 12.95 and 17.52 (Fig. 1). These extremes represent a 24-fold difference of MT mRNA levels, although comparison of the 25th percentile (15.09) and the 75th percentile (16.57) values gives a difference of 2.8, i.e., 50% of the values were less than 3-fold different. There was no significant difference in MT mRNA levels between the four rivers sampled.

The metal concentrations in water sampled from the locations where fish were captured showed that

for all rivers except the Rakaia, Cd, Cu, and Zn levels were below the limits of detection (see Materials and Methods); Rakaia River values were: Cu, 4  $\mu\text{g/litre}$ ; Zn, 34  $\mu\text{g/litre}$ ; Cd, <0.3  $\mu\text{g/litre}$ .

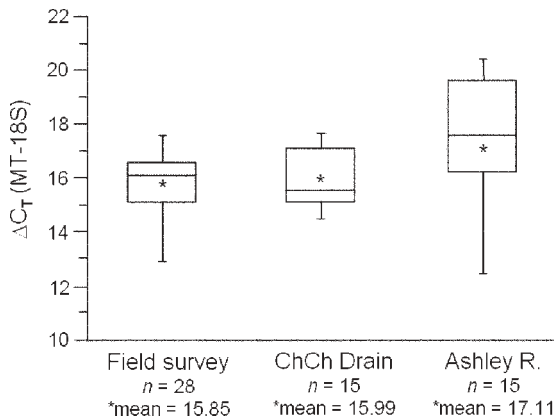
### Comparison of two populations

The Christchurch City Outfall Drain derives most of its flow from surface run-off from a 4.8 km<sup>2</sup> catchment area, much of which is zoned industrial or commercial (Robb 1988). Near its outfall to an

**Table 1** Metallothionein (MT) mRNA levels for *Gobiomorphus cotidianus* CuSO<sub>4</sub> and ZnSO<sub>4</sub> exposure experiments (48 h). MT mRNA is normalised against 18S rRNA and is expressed relative to the mean level of the 0  $\mu\text{g/litre}$  control fish for each experiment.

[Cu <sup>II</sup> /Zn <sup>II</sup> ] ( $\mu\text{g/litre}$ )	<i>n</i>	Mean $\Delta C_T$ ( $C_T$ MT – $C_T$ 18S)	$\Delta\Delta C_T$ $\Delta C_T - \Delta C_T$ (0 $\mu\text{g/litre}$ )	MT Rel. to 0 $\mu\text{g/litre}$
<b>CuSO<sub>4</sub> exposure experiment 1</b>				
0	4	17.57±0.68	0.00±0.68	1.0 (0.62–1.60)
10	4	16.09±1.57	–1.48±1.57	2.79 (0.94–8.32)
50	3	16.95±0.52	–0.62±0.52	1.53 (1.07–2.20)
100	4	16.03±1.83	–1.54±1.83	2.91 (0.82–10.36)
500	3	12.37±0.62	–5.20±0.62	36.70* (23.92–56.31)
<b>CuSO<sub>4</sub> exposure experiment 2</b>				
0	5	12.54±0.82	0.00±0.82	1.0 (0.57–1.77)
100	5	13.22±0.46	0.68±0.46	0.62 (0.45–0.85)
300	3	11.01±0.75	–1.53±0.75	2.89* (1.71–4.88)
500	4	11.36±0.86	–1.18±0.86	2.26* (1.25–4.10)
<b>ZnSO<sub>4</sub> exposure experiment 1</b>				
0	5	12.34±0.15	0.00±0.15	1.0 (0.90–1.11)
300	5	14.06±1.50	1.72±1.50	0.30 (0.11–0.86)
600	5	13.15±1.58	0.81±1.58	0.57 (0.19–1.71)
1000	5	12.00±1.08	–0.34±1.08	1.27 (0.60–2.69)
<b>ZnSO<sub>4</sub> exposure experiment 2</b>				
0	5	13.62±1.47	0.00±1.47	1.0 (0.36–2.77)
300	5	13.29±1.21	–0.33±1.21	1.26 (0.54–2.91)
600	5	12.83±1.35	–0.79±1.35	1.73 (0.68–4.40)
1000	5	12.44±0.80	–1.18±0.80	2.26 (1.30–3.95)

\*Relative MT mRNA level compared with 0  $\mu\text{g/litre}$  control was statistically significant (0.05 CI).



**Fig. 1** Boxplot showing the distribution of  $\Delta C_T$  (MT-18S) values from the field survey, and the Ashley River/Christchurch Main Drain, New Zealand comparison. Note: low  $\Delta C_T$  values indicate increased metallothionein (MT) expression.

estuary (controlled by a flat-valve) exists a sizable population of common bullies. The relative MT mRNA levels in a sample of this population ( $n = 15$ ) were compared directly with a sample of common bullies taken from the Ashley River, which served as a control. Sampling took place in December 2002, and the water temperature and pH at both sites was similar (21.7–22°C, pH 7.8). Fish were immediately killed following capture and liver tissue preserved as described above. Subsequent analysis of MT mRNA–18S rRNA  $\Delta C_T$  values for the sampled fish showed a significant (0.05 C.I.) difference in  $\Delta C_T$  values between the two populations; the mean MT mRNA levels for the Christchurch Drain population were 2.2-fold higher than for the control site ( $\Delta \Delta C_T$  between the mean  $\Delta C_T$  for each population was 1.13) (Fig. 1).

Metal analysis of the Christchurch City Outfall Drain water taken at the site was: Cu, 6  $\mu\text{g/litre}$ ; Zn, 28  $\mu\text{g/litre}$ ; Cd, 0.4  $\mu\text{g/litre}$  confirming the elevated levels, especially Zn, in this water compared with that of the Ashley River where metal levels were below detection.

## DISCUSSION

A large number of nucleotide and amino acid sequences of MT genes from a variety of fish species now exists in Genbank. The high similarity of these sequences illustrates the high level of evolutionary conservation of this gene. The MT gene of *G. cotidianus* is very similar to several recently

characterised MTs, having only 3–4 amino acid differences (c. 5%) compared to gilthead sea bream, golden grey mullet, and others. It is of interest that MT of the common bully, a freshwater fish, is most similar to that of marine species, but considering its diadromous lifestyle it may have evolved from marine ancestors. Although we could only detect a single isoform of MT in *G. cotidianus* by nucleotide sequencing of a cDNA product, we cannot discount the possibility that more than one isoform may exist. However, the presence of a single isoform has previously been observed in flatfish (George et al. 1992).

The quantitative fluorescent RT-PCR technique worked very well for this study. Analyses were highly reproducible and simple to perform, and the use of the 18S rRNA endogenous reference allowed accurate normalisation of MT mRNA values without the need to weigh tiny portions of tissue, or rely on less precise measurement of a standard. The collection of tissue samples in the field was facilitated by the use of the RNA-later preservative, avoiding the need to transport liquid nitrogen.

It could be argued that measurement of MT in gill tissue might be a more sensitive indicator of exposure to water-borne metals than hepatic MT since the gills are the main source of uptake of dissolved metals (Lelan & Kuwabara 1985). We have focused on liver MT since, according to a generalised model for metal uptake and excretion pathways in fish (Roesijadi 1992), metal ingested (e.g., as elevated levels in invertebrates) passes into the liver, as do metals absorbed through the gills (via the blood/haemolymph). Liver MT levels would therefore better represent metal exposure through both water-borne and ingested routes, which would be appropriate for measuring MT levels in field-sampled fish, but may not be as responsive as gill MT for the short-term Cu and Zn exposures.

In this study the 48 h Cu and Zn exposure experiments subjected fish to unrealistically high metal concentrations for a short time period. This situation is not representative of chronic exposure in a natural habitat, but our aim was to assess whether water-borne metals would cause transcriptional activation of MT in the liver, which could be detected as elevated hepatic MT mRNA levels. Although the first Cu exposure experiment suggested MT mRNA was a sensitive indicator of exposure to high concentrations of metals (up to 36-fold increase), subsequent exposures to Cu and Zn were less convincing. A critical property of the results obtained in these experiments was the high

variability in MT mRNA levels between fish in the same exposure sample. Such variation had a profound influence on the quality and reproducibility of the data from the Cu and Zn exposures. These results prompted investigation of the “natural” variability in hepatic MT mRNA levels in common bullies inhabiting several unpolluted rivers in the South Island. By dissecting the fish and preserving liver tissue as soon as possible after capture we hoped to obtain a snapshot of “natural” MT mRNA levels in each fish, without the stresses that transport to the laboratory, acclimatisation, and metal exposure would induce. This experiment showed highly variable natural MT mRNA levels; although half the fish had levels that differed by less than 3-fold, the most extreme variation was a 24-fold difference. Such variation did not appear to be the result of site-specific differences, but this is difficult to discern because of the low number of fish sampled from each site.

The high inter-fish variability in hepatic MT mRNA levels can be attributed to the multitude of cellular functions in which MT participates. Since its primary metabolic function is the homeostasis of Cu and Zn, MT levels could be affected by many different endogenous and environmental parameters (Roesijadi 1992; Samson & Gedamu 1998). Basal expression will fluctuate to meet cellular demands for storage and transport of these essential metals, which will be dictated by biological processes. For example, during reproduction, Zn utilisation in the liver of female rainbow trout is altered since many Zn-requiring enzymes are induced during vitellogenesis (Olsson et al. 1989). After vitellogenin synthesis is complete, MT is induced because of the release of free Zn (Olsson et al. 1987). Transcriptional induction of MT by activators such as metals, ROS, and a variety of cellular messengers such as hormones and cytokines, will be superimposed over MT basal expression. This secondary level of regulation of MT expression indicates the stress-response function of MT. Such are the processes to consider when assessing the correlation between MT mRNA levels and metal exposure. Our attempts to correlate *G. cotidianus* MT mRNA levels with variables such as fish size, sex, reproductive state, or sampling location yielded no statistically significant relationships (data not shown).

Despite the high variation between fish, the sampled population from the polluted Christchurch City Outfall Drain had significantly higher mean MT expression (2.2-fold) than the Ashley River control sample. Analysis of water-borne metal levels

showed that Cu and Zn levels were c. 6-fold and 9-fold higher, respectively, in the Drain water than in the Ashley River water. The larger sample size ( $n = 15$ ) in this analysis may have reduced the influence of the variation, allowing a difference to be discerned against the high background variability.

The results of this study suggest that, although advances in molecular biology techniques now permit accurate measurement of the expression of any given gene, caution should be exercised to ensure that the results are meaningful. In this instance, it is not clear that hepatic MT mRNA levels in the common bully are a reliable indicator of metal exposure, since expression seems primarily dictated by metabolic processes that we poorly understand. Further validation of this approach through more laboratory and field studies is necessary to assess the true utility and reproducibility of this technique. It should be stressed that the use of molecular biology approaches should be seen in the context of other measures of environmental health, and this kind of assay may complement traditional measures of water quality such as direct measurement of trace metals and other contaminants, Biological Oxygen Demand (BOD), or the Macroinvertebrate Community Index (MCI), but should not be used as a replacement.

To our knowledge this study represents the first measurement of a sub-lethal response to environmental pollution in the common bully. The work described here may lead to attempts to measure other biomarker genes in the common bully, such as vitellogenin induction, heralding the role of this native fish as a useful sentinel species for monitoring New Zealand's freshwater environment.

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