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RESEARCH ARTICLE

Tissue Specific Metallothionein Gene Expression in Air-Breathing Teleost, *Channa punctata* (Bloch)

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Abstract Cellular levels of specific transcripts or proteins are often measured in relation to a variety of biological events, using a selection of qualitative and quantitative methodologies. Changes in expression levels, measured in a native sentinel species may serve as biomarkers for the effects of environmental perturbations. A quantitative real-time reverse-transcriptase polymerase chain reaction study was conducted using freshwater air-breathing fish, *Channa punctata* (Bloch) as a test model for observing metallothionein mRNA expression pattern under the influence of varied concentrations of heavy metal, cadmium for an exposure span of 14 days. The findings revealed that metallothionein gene expression is a tissue specific phenomenon in *C. punctata* and liver is a prominent site for first-line defence during early exposure phase to cadmium, whereas kidney metallothionein mRNA indicates prolong presence of metal in the body.

Keywords Air-breathing fish · Cadmium · *Channa punctata* · Gene expression · Heavy metal · Metallothionein

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Introduction

A variety of natural and anthropogenic stressors influence adversely aquatic ecosystems in the world. Industrial discharges containing toxic and hazardous substances, including heavy metals contribute tremendously to the pollution of aquatic environment [1, 2]. On the basis of protein binding patterns of metals, several kinds of metal–protein interactions may be considered. A protein may be the target of toxicity that may play a protective role and help in reducing the activity or toxicity of the metal. In addition, proteins with specific metal binding properties play a special role in both the transport of metals from plasma to tissues and in the transport of metals across cell membranes and within the cell.

Metallothioneins (MTs) are soluble, low-molecular weight (6–7 kDa) cytosolic metalloproteins that are able to selectively bind with metals such as cadmium (Cd), zinc (Zn), and copper (Cu) due to their high cysteine content [3]. The thiol ligands of MT avidly bind a number of toxic metals as a part of defence system [4]. Binding of both essential (Cu and Zn) and nonessential (Cd and Hg) metals in MTs has a high thermodynamic but low kinetic stability. Thus, metal binding is very tight, but there is facile metal exchange with other proteins [5].

There is an increasing emphasis on the use of biomarkers of exposure, toxicity, and susceptibility to toxic metals. Biomarkers of exposure, also called as biological monitors, such as metal concentration in blood, and urine, have a long history of use, but the emergence of molecular biology has greatly expanded the possibilities for all types of biomarkers. The induction of genes is known to take part in protective role against metal toxicity for example; the metallothionein mRNA expression shows promise as marker of both the effect and susceptibility. The use of such biomarker provides guidelines for preventive measures or therapeutic intervention [6].

Although a number of studies are available that relate to the mechanism of regulation of MT expression in several fish species, studies using air-breathing teleost species as sentinels in aquatic environments are scarce. Therefore, the present investigation was conducted with the objective to study the expression profile of metallothionein gene in selected freshwater air-breathing fish, *Channa punctata* (Bloch), a potent biomarker fish species, under the influence of heavy metal, cadmium for various time durations and sublethal concentrations.

Materials and Methods

Experimental Animals

The healthy specimens of *Channa punctata* having wet weight 21.34 ± 2.79 g and length 12.05 ± 0.56 cm (mean ± S.E.) were acclimatised for 15 days under laboratory conditions prior to heavy metal exposure. The fishes were fed, ad libitum, with boiled chicken eggs. The faecal matter and other waste materials were siphoned off daily to reduce nitrogenous waste in water. Every effort was made to maintain optimal conditions during acclimatisation and no mortality occurred during this period.

Test Chemical

For the present study, analytical-grade cadmium chloride, monohydrate ($CdCl_2 \cdot H_2O$; 98.0 % EC, maximum limits of impurities, iron 0.0005 % and sulphate 0.005 %), manufactured by HiMedia Laboratories Pvt. Limited, Mumbai, India was procured and used as test compound.

In vivo Exposure Experiment

The acute toxicity bioassay to determine the 96 h median lethal concentration (96 h LC_{50}) of Cd was conducted in the semi-static system following standard methods [7]. The 96 h LC_{50} value of Cd was determined as 14.95 mg L^{-1} [8]. Based on the LC_{50} value various sublethal concentrations were determined for the study and two separate sets of experiment were designed. For the first study, a single sublethal concentration of Cd (1/4th of LC_{50} = 3.74 mg L^{-1}) was selected for exposing fish specimens in the semi-static system for 14 days and tissue sampling was done at eight exposure points—0, 1, 2, 4, 8, 24, 72 h, and 14 days at the rate of five fish per duration (n = 5). The fish maintained in tap water without the test chemical (control group) were considered as calibrator (0 h) for the study.

For the second set of experiment, fish specimens were exposed to three concentrations of Cd viz, C_1 (0 mg L^{-1}), C_2 (1/4th of LC_{50} = 3.74 mg L^{-1}), and C_3 (1/2 of LC_{50} = 7.48 mg L^{-1}) in the semi-static system for a period of 14 days and tissue sampling was done on 14th day (n = 5). The fish maintained in tap water without heavy metal (control group) were considered as calibrator (C_1 = 0 mg L^{-1} Cd). The metal concentrations selected for the study are in good concurrence to that used by other investigators [9–12].

During experiments, the test solutions were changed after every 24 h to maintain the appropriate concentration of the metal in the test aquaria. The physico-chemical properties of test water were determined according to the standard procedures [7].

At each sampling exposure point liver, kidney, and gill tissues were taken from each individual. The tissue sample were placed in sterile tubes and immediately stored in RNA*later* RNA Stabilization Reagent (Qiagen GmbH, Germany) following the manufacturer's instructions for further processing.

Primers for the Real-Time Amplification

The quantitative real-time reverse-transcriptase polymerase chain reaction (q-RT^2-PCR) primers (MT-2 F′, forward; MT-2 R′, reverse) for the real-time PCR amplifications of MT were designed on the basis of the sequence information of *C. punctata* MT gene (GenBank Accession No. FJ869867).

Beta-actin (β-actin) was used as an internal control to normalize mRNA levels in the real-time PCR amplification study. The sequence information obtained from β-actin mRNA in *C. gachua* (GenBank Accession No. GQ219743) was used for the designing of primers (β-actin F′, forward; β-actin R′, reverse).

Sequence information was submitted to Operon (Operon Biotechnologies GmbH, Germany) for primer production (Table 1). Melt curve and primer efficiency analyses were conducted for each primer for the purpose of quality control.

Total RNA Extraction

Total RNA was extracted from each sample using TRI Reagent extraction protocol (Molecular Research Center, Inc. Cincinnati, OH) [13]. RNA pellets were finally resuspended in nuclease-free water to a concentration of approximately 100 ng μl^{-1}.

To ensure quality, samples were quantified spectrophotometrically at 260 and 280 nm (PowerWave XS Microplate Spectrophotometer, Bio Tek Instruments, Inc., USA). Only samples with absorbance 260 to 280 ratios (A_{260} / A_{280}) greater than 1.7 were used in subsequent analyses. RNA integrity was checked by denaturating formaldehyde agarose (FA) gel electrophoresis with 3[N-morpholino] propanesulfonic acid (MOPS) buffer [14].

Table 1 Primer sequences used in RT-PCR for the amplification of Metallothionein and Beta-actin mRNA

Primer	Sequence (5'->3')	Length (bp)	Tm (°C)	GC %	Reference (GenBank accession no.)
MT-2	*CTGCAACTGCGGAGGA	16	57.92	62.50	FJ869867
	**GGTGTCGCATGTCTTTCCTT	20	60.12	50.00	
β-actin	*GTGCCCATCTACGAGGGTTA	20	59.96	55.00	GQ219743
	**AAGGAAGGAAGGCTGGAAGA	20	60.32	50.00	

* Forward primer, F′; ** Reverse primer, R′; FJ869867 *C. punctata* MT, 559 bp DNA; GQ219743 *C. gachua* Beta-actin 314 bp, mRNA

Expression of Metallothionein mRNA

q-RT^2-PCR amplifications were carried out using the QuantiTect SYBR Green RT-PCR kit according to the manufacturer's instructions (Qiagen). Reaction mixture included 25 µL of kit MasterMix, 1 µL of 50 µM gene specific primers, and 50 ng total RNA. Amplifications were performed in triplicates for 45 cycles at 50 °C (30 min), 95 °C (15 min), 94 °C (15 s), 56 °C (30 s), and 72 °C (30 s) for reverse transcription, PCR initial activation, denature the cDNA, anneal the primers, and extend the product, respectively in a LightCycler real-time PCR (LightCycler 480 System, Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany).

Metal Analysis

In all exposure groups Cd level was analysed using an atomic absorption spectrophotometer (AAnalyst 300 Spectrometer, Perkin Elmer, USA). Three replicate samples were analysed to obtain an average Cd concentration at each sampling. The sample digestion and analysis was done following the standard methods [7] and as per manufacturer's instructions.

Statistical Analysis

For the q-RT^2-PCR data analysis a method of comparison of C_T values, the 2^ (– $\Delta\Delta C_T$) method was used [15]. Statistical analyses were performed using SPSS (version 16.0.2, 2008) computer software [16]. Comparisons between the effects of various exposure durations and the different concentrations on mean relative tissue specific mRNA expression was done using multiple comparison technique, Tukey's HSD post hoc test. The level of significance was estimated at $\alpha = 0.01$.

Observations

Physico-Chemical Properties of the Test Medium

During the experimentation temperature of the test water varied from 19.7 to 22.9 (21.45 ± 0.40) °C, and pH values ranged from 7.19 to 7.99 (7.52 ± 0.07). The dissolved oxygen concentration was normal, varying from 6.82 to 8.13 (7.56 ± 0.16) mg L^{-1}. The conductivity of the test water ranged from 239 to 307 (282.7 ± 7.38) µS cm^{-1}. The total hardness and total alkalinity of the test water varied from 168 to 197 (185.7 ± 3.10), and 245 to 277 (263.6 ± 3.74) mg L^{-1} as $CaCO_3$ respectively during exposure.

Cadmium Concentration in Test Water

The measured concentrations of Cd in the test water using AAS at each exposure duration (0, 1, 2, 4, 8, 24, 72 h, and 14 days), and in each concentration group ($C_1 = 0$ mg L^{-1}, $C_2 = 3.74$ mg L^{-1}, and $C_3 = 7.48$ mg L^{-1} Cd) are given in Table 2 a, b. The measured concentration of Cd in test media was found to be slightly lesser in comparison to that of the contents dissolved in the samples, and showed a decrease ranging from 6.95 to 24.87 % in 0–14 days exposure span.

Metallothionein mRNA Expression

In the study tissue specific (liver, kidney, and gill) MT mRNA expression was recorded in *C. punctata* after waterborne exposure to 3.74 mg L^{-1} Cd for an exposure span of 14 days (Fig. 1). During experimentation liver shows biphasic reaction to the expression of MT mRNA. Liver MT mRNA was found to be significantly heightened with 0.67, 0.37, 0.52, and 0.58-fold induction levels after 1, 2, 4, and 8 h Cd exposure, respectively ($P < 0.01$). It has diminished significantly after a 1 h exposure ($P < 0.01$). Post 2 h Cd exposure liver shows significant up regulation of MT transcript level. Non significant up regulation in transcript level was reported on 8 h in comparison to that of 4 h post Cd exposure.

24 h after Cd exposure MT mRNA was observed below the basal level in all the tissues of test fish. From 72 h kidney shows elevated MT mRNA expression, before this at all time points it was below basal level, and was found to be significantly up regulated in duration dependent manner with 0.16 and 1.24-fold induction level post day 3 and 14 Cd exposure, respectively ($P < 0.01$).

The weakest MT mRNA expression was found in gills, where at all the time points it was and always below basal

Table 2 Cadmium concentration in aquaria test water: (a) at various exposure durations; (b) in various concentration groups

	Cd concentration (mg L^{-1})		Decrease in concentration (%)
	Dissolved	Measured (mean)*	
(a) Exposure duration			
0 h	3.74	3.48	6.95
1 h		3.45	7.75
2 h		3.41	8.82
4 h		3.38	9.63
8 h		3.21	14.17
24 h		2.91	22.19
72 h		2.81	24.87
14 d		2.91	22.19
	Cd concentration (mg L^{-1})		Decrease in concentration (%)
	Dissolved	Measured (mean)*	
(b) Exposure group			
C_1	0	N.D.	–
C_2	3.74	2.91	22.19
C_3	7.48	6.56	12.30

N.D not detected

(* triplicate samples were analyzed to obtain an average Cd concentration at each exposure point)

(* triplicate samples were analyzed to obtain an average Cd concentration from each exposure group)

level. The strongest MT mRNA expression was reported in the liver, even in the initial exposure phase *i.e.,* up to 8 h. This indicates that liver is a major site of MT transcription as an early response to Cd exposure.

In another experimental set where animals were exposed to various concentrations of waterborne Cd *i.e.,* 0, 3.74, and 7.48 mg L^{-1} for 14 days, heightened MT mRNA expression was reported only in kidney, whereas, in other tissues it was below the basal level (Fig. 2). An inversely proportionate concentration dependent MT mRNA up regulation was observed in kidney. It was found that the MT mRNA was up regulated significantly ($P < 0.01$) by 1.24 and 0.58 times at 3.74 and 7.48 mg L^{-1} Cd exposure.

The differences in the effects of metal exposure durations and the different concentrations on MT transcript level in tissues under study were found significant ($P < 0.01$). In pair wise comparison also, all differences were found significant at the level of $P < 0.01$.

Discussion

A variety of techniques and endpoints, currently employed in bio-monitoring of aquatic environments are useful for determination of genotoxic effects or impacts of pollutants. Fish MTs attracted special attention in the recent past because of increasing pollution of the aquatic environment with technogenic xenobiotics. The transcript level of MTs is usually determined by various PCR based methods. In this study, we reported tissue specific expression pattern of MT mRNA in *C. punctata* in response to heavy metal, Cd using q-RT^2-PCR.

In the study after exposing test fish for 14 days, liver shows biphasic reaction as the up regulation in MT transcript level was observed up to 8 h only, whereas kidney and gill have this below basal level (Fig. 1). The liver seems to be a major site for metal uptake in an initial defence mechanism. However, after 8 h of exposure, no significant MT transcript level was observed. It appears that liver MT complex is either degraded after 8 h of Cd exposure or is transported elsewhere to different organs.

The initial up regulation in liver MT mRNA level attests liver as a potent organ for Cd detoxification in the early exposure phase in *C. punctata*. It is in accordance with previous studies revealing that Cd treatment causes induction of MT-like protein (MTLP) in liver [17–20]. Post 24 h Cd exposure elevation in MT mRNA level was observed in kidney and transcript level was found linearly related with the exposure duration in this tissue. On day 14 the MT mRNA level was found to be heightened with >7 fold expression relative to that of on day 3 (Fig. 1).

After Cd absorption by the gills, the metal is probably transferred to the liver where it induces the synthesis of MT, which in turn is delivered to the blood and finally accumulated in the kidney [21]. In mammals, the kidney responds to Cd by both reabsorbing circulatory Cd–MT complex that has been released from the liver and gut, and filtered into the renal tubules, as well as by synthesising renal MT for Cd storage [22]. It is likely that fish also respond in a similar way, a relatively greater amount of Cd–MT complex released from extrarenal tissues reabsorbed in the kidney and more renal MT was synthesised [23].

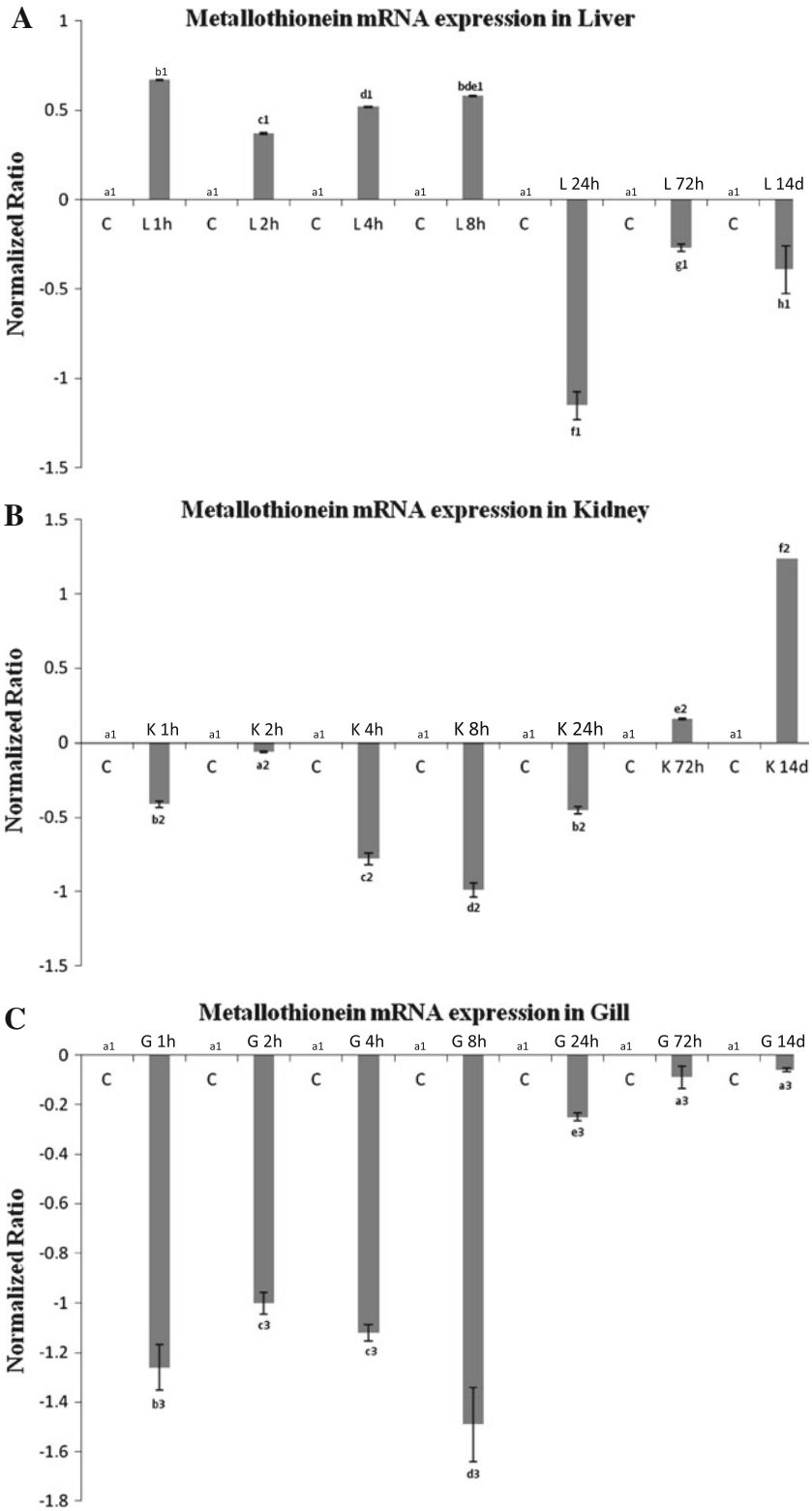

Fig. 1 Mean relative metallothionein mRNA expression (±S. D.) in liver (**a**), kidney (**b**), and gill (**c**) tissues of *C. punctata* (n = 5) after exposure to cadmium for 1, 2, 4, 8, 24, 72 h and 14 days. ***L***, ***K***, ***G*** denotes Liver, Kidney and Gill tissues, respectively. ***C*** denotes calibrator, *i.e.*, relative expression (ΔC_T) in control sample at 0 h. Different alphabet superscript letters denote significant difference ($P < 0.01$) in mRNA expression between exposure points within tissue. Different numeric superscript values denote significant difference ($P < 0.01$) in mRNA expression between tissues within exposure point

Decrease in metal concentration level was found to be directly proportionate to the duration. The decrease in the concentration after metal analysis may be attributed to the precipitation of the metal in test water as well as the hardness and relative higher pH of test aquaria solution causing lesser solubility of Cd [24, 25].

On day 14th after exposure to 3.74 and 7.48 mg L^{-1} Cd only kidney showed significant up regulation in MT mRNA

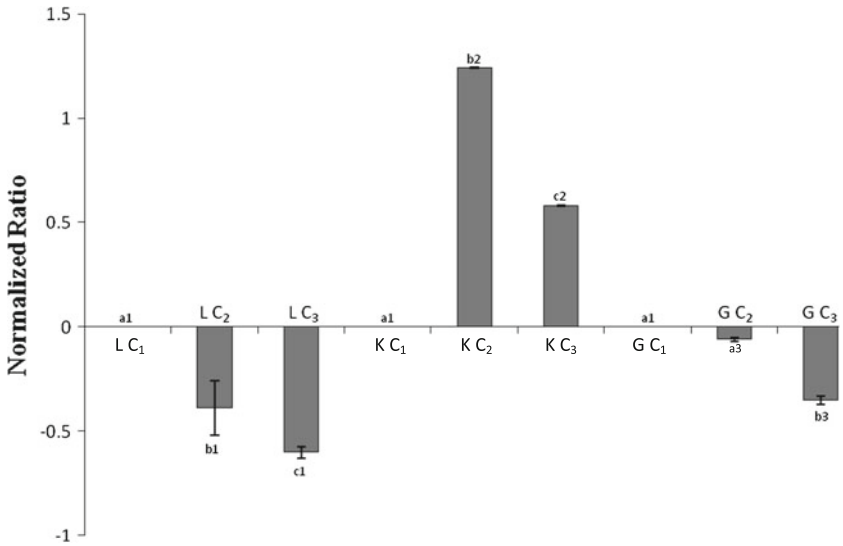

Fig. 2 Mean relative metallothionein mRNA expression (±S. D.) in liver (***L***), kidney (***K***), and gill (***G***) tissues of *C. punctata* (n = 5) after exposure to (**C_1 = 0, C_2 = 3.74, C_3 = 7.48 mg L^{-1}**) cadmium for 14 days. Relative expression (ΔC_T) in control sample (C_1 = 0 mg L^{-1} Cd) used as calibrator. Different alphabet superscript letters denote significant difference ($P < 0.01$) in mRNA expression between concentrations within tissue. Different numeric superscript values denote significant difference in mRNA expression ($P < 0.01$) between tissues within concentration

level, whereas in liver and gill it was below basal level (Fig. 2). The kidney seems to be the prominent site for MT synthesis and metal uptake in *C. punctata* after a prolonged Cd exposure. As freshwater fish can take up waterborne Cd via the gills and dietary Cd via gut, during waterborne exposure after uptake across gill, metal is probably bound to transport proteins in the blood plasma as in higher vertebrates [22]. They are distributed via the circulatory system to various internal organs where toxicological responses may be elicited [26, 27].

The dynamics of MT induction by different concentrations of Cd showed that the level of MT in the liver increased for 6 days when the fish were kept in water with Cd concentrations of 5–30 mg L^{-1} or received intraperitoneal injections at the rate of 2 mg kg^{-1} body weight [28]. In gold fish injected with Cd, the induction of MT in kidney was much lower than in liver [29]. According to this view, the Cd concentration in kidney will be higher than in liver only after a long-term exposure [30, 31].

The present study indicates a nonlinear response with the metal concentration, as two fold decrease in kidney MT mRNA level was recorded with increased metal concentration (Fig. 2). It may that the acute lethal concentrations affect the capacity of the cells to synthesise proteins and due to which MT production becomes too slow to sequester all the Cd that enter into the cells. This points out clearly that the capacity of the cells to bind Cd in MT is limited by higher accumulation of metal.

Although induction of MT in gill and kidney has been reported in fishes, liver seems to be more actively involved in the formation and storage of MT as compared to other organs [32, 33]. It may be co-related to the fact that in comparison to the liver and kidney relatively little Cd accumulated in the gills. It may be due to the lower absorption of Cd by the gills and as a consequence, low gill MT concentrations were present [34]. The lower Cd accumulation in gills with respect to other tissues is common phenomenon in fish [35, 36].

The results clearly indicated that during the initial phase of Cd exposure increased MT mRNA level was recorded in the fish liver. Subsequently, with the progression of metal exposure, the maximum heightened MT mRNA level was observed in kidney that increased up to the Cd-binding capacity of tissue, beyond that it was found to be declined at higher concentration of metal exposure. Gills were either non-inducer or a weak inducer of MT in *C. punctata* during the Cd exposure. This information can further be utilised for adopting biomarker approach for identification of contaminants in aquatic environment.

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Conflict of interest The authors declare that there are no conflicts of interest.

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