



# Duration of Induced Metallothionein is a Critical Factor in the Cadmium-Resistance Among Goldfish (*Carassius auratus*) and Common Carp (*Cyprinus carpio*)

Feng Ming Chang<sup>1</sup> · Chia Hao Lin<sup>2</sup> · Su Mei Wu<sup>1</sup>

Received: 4 July 2021 / Accepted: 29 December 2021 / Published online: 23 February 2022

© The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2022

## Abstract

According to the Environmental Protection Agency in Taiwan, the common carp (*Cyprinus carpio*) is one species of fish for acute toxic test. It has been found to be extremely sensitive to the toxicity of Cd<sup>2+</sup>; Furthermore, the goldfish (*Carassius auratus*) has a higher resistance than common carp upon Cd<sup>2+</sup> exposure, but both fish are the same family. The aim of the study was to compare the physiological and histo-pathological responses between goldfish and common carp under exposure to sublethal concentrations of Cd<sup>2+</sup> in order to understand the reasons behind the Cd<sup>2+</sup>-resistance. Results showed that metallothionein (MT) protein levels in visceral tissues were exceptionally increased and elevated at an earlier time in goldfish than in common carp. Meanwhile, the amount of Cd<sup>2+</sup> accumulation in goldfish was higher than common carp after Cd<sup>2+</sup> exposure. The histo-pathological results revealed that the density of gill mucus cells and the thickness of gill epithelium in common carp were raised earlier than in goldfish, but the histo-pathological findings resemble each other. According to the data, we suggested the efficient response of MT proteins may contribute to goldfish with a higher Cd<sup>2+</sup> tolerance.

**Keywords** Teleosts · Heavy metal · Histo-pathology · Calcium influx

Cadmium (Cd<sup>2+</sup>) is a non-essential element and has severe toxic effects on aquatic organisms. The waterborne Cd<sup>2+</sup> causes a wide range of pathological effects on fish. According to the Taiwanese Annual Report from Taiwan Environmental Protection Agency (Taiwan EPA 1994), the 96 h-LC<sub>50</sub> of Cd<sup>2+</sup> was 3.1 μM on the common carp (*Cyprinus carpio*), but the resistance of Cd<sup>2+</sup> on goldfish (*Carassius auratus*) was higher by 17.1 folds than common carp. Both fish are the same family, but the reason for such a difference is unknown. The present study is interested in finding the reason.

The distribution of Cd<sup>2+</sup> in sediment from the 6 main rivers in southern Taiwan is about 31.1 μM, and the quantity of Cd<sup>2+</sup> contained in the sediment reflect the degree of pollution for the body of water (Tsai et al. 2007). Cd<sup>2+</sup> is

easily accumulated most in the kidney, liver, gills of fish, and muscles in this order (Ambreen et al. 2015). On the other hand, metallothionein (MT) appeared most in visceral tissues including the liver, intestine, and kidney (Cretì et al. 2010). The gills are also directly exposed to an aquatic environment, and it is very sensitive to aquatic pollutants. In addition, the accumulation rate of Cd<sup>2+</sup> in the gill was faster than that in other tissues at an earlier time, but the accumulation of Cd<sup>2+</sup> had a limit to how much it can absorb after Cd<sup>2+</sup> exposure (Wu et al. 2006). In fact, the accumulation rate of Cd<sup>2+</sup> in other visceral tissues was higher compared to gills in later stages of Cd<sup>2+</sup> exposure (Cretì et al. 2010). This phenomenon reveals that the gill might have some strategies to slow down the accumulation of Cd<sup>2+</sup> in order to decrease the histological damage by free Cd<sup>2+</sup>. Therefore, the present study measured MT contents from visceral tissues, and analyzed and observed the histo-pathology in gills.

The continued stress induced by heavy metals exerts negative effects in fish. The several physiological responses of fish, such as the up regulation of MT and mucus secretion, were induced by heavy metal exposure (Alvarado et al. 2006). These responses in the gill have positive and/or negative effects to fish. For example, the increased mucus

✉ Su Mei Wu  
sumeimei@mail.nctu.edu.tw

<sup>1</sup> Department of Aquatic Biosciences, National Chiayi University, Chiayi 600, Taiwan

<sup>2</sup> Department of Marine Biotechnology, National Kaohsiung University of Science and Technology, Kaohsiung, Taiwan

secretion from the fish gill had a positive effect in removing metals from the surface of the gill epithelium. In the meantime, the increased mucus secretion covered the gill epithelium, and even reduced or blocked the gas exchange. Moreover, Liu et al. (2012) indicated that  $\text{Cd}^{2+}$  exposure disturbed the mRNA expression of epithelial  $\text{Ca}^{2+}$  transporters, decreasing the ability to uptake  $\text{Ca}^{2+}$ . On the other hand, the previous study indicated that the MT synthesis in tissues was induced by  $\text{Cd}^{2+}$  exposure, and it can increase the  $\text{Cd}^{2+}$  bioaccumulation. The increased MT was suggested to be a detoxification function for exposed tissues (Oost et al. 2003). However, its detoxification function was limited under normal physiology (Wu et al. 2016). Thus, we assumed a strategy to promote  $\text{Cd}^{2+}$ -resistance that maintains a normal physiological function rather than decreasing pathological levels. The MT induced efficiency might be related to  $\text{Cd}^{2+}$  resistance of fish.

The developmental stage of fish, waterborne factors, and fish scale functions were all related to the different  $\text{LC}_{50}$  of  $\text{Cd}^{2+}$  in fish (Zeynab et al. 2012). Hence, the aims of the study analyzed whether physiological and histo-pathological responses are both related to the different  $\text{LC}_{50}$  of  $\text{Cd}^{2+}$  in common carp (*C. carpio*) and goldfish (*C. auratus*). These parameters of physiological responses include  $\text{Cd}^{2+}$  accumulation,  $\text{Ca}^{2+}$  influx, and MT contents. The histo-pathological responses in gills includes the density of mucus cells, and the thickness of filament and hematoma levels of both fish after sublethal concentration  $\text{Cd}^{2+}$  exposure.

## Materials and Methods

In order to avoid  $\text{Cd}^{2+}$  impact levels that were influenced by fish size, developmental stage, and environmental conditions in the present study, we chose juvenile common carp (*C. carpio*) and goldfish (*C. auratus*), which has a body length/body weight of 2.5–3.5 cm/0.94 ± 0.15 g, and 2.5–3.5 cm/0.98 ± 0.23 g, respectively. Both species of juvenile fish were purchased from a private aquaculture farm in Tainan County, Taiwan. Fishes were cultured in tanks, each filled with 300 L of filtered water at 27°C, and a 12 h photoperiod with 12 h of darkness. The protocol for animal use was reviewed and approved by the Chiayi University (IACUC Approval No.: 102027).

Tissues were dried at 65°C overnight, and digested with 2 mL of 13.1 M  $\text{HNO}_3$  at 100°C overnight. The digested solutions were diluted with double deionized (dd) water and subjected to  $\text{Cd}^{2+}$  analysis from an air/acetylene flame with atomic absorption spectrophotometry (AA, Z-8000, Hitachi, Japan). Standard solutions of  $\text{Cd}^{2+}$  (Merck, Germany) were used for establishing standard curves. Addition of certain amounts of standard solutions to the test samples was used for background correction to eliminate the matrix effect.

The concentration of exposure media ( $\text{Cd}^{2+}$ ) were prepared from completely dried  $\text{CdCl}_2$  (Sigma, USA) dissolved in 1 mL concentrated HCl, and used with dd water to prepare stock solution. Exposure medium were used with local tap water from the stock solution, in which its concentration was measured using AA before exposure. The procedure in measuring  $\text{Cd}^{2+}$  followed an operation manual of the instrument and was referenced from our previous study (Wu et al. 2000). The experimental medium was changed once every day, in order to maintain the  $\text{Cd}^{2+}$  concentration and water quality.

The MT ELISA kit used is the same from our past research with minor modifications (Wu et al. 2000). The visceral tissues were collected as a sample and analyzed using MT ELISA. A synthetic peptide (MDPCECAKT-GTCNCGGS) from the N-terminal of tilapia MT antibody was coated onto 96-well microtiter plates with coating buffer (0.05 M  $\text{NaHCO}_3$ ; pH 9.6). Rabbit anti-tilapia MT serum was added as the first antibody (1:2000) and peroxidase-labeled goat anti-rabbit IgG (1:4000) was added as the secondary antibody. Color development was measured at 405 nm with an automatic microtiter plate ELISA reader (Dynex MRX, USA). Total proteins were determined using a protein assay kit (Bio-Rad Lab., USA). The coefficients of intra- and inter- assay variations were 5.04% (n = 8) and 15.05% (n = 7), respectively. MT was extracted from goldfish and carp, and the original extraction of MT was diluted with 0.01 M phosphate buffer in 1, 2, 4, and 6 folds. Both the standard MT (a synthetic peptide) and the diluted extraction of MT were run by an ELISA test at the same time.

60 juvenile goldfish (*C. auratus*) and 60 carp (*C. carpio*) were separated to 10 tanks for control and treatment groups. The two species of fish were treated with a sublethal concentration of  $\text{Cd}^{2+}$  (44.6  $\mu\text{M}$ ) for 0, 1, 2, 3, and 4 days (d), and immediately anesthetized with MS222.

The visceral tissues were excised from the body and MT was measured by ELISA following MT extraction. The Cd contents of the whole body were then measured. Both species of juvenile fish were separately treated with water containing 44.6  $\mu\text{M}$   $\text{Cd}^{2+}$  for 24 and 48 h. Six individual fish were rinsed briefly in deionized water, and then transferred to a 1 L  $^{45}\text{Ca}^{2+}$  (Amersham, Piscataway, NJ: with a final working specific activity of 5 mCi/mmol)-containing medium for a subsequent 3 h incubation. After incubation, juveniles were washed several times in an isotope-free medium in order to sufficiently remove all surface-bound calcium. Every larva was put into one vial, anesthetized with MS-222, and then digested with tissue solutions supplemented with counting solutions. Finally, samples were counted with a liquid scintillation beta counter (1211 Rack-beta, LKB, Finland). The  $\text{Ca}^{2+}$  influx was calculated using the following formula:  $J_{in} = Q_{larva} \cdot X_{out}^{-1} \cdot t^{-1} \cdot W^{-1}$ , where  $J_{in}$  is the influx (mmol/mg/h);  $Q_{larvae}$  is the radioactivity of the

larva and each individual larva is counted per minute (cpm) at the beginning and end of incubation;  $X_{out}$  is the specific activity of the incubation medium (cpm/nmol);  $t$  is the incubation time (h); and  $W$  is the average body wet weight of larvae from different stages (mg).

The gills were fixed, dehydrated, embedded, and then sectioned horizontally, perpendicular to the filaments and parallel to the lamellae for the histo-pathological observation upon fish treated with  $44.6 \mu\text{M Cd}^{2+}$  for 0–4 days. Slides were stained with periodic acid Schiff (PAS). Mucus cell (MC) numbers in gills were determined with a light microscope at  $200\times$  for each individual by examining the following: 10 filaments per section were collected from a single individual, and four sections were separated by at least two intervening sections in every individual. Three individuals in the same treatment group were collected for one sample. This represented a total of 30 measurements per treatment. Microscopic images were captured with an image analysis system (Image Pro Plus, Version 4.5, Nikon) using a color tri-charged coupled device video camera. The method for measuring the thickness of epitheliocystis in the gills were referenced from Monteiro et al. (2009), and it was assayed by the Image- Pro Plus 4.5 software (Media Cybernetics).

Data for Figs. 2 and 5B were done by static assay through one-way ANOVA analysis with Tukey’s comparison, and static analysis was done by student’s t-test for Figs. 3 and 4.

### Results and Discussion

A typical dose-related standard curve and MT extracted from the two species of fish by competitive ELISA are shown in Fig. 1. The displacement curve for Cd-induced MT extracted from goldfish and carp were parallel to that of the MT standard, indicating that the ELISA is suitable for measuring Cd-induced MT in goldfish and carp. According to the mortality from both species of fish, we find that the goldfish had a higher  $\text{Cd}^{2+}$  resistance than that in the common carp (Table s1). We also wanted to test the same  $\text{Cd}^{2+}$  treatment for both goldfish and common carp in which both species of fish have a similar mortality rate. Since there is no data for the mortality rate of goldfish between  $35.7$  and  $53.5 \mu\text{M Cd}^{2+}$ , we estimated that the concentration of  $44.6 \mu\text{M Cd}^{2+}$  would have a similar mortality (13%) for goldfish and common carp (Table s1). In the same family, the common carp exhibited a more similar level of  $\text{LC}_{50}$  with grass carp (*Ctenopharyngodon idellus*) at  $84.1 \mu\text{M}$  (Yorulmazlar and Gul 2003). Indeed, 96 h  $\text{LC}_{50}$  was observed from  $1.25$ – $159 \mu\text{M Cd}^{2+}$  in common carp (Delahaut et al. 2020). Therefore, the concentration of  $44.6 \mu\text{M Cd}^{2+}$  was used in the present study. MT mRNA was a useful biomarker for aquatic organisms upon metal exposure. Actually, the MT mRNA expression levels are positively correlated with MT

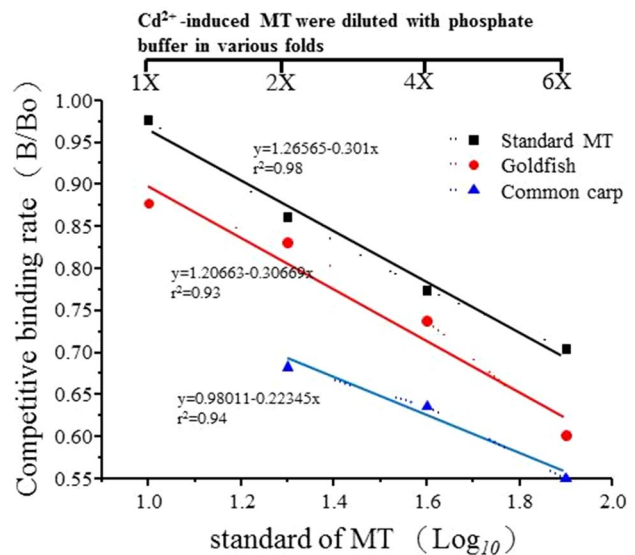


Fig. 1 Competitive binding curves of ELISA for standard MT, and  $\text{Cd}^{2+}$  induced MT in goldfish and common carp

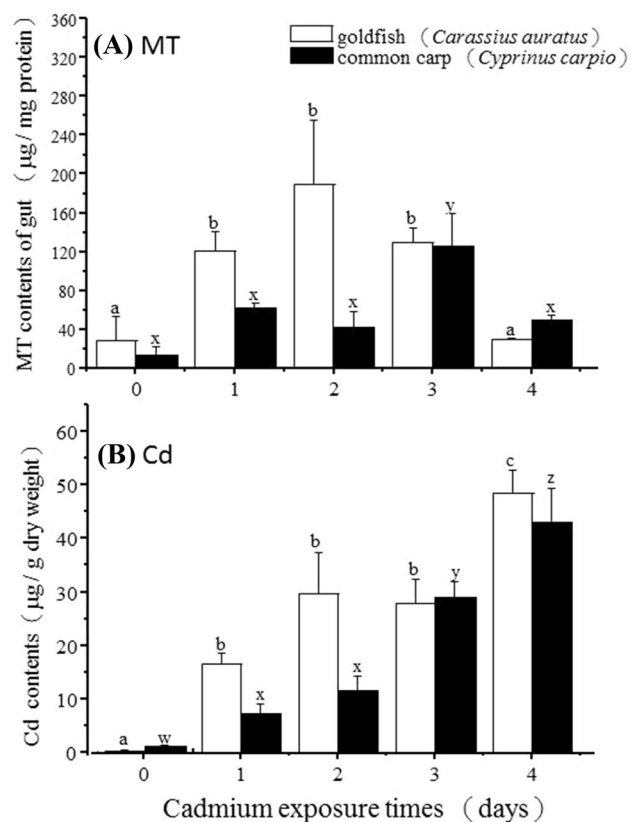
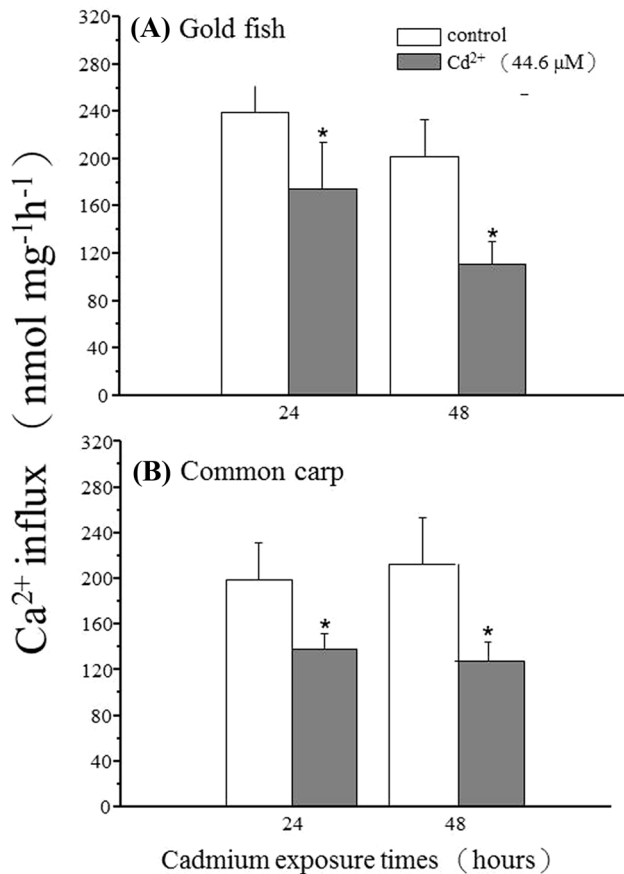


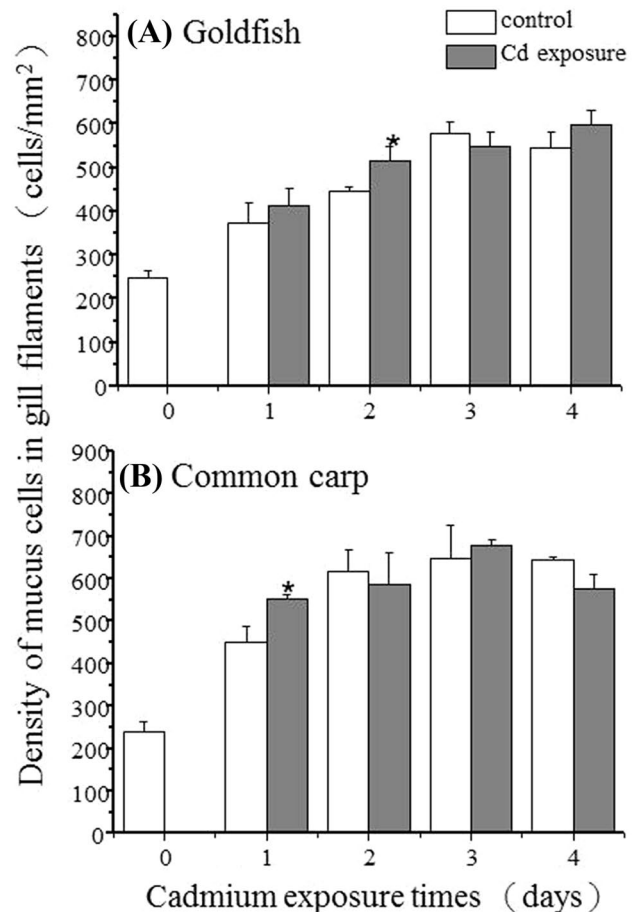
Fig. 2 Metallothionein (MT) contents and Cd contents in goldfish and common carp exposed to  $44.6 \mu\text{M}$  of Cd for 0, 1, 2, 3, and 4 days. Data shown as mean  $\pm$  SE ( $n=6$ ). Data comparison of the same species by one-way ANOVA analysis with Tukey’s comparison. Different letters indicate a significant difference among treatment time



**Fig. 3** Calcium ion influx in goldfish (A) and common carp (B) exposed to 0 (control) and 44.6  $\mu\text{M}$  of  $\text{Cd}^{2+}$  for 24 and 48 h. Data shown as mean  $\pm$  SE ( $n=5-6$ ). Comparison of data at the same treatment time by student's t-test (\* indicates a significant difference between the control and treatment group,  $p < 0.05$ )

protein contents following the treatment time in common carp (Castaldo et al. 2020). The present study used a synthetic peptide from the N-terminal of tilapia (*Oreochromis mossambicus*) MT to produce a rabbit anti tilapia MT antibody. The homology of the amino acid sequence of tilapia MT was around 80% to goldfish (*C. auratus*) or common carp (*C. carpio*). The amino acid sequence of MT in goldfish and common carp were around 92% identical (Chan 1995). We conducted the competitive binding curves of  $\text{Cd}^{2+}$  induced MT with various concentrations in goldfish and common carp, and standard MT ( $\text{Log}_{10}$ ) by ELISA. The coefficient of correlation ( $r^2$ ) values were 0.93 and 0.94. The slope of the MT standard curve was 0.301, 0.3066 in goldfish, and 0.2234 in common carp (Fig. 1). The data showed that the MT protein levels from both species of fish could be recognized by a synthetic peptide of tilapia MT antibody.

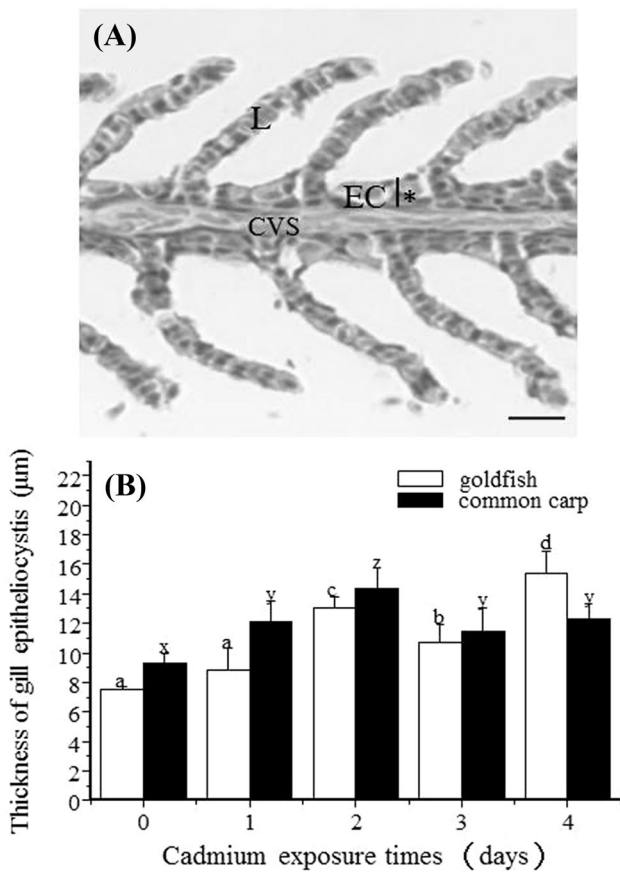
Cadmium is a MT inducer, but the sensitivity appears to be depended on the tissue and the exposure time. Thus, MT can reduce the amount of  $\text{Cd}^{2+}$  entering the cell, thereby limiting the toxicity (Divya et al. 2018). The present results



**Fig. 4** Density of mucus cells in gill filaments of goldfish (A) and common carp (B) exposed to 0 (control) and 44.6  $\mu\text{M}$  of  $\text{Cd}^{2+}$  for 0, 1, 2, 3, and 4 days. Data shown as mean  $\pm$  SE ( $n=3-6$ ). Comparison of data at the same treatment time by student's t-test (\* indicates a significant difference between control and treatment group,  $p < 0.05$ )

showed that the  $\text{Cd}^{2+}$  contents increased over time in both goldfish and common carp under  $\text{Cd}^{2+}$  exposure (Fig. 2B). Although both goldfish and common carp had similar  $\text{Cd}^{2+}$  accumulation levels, the changing trend of  $\text{Cd}^{2+}$  content was more drastic in carp (increased by 5.9 folds; 44.6/7.5  $\mu\text{g/g}$  of  $\text{Cd}^{2+}$ ) than goldfish (increased by 2.9 folds; 49.3/16.8  $\mu\text{g/g}$  of  $\text{Cd}^{2+}$ ) from day 1 to day 4 after exposing to  $\text{Cd}^{2+}$  (Fig. 2B). Meanwhile, the MT content was significantly increased in goldfish after 1 d of exposure to  $\text{Cd}^{2+}$ , but back to base levels on the 4th day. Compared to goldfish, the MT content of carp was only up-regulated on the 3rd day after exposure to  $\text{Cd}^{2+}$  (Fig. 2A). Our previous study found a similar profile that fishes had a positive relationship between MT expression and metal contents under normal physiological circulation (Wu et al. 2000, 2016).

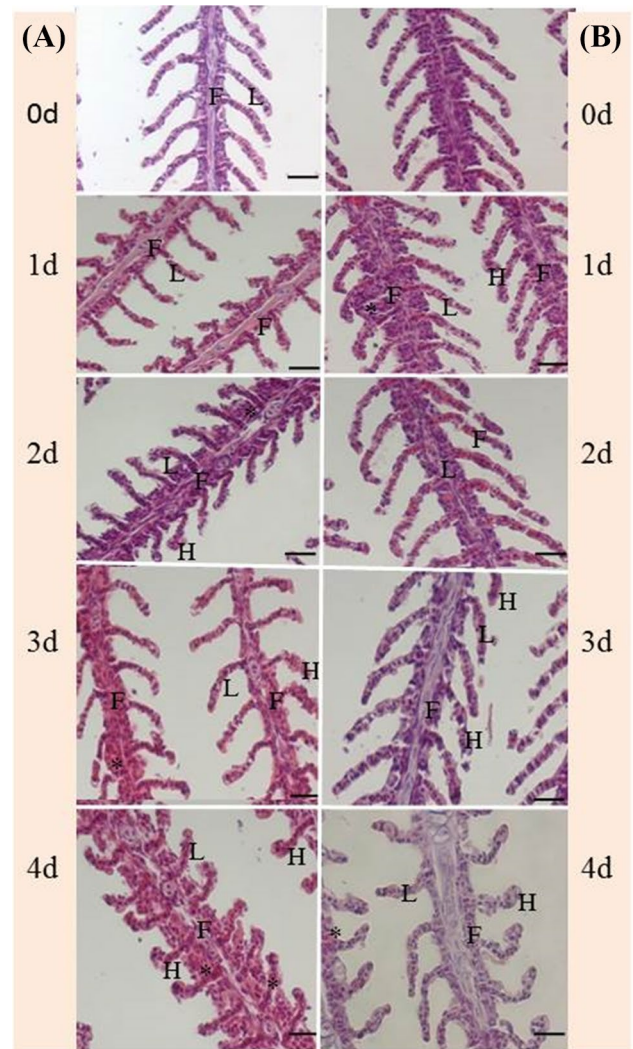
Goldfish and common carp both had around a 30%–40% decrease in  $\text{Ca}^{2+}$  uptake upon 24–48 h of 44.6  $\mu\text{M}$  of  $\text{Cd}^{2+}$  exposure (Fig. 3). The density of mucus cells in the gills of



**Fig. 5** A Schematic diagram of gill lamella (L), central venous sinus (cvs), and thickness of gill epitheliocystis (\*). The scale is 12.5 µm; (B) Comparison of the thickness of gill epitheliocystis between goldfish and common carp exposed 44.6 µM of Cd<sup>2+</sup> for 0, 1, 2, 3, and 4 days. Data shown as mean ± SE (n=6). Data by one-way ANOVA analysis with Tukey’s comparison for the same species. Different letters indicate a significant difference among treatment time (*p* < 0.05)

carp and goldfish highly increased after exposure to Cd<sup>2+</sup> for 1 and 2-day, respectively (Fig. 4). On the histo-pathological profile, the gill filament of both fish had a morphological change upon exposure to 44.6 µM Cd<sup>2+</sup> for 0–4 days. The thickness of gill epitheliums in carp and goldfish was up-regulated after 1-day and 2-day of Cd<sup>2+</sup> exposure, respectively (Fig. 5B). In addition, 36.9% hematoma was found in the gill filaments of goldfish after 4-day of Cd<sup>2+</sup> exposure, but only 14.8% hematoma was observed in common carp (Fig. 6).

According to the data, the histo-pathological responses of both fish resemble each other. Therefore, we suggested that the goldfish appeared to have a higher tolerance upon Cd<sup>2+</sup> exposure than common carp because the MT in visceral tissues was induced earlier in goldfish compared to common carp.



**Fig. 6** Histopathology comparison by HE stain between goldfish (A) and common carp (B) upon 44.6 µM Cd<sup>2+</sup> treatment for 0–4 days. (L gill lamella, F gill filaments, H hematoma). The scale is 25 µm

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00128-021-03449-4>.

**Acknowledgements** This work was supported by Grants (NSC101-2311-B-415-001-MY3) from the National Science Council, Taiwan.

**Author Contributions** FMC and SMW perform the literature search and data analysis, and Lin had a technological cooperation on the Ca<sup>2+</sup> influx test. All authors read and approved the final version of the manuscript.

**Funding** This study was supported by Ministry of science and technology, Taiwan

## References

- NE Alvarado I Quesada K Hylland I Marigómez M Soto 2006 Quantitative changes in metallothionein expression in target cell-types in the gills of turbot (*Scophthalmus maximus*) exposed to Cd, Cu, Zn and after a depuration treatment *Aqua Toxicol* 77 64 77
- F Ambreen M Javed U Batool 2015 Tissue specific heavy metals uptake in economically important fish, *Cyprinus carpio* at acute exposure of metals mixtures *Pak J Zool* 47 399 407
- G Castaldo M Pillet B Sloatmaekers L Bervoets RM Town R Blust G Boeck De 2020 Investigating the effects of a sublethal metal mixture of Cu, Zn, and Cd on bioaccumulation and ionoregulation in common carp, *cyprinus carpio* *Aqua Toxicol*. 218 105363
- KM Chan 1995 Metallothionein: potential biomarker for monitoring heavy metal pollution in fish around Hong Kong *Mar Pollut Bull* 31 411 415
- P Cretì F Trinchella R Scudiero 2010 Heavy metal bioaccumulation and metallothionein content in tissues of the sea bream *Sparus aurata* from three different fish farming systems *Environ Monit Assess* 165 321 329
- V Delahaut B Raskovic MS Salvado L Bervoets R Blust G Boeck De 2020 Toxicity and bioaccumulation of cadmium, copper and zinc in a direct comparison at equitoxic concentrations in common carp (*Cyprinus carpio*) juveniles *Plos One* 15 e0220485
- TV Divya C Pallavi A Celin 2018 NmtA, a novel metallothionein of *Anabaena* sp. strain PCC 7120 imparts protection against cadmium stress but not oxidative stress *Aqua Toxicol* 199 152 161
- CT Liu MY Chou CH Lin SM Wu 2012 Effects of ambient cadmium with calcium on mRNA expression of calcium uptake related transporters in zebrafish (*Danio rerio*) larvae *Fish Physiol Biochem* 38 977 988
- SM Monteiro E Rocha JM Mancera A Fontainhas-Fernandes M Sousa 2009 A stereological study of copper toxicity in gills of *Oreochromis niloticus* *Ecotoxicol Environ Saf* 72 213 223
- RV Oost J Beyer NPE Vermeil 2003 Fish bioaccumulation and biomarkers in environmental risk assessment: a review *Environ Toxicol Pharmacol* 13 57 149
- Taiwan EPA (1994) The study of acute toxic test on fish. Annual Report, Environmental Protection Administration of Taiwan
- LJ Tsai KC Yu ST Ho 2007 Cadmium distribution in sediment profiles of the six main rivers in southern Taiwan *J Hazard Mater* 148 630 639
- SM Wu CF Weng JC Hwang PP Hwang 2000 Metallothionein induction in early larval stages of tilapia (*Oreochromis mossambicus*) *Physiol Biochem Zool* 73 531 537
- SM Wu KJ Jangg YJ Lee 2006 Relationship among metallothionein, cadmium accumulation, and cadmium tolerance in three species of fish *Bull Environ Contam Toxicol* 76 595 600
- SM Wu LH Shu JH Liu 2016 Anti-oxidative functions of mt2 and smtB mRNA expression in the gills and brain of zebrafish (*Danio rerio*) upon cadmium exposure *Fish Physiol Biochem* 42 1709 1720
- E Yorulmazlar A Gül 2003 Investigation of acute toxicity of cadmium sulfate (CdSO<sub>4</sub>·H<sub>2</sub>O) and behavioral changes of grass carp (*Ctenopharyngodon idellus* Val., 1844) *Chemosphere* 53 1005 1010
- A Zeynab KM Kazem KE Sohrab R Hossein 2012 Comparison of lethal concentrations (LC<sub>50-96h</sub>) of CdCl<sub>2</sub>, CrCl<sub>3</sub>, and pB(NO<sub>3</sub>)<sub>2</sub> in common carp (*Cyprinus carpio*) and sutchi Ccatfish (*Pangasius hypophthalmus*) *Iran J Toxicol* 18 672 680

**Publisher's Note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.