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Relationships Among Metallothionein, Cadmium Accumulation, and Cadmium Tolerance in Three Species of Fish

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Cadmium (Cd) is a fairly common pollutant of the mining, smelting, and plating industries and is also naturally found in trace amounts in aquatic systems. The effects of waterborne Cd on fish have been well studied, and include alterations of gill structures, the destruction of neural functions, nephrotoxicity, ion disturbances, and growth inhibition (Battaglini et al. 1993). Following development of tilapia larvae, there was an inverse correlation between the 96-h LC_{50} values and the accumulation rate in the early larval stage of tilapia (Hwang et al. 1995). But, metallothionein (MT) is an important modifying agent of metal toxicity in fish (Wu et al. 2000). It can bind heavy metals and decrease their toxicity in teleosts. Therefore, both the accumulation of heavy metals and MT expression may be related to the resistance of fish to heavy metals.

MTs are cysteine-rich metal-binding proteins (Olsson et al. 1989), and have been widely suggested to play an important role in metal metabolism or detoxification of heavy metals (Olsson et al. 1989); for instance, Cd in the liver of tilapia induces synthesis of MTs (Wu 2000) and MT mRNA in the liver of teleosts (Roesijadi 1992). On the other hand, the levels of MT expression are an important indicator for comparing differential heavy-metal resistance of fish. Findings of our previous studies revealed that tilapia larvae have a higher Cd tolerance, since MT expression is induced after Cd treatment (Wu et al. 2000; Wu and Hwang 2003). Therefore, MT expression may play an important role in enhancing heavy-metal tolerance.

Fish exhibit different tolerances to Cd, and this can be species and population specific. The fish *Acrossocheilus paradoxus* possesses such characteristics and is a species native to Taiwan. It is a sensitive indicator with regard to aquatic environmental pollution (Wung 2002). Tilapia (*Oreochromis mossambicus*) is an exotic species that lives in rivers and estuaries and is a dominant species even in polluted environments in Taiwan. The milkfish (*Chanos chanos*) lives in tropical areas and is an important cultivated species in Taiwanese fisheries. These 3 species of fish appear to have different tolerances to environmental pollutants. Therefore, the objective of this study was to investigate whether different sensitivities to Cd result in the expression of MT in these 3 species of fish.

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MATERIALS AND METHODS

Tilapia (with a total length (TL) of 3~5 cm and body weight (BW) of 1.0~2.0 g) were collected from the Taiwan Fisheries Research Institute; Milkfish, (TL, 4~6 cm; BW, 4~8 g) and *A. paradoxus* (TL, 4~7 cm; BW, 4~7 g) were obtained from private fishery firms. The fish were acclimated to fresh water for 1 month before beginning the experiments. These fish were treated in the same test aquarium (80 × 60 × 60 cm) for at least 7 d before being challenged with 1.5 ppm waterborne Cd. This Cd stock solution was diluted to the desired concentrations with aerated local tap water, which had a total hardness of 146.6 ± 5.6 ppm, Na^+ of 35.6 ± 0.3 ppm, K^+ of 3.3 ± 0.1 ppm, Ca^{2+} of 30 ± 2.3 ppm, pH of 8.2 ± 0.3~8.7 ± 0.2, and a Cd concentration of < 2 ppb. The experimental medium was changed once every 2 d, in order to maintain the Cd concentration and water quality. Fish were anesthetized with MS222 during the test samplings. MT extraction and ELISA, and atomic absorption/spectrophotometric measurements of Cd in tissues and the water were carried out as described by Wu et al. (2000). The displacement curve for serial dilutions of the sample extracts was parallel to that of the MT standard. The line regression coefficient for the logarithms of the MT standard concentrations was -0.99, and the slope was -0.2. The regression coefficient for the serial dilutions of sample homogenates was -0.94. The coefficients of intra-assay and inter-assay variations were 5.04% (n = 8) and 15.05% (n = 7), respectively. Total proteins were determined using a protein kit (Bio-Rad, Hercules, CA).

The first experiment was to observe the mortality upon Cd treatment. Six juveniles each of milkfish, tilapia, and *A. paradoxus* were exposed to 1.5 ppm Cd. Every 2 d, we recorded the mortality for a total period of 7 consecutive days. This experiment was performed 3 times. In a second experiment, we compared the Cd accumulation rate in these 3 species. Six juveniles each of milkfish, tilapia, and *A. paradoxus* were exposed to 1.5 ppm waterborne Cd for 3 d. Two fish were dissected every day, and the gills, muscle, and gut, were collected to detect the Cd accumulation rate. This experiment was repeated 3 times. In the final experiment, we measured the MT content upon Cd exposure in these 3 species. The experiment design was similar to that of the second test, but the guts were excised and stored at -80 °C until the MT analysis.

In this study, all data are presented as the mean ± SD, and were evaluated using one-way ANOVA with Tukey's multiple comparison analysis. Different superscript letters (^{a,b}) indicate a significant difference between species.

RESULTS AND DISCUSSION

Environmental factors such as temperature, pH, and ion concentrations are related to the Cd tolerance of fish. For example, higher Mg^{2+} and Ca^{2+} contents enhanced the tolerance after testing with heavy metals (Perschbacher and Wurts 1999). The mortality rate was 20%~40% in milkfish and 80%~100% in tilapia and *A. paradoxus*. The milkfish appeared to have a higher tolerance to Cd exposure than either tilapia or *A. paradoxus* (Table 1). Even freshwater acclimation was more difficult for milkfish than for the other species, but none of the 3 species of fish died when acclimated in fresh water.

Table 1. Comparison of the mortality (%) among tilapia, milkfish, and *A. paradoxus* exposed to cadmium for 7 d.

Cadmium exposure (concentration)	Fish		
	Tilapia	Milkfish	*A. paradoxus*
1.5 ppm	80 ± 0.5 [a]	30 ± 5. 0[b]	90 ± 10. 0[a]
0 ppm	0	0	0

(n = 18, data were collected from 3 separate tests of 6 fish each for every species of fish). Data were evaluated by one-way ANOVA with Tukey's multiple comparison analysis. Different superscript ([a,b]) letters indicant a significant difference between species.

It has been well documented that the expression of MT mRNA and/or proteins is dose and time dependent in fish upon being exposed to certain ranges of waterborne heavy metals, suggesting the possible role of MT in heavy-metal detoxification. For example, increases in MT expression were shown in perch living in a Cd-polluted river (Olsson et al. 1989). MT synthesis and the Cd content of the MT peak increased linearly with increasing doses of $CdCl_2$ in catfish (*Heteropneustes fossilis*) (Chatterjee and Maiti 1991). The increase in MT was gradually enhanced with treatment time in tilapia larvae treated with 35 ppb Cd during 1~7 d of exposure (Wu et al. 2000). However, there are conflicting results. In turbot (*Scophthalmus maximus*), synthesis of MT mRNA and MT was attained more quickly at 100 ppb Cd; but with an acute dose exceeding 200 ppb, MT gene transcription and protein translation appeared to progressively be reduced (George et al. 1996). These results demonstrated that the dose response of MT occurs only in treatments with low doses of heavy metals, or before Cd toxicity disturbs MT synthesis in fish (Wu et al. 2000). The present findings in milkfish showed that Cd induced a 303% elevation in MT after 2 d of Cd exposure, but no significant difference was found between the treatment and control groups on the third day of Cd exposure (Table 2). Therefore, MT synthesis in milkfish may have been impacted after 3 d of Cd-exposure. The MT content of tilapia showed a rapid rise, with 389% and 360% increases after 1~3 d of Cd exposure. In addition, MT expression still did not appear to have significantly increased in *A. paradoxus* (Table 2). However milkfish showed the lowest mortality among the 3 species of fish. Results of this study demonstrate that different sensitivities to Cd may be considered another reason associated with the ability to synthesize additional MT. Some previous studies reported that relative amounts of Cd accumulation were in the order of the gut > gills > muscle (George 1996). The present study revealed similar results. The Cd contents of various tissues were compared in these fishes, and the highest accumulation of Cd appeared in whole-gut tissue. The Cd content was over 100-fold higher in gut tissue than in the gills and muscle. Because the deviation of the data was too high to achieve statistical efficiency between tests, the data were treated as relative Cd accumulation rates to show differences among

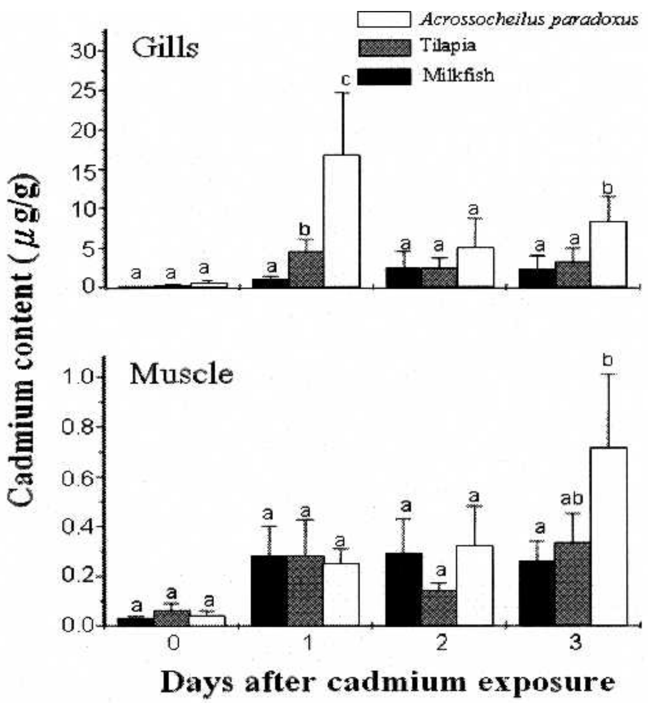

Figure 1. Comparisons of the Cd concentrations in gills and muscle among milkfish, tilapia, and *Acrossocheilus paradoxus*, exposed to 1.5 ppm Cd for 3 d. One-way ANOVA with Tukey's multiple comparison analysis. Different superscript (^{a,b}) letters indicate a significant difference between species.

these 3 species of fish at the same treatment time (Table 3). The formula was [(Cd content of an individual fish/sum of the Cd contents of all 3 fish) × 100%]. The data showed that *A. paradoxus* still had the highest relative Cd accumulation rate compared to tilapia and milkfish.

In addition, it was interesting to note that the gills contained the highest Cd content after the first day of Cd exposure, but this decreased on the second day in both tilapia and *A. paradoxus*. Finally, after 3 d of Cd exposure, both tilapia and *A. paradoxus* showed decreased Cd contents in the gills, but they had increased in the muscle (Fig. 1). We suggest that the gills are able to exchange the Cd very quickly in order to maintain their normal physiological functions. Furthermore, Cd accumulation may be another factor related to the tolerance to Cd toxicity, and this may be the reason why milkfish appeared to have a higher resistance than tilapia after Cd exposure.The highest relative Cd accumulation rate and the highest mortality were observed in *A. paradoxus*, but its MT expression was not lower than

Table 2. Comparison of MT contents among various treatment times for tilapia, milkfish, and *Acrossocheilus paradoxus.*

Species	Time of treatment (d) 0	1	2	3
Milkfish	167.4 ± 9.9^a	192.5 ± 60.1^a	507.9 ± 184.1^b	273.6 ± 55.9^a
Tilapia	259.9 ± 63.3^a	1011.8 ± 428.4^b	1007.6 ± 405.3^b	934.5 ± 292.6^b
A. paradoxus	262.8 ± 23.7^a	367.4 ± 120.2^a	383.9 ± 51.0^a	642.9 ± 343.8^a

(n = 3~5 using one-way ANOVA with Tukey's multiple comparison analysis. Different superscript (^{a,b}) letters indicate a significant difference between species.)

that of milkfish. Two reasons were considered: (1) *A. paradoxus* might contain more unbound Cd than do the other 2 species; and (2) the Cd may be bound to certain enzymes or impacted organelles. Low Cd accumulation means that the milkfish did not have to synthesize much MT and was still resistant to Cd.

Table 3. Comparison of the relative Cd accumulation rates (%) in the gut of these 3 species of fish.

Cadmium exposure (d)	Fish: Milkfish	Tilapia	*Acrossocheilus paradoxus*	F-test p level
0	37.6 ± 2.1	37.8 ± 0.7	24.4 ± 0.1	0.4
1	9.9 ± 0.4	26.1 ± 2.5	64.0 ± 3.8	0.08
2	12.9 ± 1.3	16.0 ± 0.1	71.1 ± 1.3	< 0.001
3	13.0 ± 0.6	36.0 ± 1.4	50.0 ± 0.8	0.002

Values calculated for the same length of Cd exposure, and data are compared on the same line.

In addition, tilapia showed higher MT expression levels but lower tolerance to Cd than did milkfish. Chang et al. (1997) suggested that Ca^{2+} uptake, rather than Cd accumulation, plays a major role in Cd tolerance in tilapia larvae. Other metal-binding proteins, such as superoxide dismutase, alcohol dehydrogenase, and alkaline phosphatase, were also found to bind metals (Saito and Kojima 1997).Taking all of these together, it is necessary to examine Ca^{2+} uptake or other heavy metal-binding proteins, in order to explain differences in Cd-tolerance between milkfish and tilapia.

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