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Cortisol and Copper Induce Metallothionein Expression in Three Tissues of Tilapia (*Oreochromis mossambicus*) in Organ Culture

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Su-Mei Wu, Chun-Che Chen, Yi-Chun Lee, Hsien-Tai Leu, and Nia-Sung Lin (2006) Cortisol and copper induce metallothionein expression in three tissues of tilapia (*Oreochromis mossambicus*) in organ culture. *Zoological Studies* **45**(3): 363-370. The aim of this study was to determine the major organ for metallothionein (MT) synthesis among the liver, gills, and intestines, and which organ exhibits the highest heavy metal accumulation. In a review of past studies, some researchers still doubted that the liver contains the highest MT content. We decided to perform an *in vitro* study, and hypothesized that MT expression is affected by endogenous *in vivo* factors resulting in an irregular phenomenon. Therefore, in this *in vitro* study, we compared MT expressions in these 3 important organs. The 3 organs were cultured in L15 media with 100 ng/ml cortisol and 100 μM $CuSO_4$ for 24, 48, 72, and 96 h. The MT contents of the 3 organs appeared to be time dependent from 24 to 72 h, but decreased after 96 h. Hepatic tissue exhibited the highest MT content among the 3 organs. With the cortisol and $CuSO_4$ dose-response tests, a dose-dependent response was only seen in the intestines after treatments with various concentrations of cortisol and $CuSO_4$. When organs were incubated in different culture media for 72 h, the hepatic tissue also showed the highest Cu accumulation. Summarizing these results, we suggest that the liver is the major organ synthesizing MT and accumulating Cu in comparison to the gills and intestines. http://zoolstud.sinica.edu.tw/Journals/45.3/363.pdf

Key words: Metallothionein, Cortisol, Copper, Tilapia, Organ culture.

Metallothioneins (MTs) comprise a class of inducible metal-binding proteins characterized by a low molecular weight of 6-10 kDa, a high content of cysteine (of about 30%), a lack of aromatic amino acid residues, and a wide distribution in various organisms (Kägi and Schaffer 1988). It is generally accepted that MTs play an important role in the detoxification of heavy metal ions such as Cd and Hg. MT also has a role in buffering changes in free metal ion levels in cells by binding essential metals such as Cu and Zn (Olsson 1996). In teleosts, previous studies demonstrated strong relationships between liver MT and Cu residues (Marr et al. 1995). Pretreatment with cortisol induced MT expression in a primary culture of rainbow trout (*Oncorhynchus mykiss*) hepatocytes (Hyllner et al. 1989, Olsson et al. 1990) and in adult tilapia (*Oreochromis mossambicus*) in an *in vivo* study (Wu et al. 2002).

MT acts as a housekeeping protein (George and Olsson 1994); it is most abundant in the liver, kidneys, gills (Dang et al. 2000), intestine, pancreas (Moffatt and Denizeau 1997), and brain of various fish species (Filipovic and Raspor 2003). Concentrations of MT vary with species, reproductive condition, age, and diet, and these factors have to be taken into account when MT is used as a biomarker (Livingstone 1993). Recently, attention has been focused on the use of tissue metal concentrations and especially MT as an indicator and an early warning system for heavy metal pollution (Olsson 1996, Dethloff et al. 2001). Exposure of pre-spawning juvenile catfish to 1.7 mg/l copper sulfate led to time-dependent increases in hepatic MT expression, hepatic copper content, and plasma cortisol concentrations. MT may

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thus serve as a useful indicator of acute stress as a result of acute Cu exposure (Schlenk et al. 1999). However, there are no data confirming the role of MT as an environmental monitor of heavy metals. In addition, many studies have illustrated that MT mRNA and the MT protein are highly correlated with heavy metal levels at low doses, but the expression is reduced at high doses (Chaterjee and Maiti 1987, George et al. 1996a, Wu et al. 2000). This may be ascribed to the following reasons: (1) differences in interactions of species and tissues exist for inducing MT (Lam et al. 1998, De Boeck et al. 2003); (2) concentrations and exposure times to heavy metals and MT expression are tissue-dependent (George et al. 2000, Berntssen et al. 2001); and (3) we suggest that endogenous factors do interfere with the interrelation of MT expression and heavy metal accumulation.

These endogenous factors include kinetic changes of heavy metal exposure and MT expression among tissues or different resistances to heavy metal challenge among species. In 1 study, rainbow trout (*O. mykiss*), common carp (*Cyprinus carpio*), and gibel carp (*Carassius auratus gibelio*) were exposed to 1 μM Cu^{2+} for 1 wk, and virtually no Cu accumulation was seen in rainbow trout gills. Most Cu accumulated in the liver, but there was no MT expression; both cyprinid carp gill Cu levels increased 3-4-fold, but gill MT induction was obvious only in gibel carp (De Boeck et al. 2003). In the European flounder (*Platichthys flesus*), a concentration of > 5 mg/kg Zn^{2+} induced 3-4-fold increases in MT mRNA levels in all tissues within 2-4 days. A concentration of > 80 μg/kg Cd^{2+} had no significant effect on hepatic MT levels but caused significant elevations in gill and kidney levels after 10 and 21 d (George et al. 2000). After tilapia were exposed to 70 μg/l Cd for 10 d, and then transferred to 300 μg/l Cd for 7 d, the MT content had increased about 6-fold in the intestines over levels in the gills and liver. Cd accumulation in the intestines was respectively 37- and 42-fold higher than those in the liver and gills (Wu 2001). In addition, after tilapia were immersed in 6-7 mg/l $CdCl_2$ for 14 d or injected with 0.2 mg Cd/kg body weight every 2 d for 3 doses, a higher accumulation of Cd was found in the liver then in the intestines and gills (Wu et al. 1999). Several previous papers studied MT expression in the liver, but whether the liver expresses the highest level of MT is still under debate (Chatterjee and Maiti 1987, Suzuki 1991, Wu et al. 1999). Furthermore, many proteins such as glutathione S-transferase (GST), catalase (CAT), and glutathione peroxidase (GPX) are induced by heavy metal exposure to protect organisms (Saito and Kojima 1997). Many factors indeed interfere with the relationship between heavy metal accumulation and MT expression as determined by *in vivo* studies. Therefore, the aims of this study were to compare (1) the levels of MT expression upon cortisol and Cu treatments and (2) the accumulation of copper among 3 tissues (gills, intestines, and liver) using organ cultures.

MATERIALS AND METHODS

Fish

Mature adult tilapia (*Oreochromis mossambicus*), with an average weight of 80 g, were obtained from the Mariculture Research Center of the Taiwan Fisheries Research Institute, Tainan, Taiwan. The tilapia were reared in 182-L glass aquaria with plastic chips for gravel. Each tank was supplied with dechlorinated, circulating, and aerated local tap water (FW) at 26-28°C under a photoperiod of 12-14 h. Water quality parameters included a total hardness of 146.6 ± 5.6 mg/l; Na^+ of 35.6 ± 0.3 mg/l; K^+ of 3.3 ± 0.1 mg/l; Ca^{++} of 30 ± 2.3 mg/l, and a pH of 8.2 ± 0.3. Fish were fed commercial fish food pellets. All fish were acclimated to the laboratory conditions for a minimum of 1 mo before commencement of the experiment.

Enzyme-linked immunosorbent assay (ELISA) for MT

The same tissues were collected as a group sample for MT ELISA with minor modifications from our past research (Wu et al. 2000). Fish were anesthetized with MS222 immediately after removal from the experimental tanks during sampling. The gills, intestines, and liver were excised from the body, and soluble extracts of tissues were prepared by individually homogenizing the tissues in homogenization buffer (10 mM Tris-HCl and 5 mM 2-mercapto-ethanol; pH 7.0) in a 1: 2.5-3.0 (w/v) volume using a Teflon homogenizer at 1000-1200 rpm. The homogenates were centrifuged at 12,000 x*g* for 40 min at 4°C. The supernatant was inactivated at 80°C for 10 min, and then was centrifuged again at 12,000 x*g* for 40 min at 4°C; the final supernatants were subjected to MT ELISA as described below.

A synthetic peptide (AA sequence) from the N-terminal of tilapia MT (Wu et al. 1999) was coat-

ed onto micro-titer plates (96 wells) with coating buffer (0.05 M $NaHCO_3$; pH 9.6) at 50 µl/well. After incubation for 1.5 h at 37°C, plates were washed 3 times with washing buffer (PBS buffer with 1% Tween 20), and BSA was added (100 µl/well) for 1 h to block non-specific binding. Rabbit anti-tilapia MT serum (diluted 1: 2000, 50 µl/well) and tissue extract or standard solution (50 µl/well) were mixed and incubated 1.5 h at 37°C. The plate was washed 3 times, and then HRP (peroxidase-labeled goat anti-rabbit IgG, diluted 1: 4000) was added as the secondary antibody. After 3 washes, 100 µl/well of the ABTS peroxidase substrate (Kirkegaard and Perry Lab, USA) was added followed by incubation at room temperature for 20-30 mins. Color development was measured at 405 nm with an automatic microtiter plate ELISA reader (Dynex MRX, Chantilly, VA, USA). Total proteins were determined using a protein assay kit and a dye reagent concentrate (Bio-Rad Lab, USA). The line regression coefficient (Microsoft Excel 97 SR-1, 1997; Microsoft Corp., Seattle, WA, USA) for the logarithms of the MT standard concentrations was -0.99, the range of standard MT levels was from 10 to 200 ng/well, and the coefficients of intra-assay and interassay variations were 5.04% (n = 8) and 15.05% (n = 7), respectively. In addition, the MT contents of the 3 tissues were measured by this ELISA system by confirming the parallel curves of the MT standard and serial dilutions (1x, 2x, and 4x) of MT extracted from gills, intestines, and liver, and the anti-rabbit MT antiserum was confirmed with Western blot analysis (Wu 2001).

Measurement of copper

Tissues were washed in fresh water 3 times and briefly rinsed in double-deionized water (ddH_2O); water left on the tissue surface was blotted dry with filter paper. Tissues were dried at 65°C overnight and digested with 200 µl 13.1 N HNO_3 at 40°C overnight. The digested solutions as well as water samples from the incubation media were diluted with ddH_2O and subjected to atomic absorption spectrophotometry (Z-5000, Hitachi, Japan), using a graphite furnace for Cu^{2+} analysis. Cu standard solutions from Merck (Darmstadt, Germany) were used as a reference. The addition of certain amounts of standard solutions to the test samples was used to correct the matrix effect (according to the User Instructions of the Hitachi spectrophotometer).

Culture and wash media

The culture medium contained 0.16 g $CuSO_4$ (Sigma, St. Louis, MO, USA) dissolved in 10 ml of ddH_2O to prepare the 10 mM Cu^{2+} stock solution; 10 mg hydrocortisone-21 Hemisuccinate (Sigma) was dissolved in 1 ml ddH_2O to prepare the stock solution, and both were stored at -20°C until the start of the experiment. Leibovitz-15 (L15) medium was supplemented with glutamine (Gibco, Grand Island, USA) containing 100 IU penicillin, 100 IU streptomycin, and 2.5 µg amphotericin B (ICN Biomedicals, USA) in sterile 24-well culture dishes. Washed media contained 200 IU penicillin, 200 IU streptomycin, and 5 µg amphotericin B in 1 mL 0.01M PB buffer (2.6 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, and 8 mM Na_2HPO_4-$2H_2O$, dissolved in 100 mL ddH_2O).

Organ culture

Preparation of gill filaments for organ culture followed methods similar to those in McCormick and Bern (1989). The liver was cut into 1 mm^3 pieces, and the middle of the intestines was cut into 4 mm^3 fragments. These tissues were washed 3 times with wash medium, and then 4 gill filaments, 5 pieces of hepatic tissue, or 1 fragment of the intestines were placed in 2 ml culture medium. The Cu 96-h LC_{50} values of blue tilapia (*Oreochromis aureus*) are 16-43 mg/l (Straus 2003). A previous study reported that 50-250 mg/kg of cortisol and 300 µg/l of Cd significantly enhanced MT synthesis in tilapia (Wu et al. 2002), and 100 µM Cu was used to induce MT expression in the hepatocyte primary culture of rainbow trout (Olsson et al. 1990). Cortisol was added to the incubation media in order to enhance the Cu-induced MT expression. Based on these previous studies, 0-200 µM Cu^{2+} (equivalent to 0-12.7 mg/l) and 0-300 ng/ml cortisol were used in the following experiments.

Experiment 1. Time dependence of MT expression

Organs were incubated at 28°C for 24, 48, 72, and 96 h in culture medium containing 100 µM $CuSO_4$ and 100 ng/ml cortisol. Tissues were collected and stored at -80°C, and MT levels were measured by ELISA immediately after the end of the test.

Experiment 2. Dose response of cortisol and $CuSO_4$ for inducing MT

Organs were incubated at 28°C for 72 h in culture medium containing 100 μM $CuSO_4$ (control) or culture medium containing 100 μM $CuSO_4$ with 50, 150, and 300 ng/ml cortisol to detect the dose response of cortisol-induced MT. In the same culture conditions, the culture medium contained 150 ng/ml cortisol or 0 (control), 50, 100, and 200 μM $CuSO_4$ to detect the effect of the dose response of $CuSO_4$ -induced MT expression.

Experiment 3. Comparison of Cu accumulation among the 3 organs

Organs were incubated at 28°C for 72 h in various culture media (L15, L15 with 100 μM $CuSO_4$ and 100 ng/ml cortisol, or L15 with 100 μM $CuSO_4$). Organs were collected, and Cu accumulation was measured by atomic absorption spectrophotometry.

Statistical analysis

Results were compared by a one-way or two-way ANOVA followed by Tukey's multiple comparison test at various concentrations or time courses of the same organ culture.

RESULTS

Experiment 1. The MT contents of all three of these tissues appeared to be time dependent during the 24-72-h period for tissues cultured with 100 ng/ml cortisol and 100 μM $CuSO_4$, but the MT content decreased after 96 h. The contents had increased 3.21-, 1.63-, and 1.59-fold in the hepatic tissue, gills, and intestines, respectively, after 72 h of treatment (Fig. 1).

Experiment 2. Treatments with 50, 100, and 200 μM of $CuSO_4$ caused 1.4-1.84-fold, 0.8-2.02-fold, and 2.29-8.37-fold MT expression enhancement in hepatic tissue, gill fragments, and intestines, respectively (Table 1). The MT contents showed a dose-dependent response after various cortisol treatments in the gills and intestines, but this was not found in hepatic tissue (Table 2). All of the MT contents showed dose-dependent responses whether the intestine culture was treated with cortisol or $CuSO_4$. The hepatic tissue and intestines contained higher MT contents than the gill filaments. The MT contents exhibited a large range of variation in the intestine culture with 150 ng/ml cortisol and 100 μM $CuSO_4$, which increased 2.07- and 5.23-fold in different experiments (Tables 1, 2).

Experiment 3. The Cu^{2+} levels were 17.0, 3.0, and 0.7 μg/g (dry weight of tissue), in the hepatic tissue, gill filaments, and intestines, respectively, before culturing. However, after treatment with 100 μM $CuSO_4$ and 150 ng/ml cortisol for 72 h, levels increased 213.7-, 39.2-, and 150.9-fold in the liver, gills and intestines, respectively. The Cu contents of hepatic tissue were 31.3- and 35.4-fold higher than those of the gills and intestines. These three tissues didn't appear significantly difference between tissues cultured in L15 medium with cu and cortisol, and did in L15 medium only with cu (Table 3).

DISCUSSION

Much research has shown that the MT content appears to have time- and dose-dependent responses in *in vivo* studies, but it always decreases when the heavy metal treatment dose or time exceeds the fish's resistance, and similar effects are not specifically limited to heavy metals. In tilapia larvae, the MT content decreased when fish were exposed to Cu for over 72 h or over 200 μg/l Cu^{2+} (Wu and Hwang 2003). In turbot

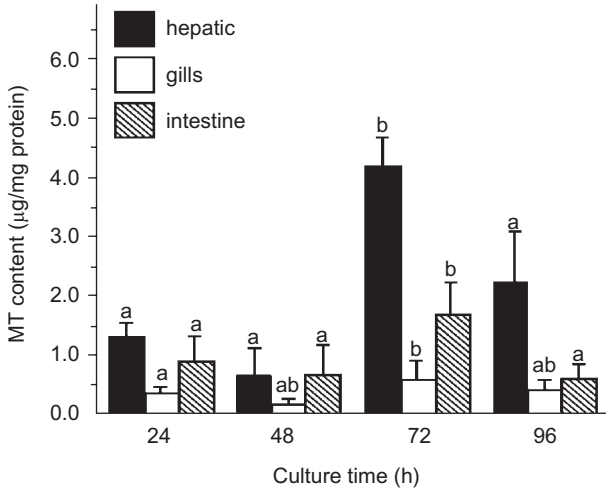

Fig. 1 Comparison of the MT content (μg/mg protein) between various culture times for the 3 organs treated with 100 ng/ml cortisol and 100 μM $CuSO_4$ for 24-96 h. Mean ± SD; different superscripts indicate a significant difference among time courses in the same organ ($p < 0.05$, ANOVA with Tukey's comparisons).

(*Scophthalmus maximus*), syntheses of MT mRNA and MT were attained more quickly at 100 μg Cd^{2+}/kg; but at acute doses of > 200 μg/kg, MT gene transcription and protein translation appeared to progressively be reduced (George et al. 1996a). The relationship between MT mRNA and protein expressions was only found after exposure to low doses of cadmium in turbot (*S. maximus*) (Georgy et al. 1996b). All these results imply that dose-related responses of MT expression only occur with doses of heavy metals that do not cause detrimental effects to the physiological functioning of the fish (Wu et al. 2000). In fact, correlations among MT expression, heavy metal accumulation, and tolerance of fish to heavy metals are complicated, especially in *in vivo* systems. It is interesting that similar changes appeared in this *in vitro* study. Levels of MT increased when hepatic tissues were cultured with cortisol and $CuSO_4$ for 24-72 h, but they decreased after 96 h of culture. We suggest that cortisol and $CuSO_4$ validly induced MT synthesis levels in the 3 organs within 72 h in this *in vitro* study. However, the MT contents of all three of these organs showed differences in expression in the dose-response study to $CuSO_4$ and cortisol treatments. The highest content of MT appeared in the liver after treatments with $CuSO_4$ and cortisol at various concentrations (Tables 1, 2).

Cortisol is important for seawater adaptation and Na^+/K^+-ATPase activity in gills and intestines. Cortisol mediates the increase in intestinal fluid absorption in Atlantic salmon during the parr-smolt transformation (Veilette et al. 1995), and it is also involved in carbohydrate metabolism in the liver (Vijayan et al. 2003). Therefore, liver, gills, and intestines are all cortisol target organs. Either mineralocorticoid-like receptor or glucocorticoid receptor cDNA is cloned from the liver and gills (Greenwood et al. 2003), and the cortisol receptor has been demonstrated in salmonid species by the specific binding of radiolabeled cortisol in the intestines (DiBattista et al. 1984). Although glucocorticoid response elements (GREs) have not been identified in teleost MT promoter regions, it is hypothesized that cortisol apparently indirectly induces hepatic MT causing a redistribution of Zn and Cu into hepatocytes which interact with tran-

Table 1. Comparison of the MT contents (ng/mg protein) among the 3 organs treated with various doses of $CuSO_4$ and 150 ng/ml cortisol after 72 h

Organ	Treatment concentration of $CuSO_4$ (μM)			
	0	50	100	200
Liver	1312 ± 194^a	1846 ± 331^{ab}	2425 ± 254^b	2423 ± 344^b
Gills	504 ± 101^a	404 ± 30^a	373 ± 45^a	1018 ± 115^b
Intestine	551 ± 102^a	1262 ± 285^{ab}	2882 ± 240^{bc}	4617 ± 1125^c

Data showed Mean ± SE. ^{a,b,c}Different superscripts indicate a significant difference among treatment doses of $CuSO_4$ in the same organ culture ($p < 0.05$, ANOVA with Tukey's comparisons).

Table 2. Comparison of the MT contents (ng/mg protein) among the 3 organs treated with various doses of cortisol and 100 μM $CuSO_4$ after 72 h

Organ	Cortisol (ng/ml)			
	0	50	100	200
Liver	4128 ± 173^a	2653 ± 1224^{ab}	1987 ± 105^b	2453 ± 212^{ab}
Gills	142 ± 2^a	141 ± 54^a	461 ± 44^b	226 ± 45^a
Intestine	155 ± 69^a	263 ± 94^{ab}	320 ± 9^b	740 ± 38^c

Data showed Mean ± SE. ^{a,b,c}Different superscripts indicate a significant difference among treatment doses of cortisol in the same organ culture ($p < 0.05$, ANOVA with Tukey's comparisons).

scription factors and bind metal response elements (MREs) on the MT promoter (Olsson 1993). In fact, many reports have provided evidence that MT is enhanced by cortisol treatment. For example, pretreatment with cortisol was found to enhance the induction of MT in primary culture of rainbow trout hepatocytes (Hyllner et al. 1989, Olsson et al. 1990). Treatment of adult tilapia with 250 mg/kg cortisol followed by Cd^{2+} exposure significantly stimulated the expression of MT (Wu et al. 2002). Therefore, it was predicted that cortisol would have actions in these 3 organs, and the culture media containing cortisol and copper sulfate.

In a review of previous studies, the order of Cu accumulation in the marine gulf toadfish (*Opsanus beta*) exposed to 55.2 μM Cu^{2+} for 30 h was bile > liver > intestines > gills > kidneys (Grosell et al. 2004). Common carp (*C. carpio*) were exposed to various concentrations of Cd and Zn for 29 d. Control levels of hepatic MT were 4 times higher compared to those of the gills and kidneys, but Cd accumulation was in the order of kidneys > liver > gills (De Smet et al. 2001). The exposure of red sea bream (*Pagrus major*) to Cd^{2+} resulted in the highest accumulation in the liver, followed in descending order by the kidneys, intestines, and gills. However, the highest proportion of MT appeared in the intestines (Kuroshima 1992). Many inconsistent results indeed appeared in past papers, which were *in vivo* studies. In the present study, 3 organs were incubated in different culture media for 72 h, and then their Cu accumulations were compared. This resulted in the highest accumulation in the liver, followed by the intestines and gills. This differed from an *in vivo* Cd^{2+} study (Wu 2001). In addition, by comparing tables 1-3, some information is revealed. The 3 organs were incubated for 72 h after being cultured with $CuSO_4$ and cortisol. The highest Cu accumulation was shown in the liver instead of the gills and intestines before culturing. Even after Cu incubation, the liver still contained the highest Cu levels compared to the others. These results demonstrate that the liver is a major organ accumulating heavy metals in native habitats and with artificial treatments. In addition, the Cu contents of the liver did not show significant differences between that incubated with L15 media (without Cu^{2+}) and before culturing (initiation). In addition, the Cu content did not show a significant difference between these 3 organs incubated in L15 with Cu and cortisol, but it did when incubated in L15 with Cu only (Table 3). We suggest that cortisol cannot enhance Cu uptake in this *in vitro* system. It is interesting to compare tables 1 and 2, in which the MT expression of hepatic tissue was 3.1-fold higher with $CuSO_4$ induction (0 ng/ml cortisol with 100 μM $CuSO_4$) than with cortisol induction (0 μM $CuSO_4$ with 150 ng/ml cortisol). However, this circumstance was reversed in the gills and intestines, in which MT expression in organs after 150 ng/ml cortisol incubation was higher than that in organs incubated with 100 μM $CuSO_4$. We suggest that both Cu and cortisol are important factors for MT induction, but copper is more effective than cortisol. This may be the reason why cortisol did not exhibit a dose-responsive effect on liver MT induction. On the other hand, in all doses of cortisol and Cu, MT expression in the liver was always higher than those in the intestines and gills, even though cortisol seemed to downregulate the MT content in the liver but to upregulate

Table 3. Comparison of Cu^{2+} accumulation (μg/g tissue dry weight) ^{ab} among organs in the same culture medium after 72 h of culture, and ^{xy} between different culture media (L15, L15 with 100 μM Cu but no cortisol (F), and L15 with 100 μM Cu and 100 ng/ml F) or before culturing in the same organ. Mean ± SD; different superscripts indicate a significant difference ($p < 0.05$, two-way ANOVA)

Organ	Before culture	Culture medium		
		L15	L15+Cu	L15+Cu+F
Gills	3.0*	17.3*	62.7 ± 13.8^{a}	116.6 ± 21.9^{a}
Intestines	0.7*	30.6 ± 8.4^{ax}	219.4 ± 73.1^{ay}	102.6 ± 24.8^{ay}
Liver	17.0 ± 1.0^{x}	111.3 ± 13.7^{bx}	2125.5 ± 358.5^{by}	3633.4 ± 651.9^{by}

*The Cu content was too low to measure in gills or intestines, therefore tissues from 5 wells were collected as 1 sample.

MT contents in gills and intestines (Table 2). We suggest that levels of the glucocorticoid receptor differed in these 3 organs because the glucocorticoid receptor is more abundant in the liver (Vijayan et al. 2003). Furthermore, evident variations in MT expression were found in the intestines between different experiments (Tables 1, 2). This may have been due to the unequal distribution of MT synthetic cells in different parts of the intestine, since only 3% of the total intestine was sampled for the culture experiments.

In summary, the major findings of the present study are that (1) the increase in MT synthesis following the dose response of cortisol and $CuSO_4$ treatment only appeared in the intestines; (2) the hepatic tissue showed the highest MT synthesis and the highest Cu accumulation compared with the intestines and gills, but it was limited by the culture time and the substrate of the medium; and (3) in this *in vitro* study, MT expression also appeared to decrease after a longer Cu exposure, which is similar to results of *in vivo* studies.

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