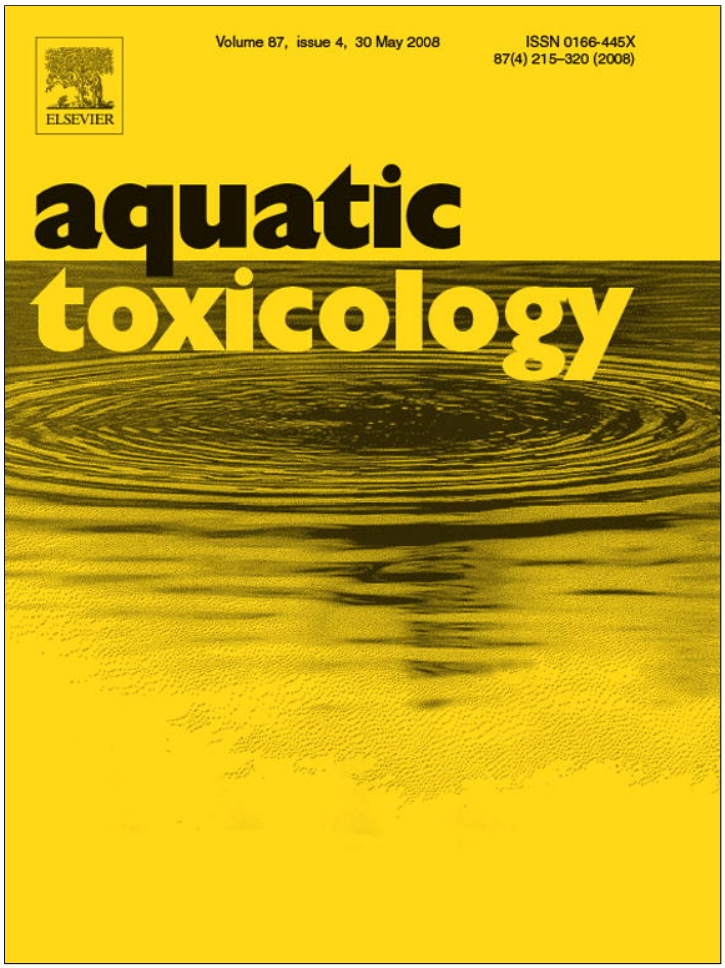

Aquatic Toxicology 87 (2008) 296–302

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

The effects of maternal Cd on the metallothionein expression in tilapia (*Oreochromis mossambicus*) embryos and larvae

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ARTICLE INFO

Article history:
Received 9 January 2008
Received in revised form 20 February 2008
Accepted 20 February 2008

Keywords:
Metallothionein
Maternal effect
Offspring
Cadmium

ABSTRACT

The purpose of this study was to identify the factor(s) which would enhance the Cd resistance as assessed by the metallothionein (MT) expression in tilapia larvae. Larvae were collected from parents that were pretreated respectively with Cd or saline. At the end of the 12-week experiment, the hepatic MT and Cd contents in the breeding female fish were recorded. Our results indicated that a significant relationship between Cd and MT contents can be found in the offspring from the parent fish treated with Cd. However, the higher Cd resistance, Cd contents, and MT expression were limited to those larvae from parent fish bred within 4 weeks of the injection. By week 12, the Cd-treated fish still contained high levels of MT in their hepatic tissues. However, the MT and Cd contents in the larvae from these adult fish were not significantly different from those from the controls. In summary, we suggest that the higher Cd resistance of larvae from the egg stage was a result of the Cd contamination of the parent female, as evidenced by an increase in MT expression induced in tilapia embryos and larvae.

1. Introduction

Fish exhibit different tolerances to Cd, and although this can be species- and population-specific (Wu et al., 2006), it can also be due to metal pre-exposure (Wu and Hwang, 2003), water hardness (Hollis et al., 2000), and even maternal effects (García et al., 1999; Lin et al., 2000). It is known from studies on the maternal effects that a parent treated with heavy metals resulted in higher metal resistance in the offspring. There were many different suggested explanations for this phenomenon. For example, in Nile tilapia (*Oreochromis niloticus*), the first generation of progeny (F1) from a parent who had been exposed to a metal appeared more resistant to heavy metals than those who had not. The authors suggested that this was due to the rapid physiological acclimation to the metal in the F1 from the parent who had been exposed to a metal or due to the results of genetic selection (Lourdes et al., 1995). In addition, higher Cd resistance in Mozambique tilapia larvae (*Oreochromis mossambicus*) was noted after the female parent had been treated with a Cd injection (Lin et al., 2000). It was proposed that the larvae contained higher metallothionein (MT) contents through the maternal effect (Lin et al., 2000). A higher Cu tolerance and increased whole-body Cu and Na^+ contents were also described in larval minnows (*Pimephales promelas*) from exposed parents (Kolok and L'Etoile-lopes, 2005). It is suggested that the MT or ion regulatory capacity of the parents was transferred to larvae *via* the maternal effect and the pre-exposed gravid females produced offspring with higher metal resistance. In fact, our previous study reported that a direct Cd treatment to the larvae would lead to a higher tolerance to Cd and these larvae had higher levels of MT (Wu and Hwang, 2003), and additional Ca^{2+} would enhance the resistance to Cd *via* increased MT production (Wu et al., 2007). Therefore, this raises the possibility of MT in the larvae being extrinsically taken up along with Cd contamination in the ovary.

Maternal effects is suggested when the phenotype of an individual is influenced by environmental factors experienced by the mother rather than by the individual's own genotype. The best model system to recognize this is the effects of maternal nutrition on offspring size in fish (Reznick et al., 1996) and on the spawning population in determining the level of recruitment (Solemdal, 1997). Some hormones, like thyroxine and cortisol, have been reported to be transferred to embryos from the mother in Japanese flounder, *Paralichthys olivaceus* (De Jesus et al., 1991), Mozambique tilapia (Hwang et al., 1992), and rainbow trout, *Oncorhynchus mykiss* (Pottinger and Mosuwe, 1994). In addition, some researchers also reported that MT may be transferred to offspring in Mozambique tilapia (Lin et al., 2000) and zebrafish (*Danio rerio*) (Riggio et al., 2003) through a maternal effect. However, only the first generation of progeny of Cd-pretreated parents possessed this higher Cd tolerance (Lourdes et al., 1995). In addition, larvae pretreated with Cd could induce MT over-expression (Wu and Hwang, 2003). In this

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doi:10.1016/j.aquatox.2008.02.012

study, we investigated the reason for MT over-expression in newly hatched larvae and examined whether the eggs were contaminated with Cd which induced self MT expression or MT transfer by the maternal effect.

Metallothioneins comprise a class of inducible metal-binding proteins characterized by a low molecular weight of 6–10 kDa. It is known that MTs play an important role in the detoxification of heavy metals such as Cd, Zn, and Cu in teleosts. In tilapia larvae, MTs can be induced by Cd (Wu et al., 2000). In zebrafish, a 30-fold increase of the MT content in oocytes during vitellogenesis and an induction of MT synthesis at the blastula stage of embryos were found when they were treated with 20 μM Cd (Riggio et al., 2003). The roles of MT during vitellogenesis are to induce vitellogenin and to regulate essential metals (like Zn and Cu) in lake trout (*Salvelinus namaycush*) eggs (Werner et al., 2003). In fact, the Cd uptake by eggs was found to be rapid (Rombough and Garside, 1982), and Cd significantly accumulated in ovaries and testes after 1 week of 0.1 mg/L Cd exposure in blue tilapia (*O. aureus*) (Allen, 1995). However, Cd content in the ovary of a marine teleost (*B. pholis*) increased and then rapidly reduced after 2 months (Shackley et al., 1981).

These previous studies demonstrated that: (1) maternal pre-exposure to Cd increased the survival of newly hatched larvae exposed to Cd treatment (Lourdes et al., 1995); (2) Cd may be transferred to the ovaries and testes of adult fish after several days of Cd exposure (Allen, 1995), and Cd is rapidly taken up by eggs (Rombough and Garside, 1982) and decreased rapidly (Shackley et al., 1981); (3) MT can be detected in embryos and can be induced in larval fish upon Cd exposure (Wu et al., 2000); and (4) MTs appeared to be over-expressed in larvae once a parent had been pretreated with a metal (Lin et al., 2000). Therefore, it is suggested that MTs are similarly induced in larvae *via* the maternal effect. Nevertheless, the eggs in the ventral cavity of the intraperitoneally (i.p.)-injected female may be immersed in the body fluid containing Cd. This may create a similar effect to the MT expression in larvae pre-exposed with Cd. The question we posed was whether the MT expression of larvae was initiated by metal contamination since its egg stage or was affected *via* the maternal effect. In the first experiment, both male and female tilapia were injected with Cd. The levels of MT mRNA, MT protein, and Cd in pre-hatched embryos, newly hatched, and 1-day post-hatched larvae were determined. Embryos and larvae bred at different times after their parents had been treated with Cd were collected. The objective of this work was to compare the duration of the influence of Cd treatment to their offspring between male and female. Next, we compared the hepatic MT contents and Cd accumulation between Cd-treated and untreated females, to examine the current hypothesis of parental MT transfer to offspring.

2. Materials and methods

2.1. Fish

Mature Mozambique tilapia (body weight 100 ± 9.6 g; total length 10–12 cm) were collected from the Mariculture Research Center of the Taiwan Fisheries Research Institute, and were reared in 182-L glass aquaria using plastic chips for gravel. Each tank was supplied with dechlorinated, circulated, and aerated local tap water (FW) at 26–28 °C under a photoperiod of 12–14 h. Fish were fed commercial fish food pellets. The water quality parameters included a total hardness of 146.6 ± 5.6 mg/L $CaCO_3$, Na^+ of 1.55 ± 0.01 mM, K^+ of 0.08 ± 0.003 mM, Ca^{2+} of 0.75 ± 0.06 mM, and pH of 8.2 ± 0.3; Cd concentration was less than 1 μg/L.

2.2. Comparisons of Cd accumulation and MT contents in larvae from parents with different treatments

Ten mature male and 20 females Mozambique tilapia were used in this part of the experiment. Each of the five glass aquaria (90 cm × 45 cm × 45 cm) had two males and four females for every treatment. They received either saline (0.9% NaCl) or Cd (1 mg $CdCl_2$/kg body weight, Sigma) *via* an i.p. injection. The experiment consisted of five treatments: treatment 1, both the male and females were untreated (blank); treatment 2, both the male and females were injected with saline (control); treatment 3, both the male and females were injected with Cd (IF/IM); treatment 4, the male was injected with Cd while the females were injected with saline (IM); and treatment 5, the male was injected with saline while the females were injected with Cd (IF). Four females tilapia were allowed to mate with the two males over the next 12 weeks. Fertilized eggs from the mouth of the brooding females were collected 1 day before hatching and were incubated in a gently aerated 1-L container at 28 °C. The dry weights, Cd contents, and MT contents and mRNA expression levels were measured in pre-hatched (Pre-H0), H0 (within 12 h post-hatching), and H1 (1-day post-hatching) larvae. Larvae were not fed during the experiments. After 12 weeks, all females were sacrificed to measure the Cd and metallothionein contents of the hepatic tissues ($n = 4$).

2.3. Enzyme-linked immunosorbent assay (ELISA) analysis of MT

Hepatic tissues were collected as a sample for MT ELISA with minor modifications from our previous study (Wu et al., 2000). Females and larvae were anesthetized with MS222 immediately after removal from the experimental tanks during sampling. The liver or ovary of adult females was excised from the body. Fifteen to 20 larvae were collected and pooled as one sample, and soluble tissue extracts 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 × *g* for 40 min at 4 °C. The supernatant was inactivated at 80 °C for 10 min, and centrifuged again at 12,000 × *g* for 40 min at 4 °C. The final supernatants were subjected to MT ELISA for larvae (Wu et al., 2000) and tissues (Wu et al., 2006).

2.4. Cd measurements in the whole body of larval fish and adult tissues

The Cd assays of the whole body of larval fish and adult tissues were performed as previously described (Chang et al., 1998; Wu and Hwang, 2003; Wu et al., 2007). Briefly, tilapia larvae and adult tissues were weighed, dried at 65 °C overnight, and digested in 13.1 N HNO_3 at 45 °C overnight. Digested solutions were diluted with double-deionized water (DI-S4, Millipore, Ireland REV) for subsequent analysis. Digested samples, test media, and appropriate blanks, including deionized water, were subjected to atomic absorption spectrophotometry (Z-5000, Hitachi, Japan), with a graphite furnace for the Cd^{2+} analyses. The standard solution of Cd^{2+} (Merck) was used to create a standard curve for the measurements. The addition of certain amounts of the standard solution to the test samples was used for background correction to estimate the matrix effect following the user instructions for the Hitachi spectrophotometer. The detection limit of Cd^{2+} was 0.1 μg/L.

2.5. Isolation of total RNA and quantitative comparison with RT-PCR of MT mRNA

Total RNA was extracted using an RNA zol B kit (Teltest, USA). Ten tilapia eggs or larvae were homogenized with 4 mL RNA zol

Table 1
Comparison of the Cd accumulation (ng/g dry weight) and metallothionein (MT) contents (ng/mg protein) in the whole bodies of pre-H0 larvae, the parents of which either the female (IF) or male (IM) or both (IF/IM) were injected with 1 mg/Kg body weight Cd or saline (control), or were untreated (blank)

Sampling time after the parental injection (week)	Treatment condition				
	Blank	Control	IM/IF	IM	IF
Cd contents in pre-H0 stage larvae					
0–1	98.9 ± 21.68[a]	[A]		146.4 ± 20.93[b]	203.7 ± 44.69[b]
1–2		103.1 ± 22.72	235.1 ± 46.67[*]		
2–3	97.8 ± 35.26				156.6 ± 12.53[*]
3–4		95.8 ± 14.42[a]	161.4 ± 18.29[b]	128.1 ± 13.48[b]	
5–6		103.2 ± 10.18			106.2 ± 9.99
6–7	100.8 ± 38.09[a]		106.3 ± 17.53[a]	110.5 ± 25.36[a]	
8–9	96.9 ± 24.7[a]		101.7 ± 16.88[a]		110.7 ± 20.36[a]
9–10		98.5 ± 14.9		104.9 ± 18.2	
10–11	100.5 ± 15.84[a]		99.4 ± 19.04[a]		100.6 ± 11.31[a]
11–12		104.1 ± 14.7		108.6 ± 19.14	
MT contents in pre-H0-stage larvae					
0–1	452.8 ± 34.03[a]	[A]		756.7 ± 62.44[b]	689.3 ± 83.31[b]
1–2		418.4 ± 67.46	722.3 ± 24.42[*]		
2–3	465.4 ± 31.78				589.5 ± 22.08[*]
3–4		489.0 ± 20.44[a]	663.5 ± 13.94[b]	553.6 ± 50.49[a]	
5–6		428.4 ± 56.46			493.8 ± 14.38
6–7	433.4 ± 81.84[a]		419.8 ± 93.18[a]	482.4 ± 105.91[a]	
8–9	433.1 ± 56.46[a]		485.5 ± 30.83[a]		466.8 ± 8.05[a]
9–10		436.3 ± 18.79		453.2 ± 24.6	
10–11	438.1 ± 31.61[a]		448.3 ± 27.19[a]		479.9 ± 22.95[a]
11–12		472.2 ± 18.01		468.0 ± 20.87	

Mean ± S.E. ($n = 9$ for Cd and $n = 4$ for MT). ([ab]) Comparison of data among each row collected from the same sampling time after a parent was injected; by one-way ANOVA analysis with Tukey's comparisons. Different letters indicate a significant difference ($p < 0.05$); in addition, two groups were compared by t-test.

[*] Indicates a significant difference at $p < 0.05$.

[A] No sample was collected because no larvae were born at that time.

B reagent. The homogenate was mixed with 120 μL chloroform, shaken gently for 15 s, and kept on ice for 15 min before centrifugation at 11,000 rpm for 15 min at 4 °C. An aliquot of 600 μL of supernatant was mixed well with 600 μL isopropanol and 3 M acetic acid, and stored at −80 °C for over 30 min before another centrifugation at 11,000 rpm for 15 min at 4 °C. The supernatant was removed, and the precipitate was washed with 75% ethanol. The precipitate was allowed to dry for 30 min and dissolved again in an adequate amount of DEPC (diethylpyrocarbonate, Sigma, USA) to obtain total RNA. The RNA extract was stored at −80 °C before measurement for its total RNA content in a spectrophotometer (Hitachi U-2000) at OD 260/280 nm.

The total RNA from the gills was extracted using REzol™ C&T (PROtech, Taiwan) with a ratio of 0.1 mg of tissue to 1 mL of reagent.

Table 2
Comparison of the Cd accumulation (ng/g dry weight) and metallothionein (MT) contents (ng/mg protein) in the whole bodies of H0 larvae, the parents of which either the female (IF) or male (IM) or both (IF/IM) were injected with 1 mg/Kg body weight Cd or saline (control), or was untreated (blank)

Sampling time after parental injection (week)	Treatment condition				
	Blank	Control	IM/IF	IM	IF
Cd contents in H0-stage larvae					
0–1	86.2 ± 20.27[a]	[A]		151.3 ± 54.12[b]	227.5 ± 36.96[b]
1–2		91.3 ± 14.33	221.1 ± 39.03[*]		
2–3	96.4 ± 24.6				160.6 ± 14.7[*]
3–4		101.8 ± 20.27[a]	172.9 ± 37.9[b]	134.2 ± 14.24[ab]	
5–6		105.3 ± 23.76			102.6 ± 5.0
6–7	97.1 ± 28.57[a]		114.9 ± 19.23[a]	105.3 ± 14.33[a]	
8–9	108.2 ± 22.06[a]		104.9 ± 12.63[a]		105.8 ± 16.22[a]
9–10		105.3 ± 19.14		95.2 ± 19.14	
10–11	99.4 ± 20.36[a]		98.7 ± 12.35[a]		103.7 ± 14.9[a]
11–12		102.6 ± 14.52		102.3 ± 14.9	
MT contents in H0-stage larvae					
0–1	351.8 ± 59.76[a]	[A]		579.6 ± 45.55[b]	643.5 ± 11.69[b]
1–2		331.6 ± 49.1	734.6 ± 89.46[*]		
2–3	261.5 ± 57.59				512.7 ± 84.96[*]
3–4		304.5 ± 31.35[a]	557.0 ± 114.92[b]	387.1 ± 75.26[ab]	
5–6		302.4 ± 42.09			358.3 ± 45.29
6–7	383.0 ± 49.19[a]		434.9 ± 60.79[a]	343.9 ± 28.06[a]	
8–9	363.4 ± 18.71[a]		367.7 ± 45.81[a]		358.3 ± 45.29[a]
9–10		337.9 ± 44.43		304.6 ± 59.15	
10–11	343.2 ± 17.49[a]		355.2 ± 33.34[a]		363.2 ± 24.68[a]
11–12		383.6 ± 32.39		375.0 ± 18.45	

Mean ± S.E. ($n = 9$ for Cd and $n = 4$ for MT). ([ab]) Comparison of data among each row which were collected from the same sampling time after a parent was injected; by one-way ANOVA analysis with Tukey's comparisons. Different letters indicate a significant difference ($p < 0.05$). In addition, two groups were compared by t-test.

[*] Indicates a significant difference at $p < 0.05$.

[A] No sample was collected because there were no newly hatched larvae at this time.

Table 3
Comparison of the Cd accumulation (ng/g dry weight) and metallothionein (MT) contents (ng/mg protein) in the whole bodies of H1 larvae, the parents of which either the female (IF) or male (IM) or both (IF/IM) were injected with 1 mg/Kg body weight Cd or saline (control), or were untreated (blank)

Sampling time after parental injection (week)	Treatment condition				
	Blank	Control	IM/IF	IM	IF
Cd contents in H1-stage larvae					
0–1	90.9 ± 15.74[a]	[A]		146.5 ± 21.59[b]	228.6 ± 37.43[b]
1–2		91.8 ± 12.63	252.9 ± 64.87		
2–3	99.3 ± 26.59				168.2 ± 14.05
3–4		103.4 ± 16.69[a]	178.3 ± 10.75[b]	134.6 ± 19.14[a]	
5–6		98.1 ± 12.45			114.8 ± 6.69
6–7	90.4 ± 26.21[a]		111.7 ± 15.46[a]	103.4 ± 18.67[a]	
8–9	98.8 ± 19.42[a]		106.7 ± 12.54[a]		114.2 ± 19.33[a]
9–10		101.8 ± 16.31		109.3 ± 15.56	
10–11	104.1 ± 12.82[a]		102.7 ± 16.69[a]		105.2 ± 22.9[a]
11–12		105.8 ± 19.33		99.4 ± 14.9	
MT contents in H1-stage larvae					
0–1	605.7 ± 62.09[a]	[A]		826.3 ± 102.62[b]	1388.2 ± 116.57[c]
1–2		616.4 ± 74.74	1259.8 ± 56.03[*]		
2–3	539.3 ± 128.69				1128.6 ± 94.83[*]
3–4		566.0 ± 23.9[a]	1097.4 ± 98.03[c]	807.6 ± 45.9[b]	
5–6		605.9 ± 27.19			749.7 ± 83.31
6–7	575.0 ± 16.54[b]		737.7 ± 27.02[a]	560.7 ± 38.97[b]	
8–9	572.8 ± 18.62[a]		594.3 ± 41.92[a]		578.4 ± 33.08[a]
9–10		600.3 ± 40.27		510.0 ± 55.69	
10–11	584.7 ± 40.27[a]		595.2 ± 27.11[a]		566.3 ± 30.74[a]
11–12		593.4 ± 36.11		584.2 ± 39.84	

Mean ± S.E. ($n = 9$ for Cd and $n = 4$ for MT). ([ab]) Comparison of data among each row collected from the same sampling time after a parent was injected; by one-way ANOVA analysis with Tukey's comparisons. Different letters indicate a significant difference ($p < 0.05$). In addition, two groups were compared by *t*-test.

[*] Indicates a significant difference at $p < 0.05$.

[A] No sample was collected because there were no newly hatched larvae at this time.

Table 4
The relative metallothionein (MT) mRNA expression (MT/β-actin) in pre-H0, H0, and H1 larvae, the parents of which either the female (IF) or male (IM) or both (IF/IM) were injected with 1 mg/Kg body weight Cd or saline (control), or were untreated (blank)

Sampling time after parental injection (week)	Treatment condition				
	Blank	Control	IM/IF	IM	IF
Pre-H0 stage					
0–2	1.36 ± 0.06[a]	1.37 ± 0.07[a]	2.16 ± 0.07[d]	1.60 ± 0.04[b]	1.83 ± 0.03[c]
2–3	1.27 ± 0.06	[A]			1.55 ± 0.03[*]
3–4		1.30 ± 0.1[a]	1.80 ± 0.07[b]	1.37 ± 0.11[a]	
5–6		1.41 ± 0.11			1.57 ± 0.15
6–7	1.37 ± 0.05[a]		1.45 ± 0.07[a]	1.46 ± 0.14[a]	
8–9	1.40 ± 0.11[a]		1.54 ± 0.15[a]		1.49 ± 0.16[a]
9–10		1.53 ± 0.16		1.47 ± 0.13	
10–11	1.33 ± 0.07[a]		1.47 ± 0.11[a]		1.49 ± 0.16[a]
11–12		1.51 ± 0.16		1.45 ± 0.12	
H0 stage					
0–2	1.30 ± 0.07[a]	1.23 ± 0.08[a]	1.95 ± 0.07[c]	1.50 ± 0.05[b]	1.86 ± 0.02[c]
2–3	1.25 ± 0.07	[A]			1.61 ± 0.04[*]
3–4		1.28 ± 0.01[a]	1.67 ± 0.02[b]	1.32 ± 0.07[a]	
5–6		1.31 ± 0.05			1.44 ± 0.04
6–7	1.35 ± 0.07[a]		1.36 ± 0.11[a]	1.36 ± 0.11[a]	
8–9	1.38 ± 0.11[a]		1.28 ± 0.13[a]		1.32 ± 0.07[a]
9–10		1.31 ± 0.1		1.27 ± 0.05	
10–11	1.38 ± 0.11[a]		1.42 ± 0.11[a]		1.32 ± 0.07[a]
11–12		1.31 ± 0.09		1.33 ± 0.04	
H1 stage					
0–2	1.34 ± 0.08[a]	1.38 ± 0.1[a]	2.12 ± 0.15[c]	1.53 ± 0.1[ba]	1.98 ± 0.17[b]
2–3	1.31 ± 0.05	[A]			1.71 ± 0.06[*]
3–4		1.34 ± 0.1[a]	1.72 ± 0.05[b]	1.35 ± 0.11[a]	
5–6		1.46 ± 0.1			1.54 ± 0.11
6–7	1.53 ± 0.13[a]		1.43 ± 0.03[a]	1.47 ± 0.07[a]	
8–9	1.43 ± 0.11[a]		1.31 ± 0.05[a]		1.41 ± 0.11[a]
9–10		1.42 ± 0.08		1.56 ± 0.11	
10–11	1.46 ± 0.14[a]		1.42 ± 0.19[a]		1.54 ± 0.12[a]
11–12		1.31 ± 0.02		1.52 ± 0.16	

Mean ± S.E. ($n = 3$). ([ab]) Comparison of data from the same line by one-way ANOVA analysis with Tukey's comparisons; different letters indicate a significant difference ($p < 0.05$). Two group were compared by *t*-test.

[*] Indicates a significant difference at $p < 0.05$.

[A] No sample was detected because no larvae were newly born at this time.

The mRNA was separated, and 1 μL Random primer (PROtech) was added to 3 μg of total RNA, and then approximately 15 μL of DEPC-treated water was added. This mixture was denatured at 75 °C for 5 min then placed on ice for 5 min so that the primers could anneal to the template. The components of the first-strand synthesis were supplemented with 3 μL of an imProm-II™ Reverse Transcriptase kit (Promega, Taipei, Taiwan), 1 μL RNase inhibitor, 10 mM dNTP, 25 mM $MgCl_2$, and 6 μL of 5× Rxn buffer. The 30-μL mixture was reacted at 25 °C for 5 min for annealing, then at 55 °C for 1 h for extending the first strand, with a final increase in temperature to 70 °C for 15 min in order to terminate the enzyme reaction. The single-stranded cDNA was kept at 4 °C.

The MT-F primer was 5′-GCCAAGACTGGAACCTGC-3′, and the MT-R primer was 5′-GCACACGCAGCCAGAGGC-3′. The β-actin-F primer was 5′-ACCACCACAGCCGAGAGGGA-3′, and the β-actin-R primer was 5′-CCCAACCAAACGCCCAACAA-3′. The PCRs were performed using buffer (100 mM Tris–HCl, 500 mM KCl, and 15 mM $MgCl_2$), 2.5 μL of the dNTP mixture, primers (0.1 μL for β-actin and 0.9 μL for MT), 2 μL of the cDNA template, 1.25 U Taq DNA polymerase, and distilled H_2O. All reactions were performed in a 25-μL volume. After denature with 95 °C for 2 min, one thermal cycling program consisted of 40 cycles (saturation point), 95 °C for 1 min, 55 °C for 30 s for annealing, and 72 °C for 30 s before being polymerized at 72 °C for 10 min, and final cooling at 4 °C.

Each PCR product was resolved on a 1.2% agarose gel, photographed, and analyzed using image analysis (Kodak Digital Science, USA). The relative MT mRNA expression was calculated from the ratio of MT cDNA and β-actin cDNA.

2.6. Statistical analysis

Data are presented as mean ± S.E. Results were analyzed by Student's *t*-test, one-way or two-way ANOVA with Tukey's multiple comparisons. Statistical significance was set at $p < 0.05$.

3. Results

In the preliminary study, tilapia larvae from Cd-pretreated males and females had a significantly higher Cd tolerance in comparison with those from untreated males and females. However, the effect can only be found in those bred from adults within 4 weeks of Cd injections. In addition, there was a linear relation between the mortality and the length of time after the parent fish were Cd-treated ($Y = 3.65 + 0.70X$; $r^2 = 0.94$). Our previous study found that the H3 tilapia larvae caused greater than 25% mortality after 24 h with 35 μg/L Cd exposure (Wu et al., 2000). Thus, the H0 larvae challenged with 35 μg/L Cd for 24 h were chosen in the preliminary study, and the data appeared to have approximately 4%, 7% and 11.5% mortality when their parents were bred at 2, 4, and 12 weeks, respectively. In contrast, if the parent fish received saline treatment and bred within 2–4 weeks, their larvae exposed to 35 μg/L Cd had a higher mortality (approximately 11–12%) as compared to those from the Cd-treated parent.

The Cd and metallothionein contents of pre-H0, H0, H1 larvae were showed in Tables 1–3, respectively. At all of these stages, the offspring that were bred within 4 weeks after the parents were pretreated with Cd showed significant higher levels of body Cd and MT contents in comparison with the blank group (untreated) and control group (the female treated with saline). Both Cd and MT contents decreased through time and became indifferent from the blank and the control by week 4. The pre-H0 stage larvae bred within 1 week from IM and IF contained about 1.48- and 2.06-fold (146.4/98.9; 203.7/98.9 in Table 1) more Cd than those bred from the blank treatment parents, respectively. Their MT contents were 1.67- (756.7/452.8) and 1.52- (689.3/452.8) fold higher (Table 1).

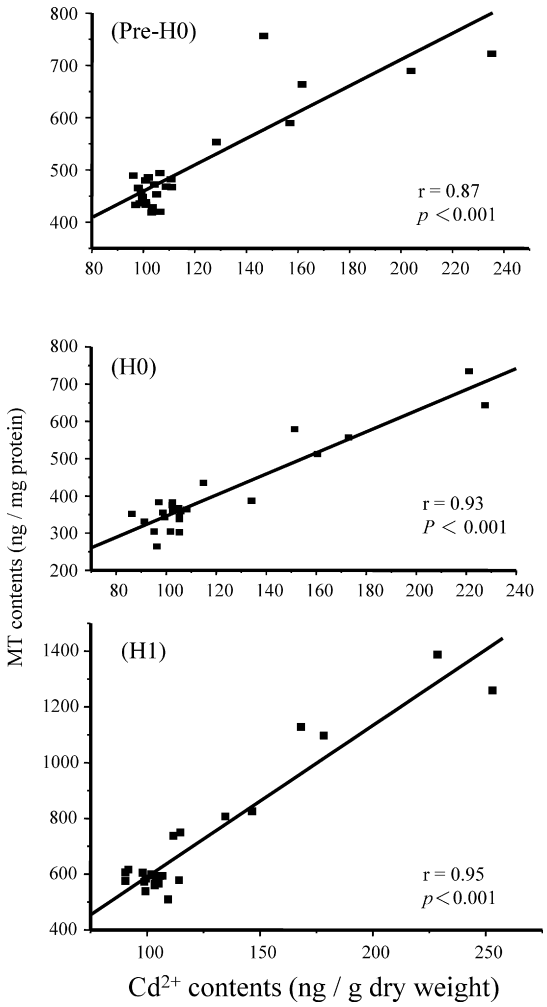

Fig. 1. Linear regression comparing the relationship between Cd contents and MT levels which were measured from the whole bodies of tilapia larvae (*O. mossambicus*). Data were collected from various growth stages with different Cd treatments.

We collected the pre-H0 larvae for Cd and MT contents, and the rest of the broods were kept until hatching when they were sampled within 12-h post-hatching (H0) and 1-day post-hatching (H1). At the H0 stage, it was 1.76- and 2.64-fold in Cd and 1.65- and 1.83-fold in MT contents (Table 2) from IM and IF treatment larvae, respectively compared to those from the blank treatment larvae. Similar profile in the ratios of Cd and MT to their respective controls was found in the H1-stage larvae, particularly in those broods produced within the first 4 weeks of Cd application (Table 3). By the end of the 12-week experiment, the MT contents were persistently higher than those from pre-H0- and H0-stage larvae. Besides, the data were collected from different breeding times after the injection and they were analyzed for the relation between the changes of MT and Cd contents at different developmental stages. Our results indicated a high level of correlation among all three stages (Fig. 1, $r = 0.87$–0.95). Furthermore, the MT mRNA expression had a similar profile (Table 4; Fig. 2) as did the Cd and MT contents and most MT mRNA dropped to background levels within 4–6 weeks.

At the end of the 12-week experiment, all females were collected for their hepatic tissues. We found females received Cd treatment had significantly higher hepatic Cd and hepatic MT contents. As compared with the blank and control groups, the hepatic tissues of the Cd-treated group contained 2487-fold Cd content and 4.85- and 5.23-fold MT contents, respectively (Table 5). No difference was

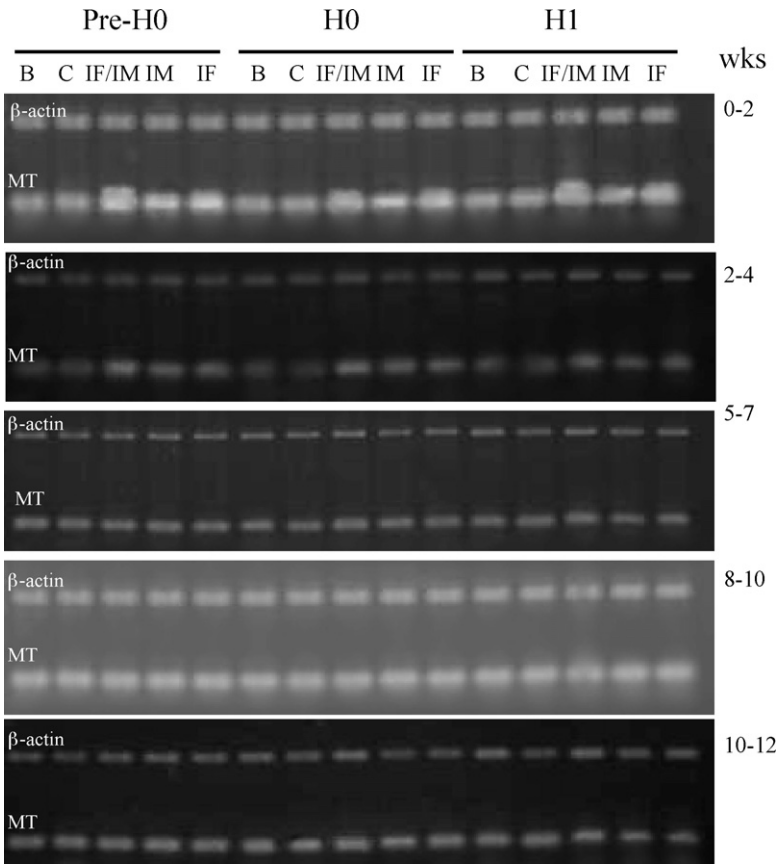

Fig. 2. RT-PCR products of MT mRNA and β-actin expression of pre-hatching (Pre-H0), newly hatched (H0), and 1-day post-hatching (H1) tilapia larvae (*O. mossambicus*) which had been bred at different times (weeks) after different treatments of their parents (B, untreated; C, parents were intraperitoneally (i.p.) injected with saline; IM/IF, both the female and males were i.p. injected with Cd; IM, males were i.p. injected with Cd; IF, the female was i.p. injected with Cd).

Table 5
Comparison of metallothionein (MT) contents (ng/mg protein) and Cd accumulation (μg/g dry weight) in hepatic tissue of females ($n = 4$) injected with saline, 1 mg/Kg body weight Cd, or a blank (untreated) after 12 weeks

Parameter	Treatment condition		
	Blank	Saline-injection	Cd-injection
Cd content	0.1 ± 0.01^{a}	0.1 ± 0.11^{a}	248.7 ± 35.25^{b}
MT content	1603.4 ± 155.8^{a}	1489.0 ± 241.45^{a}	7786.0 ± 628.47^{b}

Data are presented as mean ± S.E. (^{ab}) Comparison of data on the same line by one-way ANOVA analysis with Tukey's comparisons; different letters indicate a significant difference ($p < 0.05$).

found between the blank and the control females in either hepatic Cd or hepatic MT contents.

4. Discussion

It is known that some proteins and endocrine factors are transferred to larvae by the maternal effect (Bernardo, 1996). Wade (1998) reported that parental effects can manifest themselves at three different stages of offspring development: (1) a prezygotic maternal effect in which the maternal nutritional condition affects gametic size and quality; (2) postzygotic-prenatal maternal effects that arise from several intimate prenatal nutritional relationships between the mother and developing embryos; and (3) postzygotic-postnatal maternal effects, wherein maternal care and maternal competition affect the offspring's phenotype. Our previous study indicated that MT contents rapidly reached a peak at H1 (Wu et al., 2000). Therefore, the MT and Cd contents were measured in pre-hatching (pre-H0), newly hatched (H0), and 1 day post-hatching larvae (H1). In the present study, no significant difference was found among the three stages of larvae in body Cd contents in either control or blank groups. However, MT content in the H1-stage larvae was significantly higher than pre-H0- and H0-stage larvae and this was also reported in one of our previous studies (Wu et al., 2000). It is irrefutable that the difference in heavy metal resistance among larvae is related to their own parental effects, and this explains why larvae show a higher resistance to metals and higher MT expression when they or their parents are treated with metals (Chang et al., 1997; Lin et al., 2000; Peake, 2002). It is possible that some materials were transferred to the larvae by Cd-pretreated females.

The major function of MT is to detoxify metals in fish, and it enhances resistance to metals. However, many factors are related to the Cd resistance of larvae, including an increase in Ca^{2+} uptake (Chang et al., 1997), genetic adaptations (Knapen et al., 2004), pre-treatment with a low dose of Cd (Wu and Hwang, 2003), and chronic exposure to Cd in the parent fish (García et al., 1999). Therefore, it is presumable that a higher Cd resistance of larvae may result from the maternal effect, and the oocytes may be contaminated by the metal. We traced and measured changes in Cd contents and MT levels in larvae and parents from the breeding time of larvae after the parents were injected with Cd. In a comparison of differences in Cd-pretreated females and males, two major results were found. First, there was a positive correlation between the Cd content and MT expression in larvae (Fig. 1). It implies a higher MT expression in pre-hatched, new-born and hatched larvae which was induced by higher Cd levels. Second, Cd accumulation and MT expression were evidently higher in newly born larvae than larvae produced later by Cd-treated parents (Tables 1–3). The evidence suggests that the

higher MT expression was more affected by maternal-Cd induction than by maternal-MT transfer.

During vitellogenesis, many exogenous and endogenous materials are transferred from the mother fish to the oocytes and subsequently to the eggs and yolk sac of larvae (Mommsen and Walsh, 1988). The major vitellogenic steps included the exogenous step that is through the gonadotrophin to stimulate estrogen activity and to induce vitellogenin from hepatic to oocyte. The endogenous step is the direct induction of gonadotrophin for the vitellogenesis in oocyte. Some factors including Cd, MT mRNAs, and MTs are transferred to oocytes of various organisms. For example, in the zebrafish (*D. rerio*), it was found that both Zn and Cu accumulations increased and the MT content was also higher during oocyte growth (Riggio et al., 2003). It is possible that the entry of Zn into zebrafish oocytes is mediated by the uptake of vitellogenin, a protein that is known to bind with metal ions (Montorzi et al., 1995). Lin et al. (2000) reported that the low-molecular-weight MT mRNA was possibly extrinsically taken up along with other maternal materials into the oocytes. If this is the case, offspring with a higher MT content would be from parental females containing more MT. In the present study, we sacrificed all females for hepatic Cd and MT measurements 12 weeks after the Cd injection. Indeed, high levels of Cd and MT were found in Cd-pretreated females 12 weeks after the Cd injection (Table 5), but the offspring did not show a similar profile at this time point (Tables 1–3). Obviously, it is necessary to confirm whether the MT mRNA was transferred from Cd-treated females to the larvae. Similarly, Lin et al. (2000) reported that a high level of MT mRNA was found in newly hatched larvae from Cd-treated mothers. However, the MT mRNA expression of larvae had decreased to a non-significant difference compared with the control and blank groups in larvae bred after 4 weeks of parental injection (Table 4; Fig. 2). Therefore, we concluded that the higher level of MT expression in larvae is from the Cd-treated mothers, since the Cd had contaminated the ovary rather than MT being transferred by the maternal effect.

5. Conclusions

The present results indicated that a significant relationship between Cd and MT contents can be found in the offspring from the parent fish treated with Cd. However, the higher Cd resistance, Cd contents, and MT expression were limited to those larvae from parent fish bred within 4 weeks of the injection. According to Lin et al. (2000), a high level of MT mRNA was found in newly hatched larvae from Cd-treated mothers, and they suggested that the low-molecular-weight MT mRNA was possibly extrinsically taken up along with other maternal materials into the oocytes. The present study showed that the MT mRNA expression of larvae had decreased to a non-significant difference compared with the control and blank groups in larvae bred after 4 weeks of parental injection (Table 4; Fig. 2). In addition, by week 12, the Cd-treated fish still contained high levels of MT in their hepatic tissues. However, the MT and Cd contents in the larvae from these adult fish were not significantly different from those from the controls. In summary, we suggest that the higher Cd resistance of larvae from the egg stage was a result of the Cd contamination of the parent female, as evidenced by an increase in MT expression induced in tilapia embryos and larvae.

Acknowledgements

This work was supported by grant nos. NSC 94-2317-B-001-015 and NSC-96-2311-B-415-001-MY3 from the National Science Council, Executive Yuan, Taiwan and in part supported by National Chiayi University, Taiwan.

References

Allen, P., 1995. Accumulation profiles of lead and cadmium in the edible tissues of *Oreochromis aureus* during acute exposure. J. Fish Biol. 47, 559–568.
Bernardo, J., 1996. Maternal effects in animal ecology. Am. Zool. 36, 83–105.
Chang, M.H., Lin, H.C., Hwang, P.P., 1997. Effects of cadmium on the kinetics of cadmium uptake in developing tilapia larvae, *Oreochromis mossambicus*. Fish Physiol. Biochem. 16, 459–470.
Chang, M.H., Lin, H.C., Hwang, P.P., 1998. Ca^{2+} uptake and Cd^{2+} accumulation in larval tilapia (*Oreochromis mossambicus*) acclimated to waterborne Cd^{2+}. Am. J. Physiol. 274, R1570–R1577.
De Jesus, E.G.T., Hirano, T., Inui, Y., 1991. Changes in cortisol and thyroid hormone concentrations during early developmental and metamorphosis in the Japanese flounder, *Paralichthys olivaceus*. Gen. Comp. Endocrinol. 82, 369–376.
García, M.E., Cappelletti, C.A., Salibián, A., 1999. Sublethal maternal pre-exposure of fish to cadmium. Effect on the survival of the newly hatched alevins. Arch. Physiol. Biochem. 107, 152–158.
Hollis, L., McGeer, J.C., McDonald, D.G., Wood, C.M., 2000. Protective effects of calcium against chronic waterborne cadmium exposure to juvenile rainbow trout. Environ. Toxicol. Chem. 19, 2725–2734.
Hwang, P.P., Wu, S.M., Lin, J.H., Wu, L.S., 1992. Cortisol content of eggs and larvae of teleosts. Gen. Comp. Endocrinol. 86, 189–196.
Knapen, D., Bervoets, L., Verheyen, E., Blust, R., 2004. Resistance to water pollution in natural gudgeon (*Gobio gobio*) populations may be due to genetic adaptation. Aquat. Toxicol. 67, 15–165.
Kolok, A.S., L'Etoile-lopes, D., 2005. Do copper tolerant fathead minnows produce copper tolerant adult offspring? Aquat. Toxicol. 72, 231–238.
Lin, H.C., Hsu, S.C., Hwang, P.P., 2000. Maternal transfer of cadmium tolerance in larval *Oreochromis mossambicus*. J. Fish Biol. 57, 239–249.
Lourdes, M., Cuvin-Aralar, A., Aralar, E.V., 1995. Resistance to a heavy metal mixture in *Oreochromis niloticus* progenies from parents chronically exposed to the same metals. Chemosphere 30, 953–963.
Mommsen, T.P., Walsh, P.J., 1988. Vitellogenesis and oocyte assembly. In: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology: The Physiology of Developing Fish. Part A. Eggs and Larvae, Vol. 11. Academic Press, San Diego, pp. 347–406.
Montorzi, M., Falchuk, K.H., Vallee, B.L., 1995. Vitellogenin and lipovitellin: zinc proteins of *Xenopus laevis* oocytes. Biochemistry 34, 10851–10858.
Peake, E.B., 2002. Maternal transfer of copper resistance in fathead minnows. In: Kennedy, C., Kolok, A., Kinlay, D.M. (Eds.), Aquatic Toxicology Mechanisms and Consequences. University of British Columbia, Vancouver, Canada, pp. 87–91.
Pottinger, T.G., Mosuwe, E., 1994. The corticosteroidogenic response of brown and rainbow trout alevins and fry to environmental stress during a "critical period". Gen. Comp. Endocrinol. 95, 350–362.
Reznick, D., Callahan, H., Llaurdo, R., 1996. Maternal effects on offspring quality in poeciliid fishes. Am. Zool. 36, 147–156.
Riggio, M., Filosa, S., Parisi, E., Scudiero, R., 2003. Changes in zinc, copper and metallothionein contents during oocyte growth and early development of the teleost *Danio rerio* (zebrafish). Comp. Biochem. Physiol. 135C, 191–196.
Rombough, P.J., Garside, E.T., 1982. Cadmium toxicity and accumulation in eggs and alevins of Atlantic salmon *Salmo salar*. Can. J. Zool. 60, 2006–2014.
Shackley, S.E., King, P.E., Gordon, S.M., 1981. Vitellogenesis and trace metals in a marine teleost. J. Fish Biol. 18, 349–352.
Solemdal, P., 1997. Maternal effects—a link between the past and the future. J. Sea Res. 37, 213–227.
Wade, M.J., 1998. The evolutionary genetics of maternal effects. In: Mousseau, E.T., Fox, C.W. (Eds.), Maternal Effects as Adaptations. Oxford University Press, New York, pp. 5–21.
Werner, J., Wautier, K., Evans, R.E., Baron, C.L., Kidd, K., Palace, V., 2003. Waterborne ethynylestradiol induces vitellogenin and alters metallothionein expression in lake trout (*Salvelinus namaycush*). Aquat. Toxicol. 62, 321–328.
Wu, S.M., Hwang, P.P., 2003. Copper or cadmium pretreatment increases the protection against cadmium toxicity in tilapia larvae (*Oreochromis mossambicus*). Zool. Stud. 42, 179–185.
Wu, S.M., Weng, C.F., Hwang, J.C., Huang, C.J., Hwang, P.P., 2000. Metallothionein induction in early larval stages of tilapia (*Oreochromis mossambicus*). Physiol. Biochem. Zool. 73, 531–537.
Wu, S.M., Jong, K.J., Lee, Y.J., 2006. Relationships among metallothionein, cadmium accumulation, and cadmium tolerance in three species of fish. Bull. Environ. Contam. Toxicol. 76, 595–600.
Wu, S.M., Ho, Y.C., Shih, M.J., 2007. Effects of Ca^{2+} or Na^{+} on metallothionein expression in tilapia larvae (*Oreochromis mossambicus*) upon cadmium or copper exposure. Arch. Environ. Contam. Toxicol. 52, 229–234.