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# Effects of Exogenous Cortisol and Progesterone on Metallothionein Expression and Tolerance to Waterborne Cadmium in Tilapia (*Oreochromis mossambicus*)

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**Su-Mei Wu, Yi-Ying Chou and Am-Ni Deng (2002)** Effects of exogenous cortisol and progesterone on metallothionein expression and tolerance to waterborne cadmium in tilapia (*Oreochromis mossambicus*). *Zoological Studies* **41**(1): 111-118. The object of the present study was to test the hypothesis that glucocorticoids can induce the expression of metallothionein (MT) which consequently enhances the tolerance to metal toxicity in tilapia (*Oreochromis mossambicus*). Adult tilapia (4-5 cm in total length and 5.0-6.0 g in body weight) and larval tilapia (0.5-0.7 cm in total length and 0.012-0.017 g in body weight) were reared with artificial feed containing 0 (control), 50 (low dose), 125 (medium dose), or 250 mg/kg (high dose) of cortisol or progesterone for 10 d. Liver and whole-body contents of MT and mortality in tilapia upon $Cd^{2+}$ exposure were examined after the steroid-rearing experiments. Both cortisol and progesterone significantly increased survival rates in adult fish after $Cd^{2+}$ challenge. Treatment with middle and high doses of progesterone significantly stimulated the expression of MT in adult fish, but stimulation by cortisol showed no significant effect on MT expression. More MT was expressed after $Cd^{2+}$ challenge in both cortisol- and progesterone-treated groups. However, exogenous steroids caused no significant effect on MT contents or survival upon $Cd^{2+}$ challenge in tilapia larvae. This suggests that cortisol and progesterone are involved in metal-detoxification mechanisms in adult tilapia via regulating the expression of MT, but the occurrence of this pathway in developing fish is unclear. http://www.sinica.edu.tw/zool/zoolstud/41.1/111.pdf

**Key words:** Corticosteroid, Metallothionein, Cadmium, Tolerance, Tilapia.

Cadmium ($Cd^{2+}$), a highly toxic metal, is present throughout the environment and accumulates primarily in liver and kidney of mammals through the food chain. Acute exposure to cadmium results in toxic lesions in the liver, kidney, lung, gastrointestinal tract, central nervous system, ovaries, placenta, and testes (Friberg et al. 1986, Waalkes et al. 1991, Goering et al. 1994). Cadmium is also an important xenobiotic, non-degradable cumulative pollutant in aquatic ecosystems, and freshwater fish are particularly vulnerable to cadmium exposure (Sorensen 1991). With regard to the toxic effects of metals on aquatic organisms, much research focused on the $LC_{50}$ or $LT_{50}$ of toxic metals, and some studies also reported the impacts of toxic metals on physiological performances of fish (Heath 1995). Cadmium and zinc inhibit calcium channels of the myocardium and affect the function of the heart (Tort and Madsen 1991). Cadmium, lead, and mercury cause anemia in fish (Fletcher and White 1986, Tewari et al. 1987, Houston et al. 1993). Heavy metals produce adverse effects by causing osmoregulatory stress in fish (Reid and McDonal 1988, Pratap et al. 1989).

Metallothionein (MT) is a low-molecular-weight protein that binds to heavy metals such as cadmium, mercury, zinc, and copper with high affinity. Therefore, MT has been considered an important factor in the detoxification of heavy metals. In addition, MT is a multi-regulated protein in mammals (Kagi and Kojima 1987), and a variety of hor-

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mones and factors may induce its expression in tissue. Moreover, a number of stressors are also known to induce rat liver MT (Hidalgo et al. 1986).

The plasma corticosteroid level is generally used as an indicator to determine the magnitude of stress responses in vertebrates, including fish (Mazeaud and Mazeaud 1981, Donaldson et al. 1984). The major corticosteroid is cortisol, which has mineralocorticoid as well as glucocorticoid properties (Donaldson 1981). Cortisol levels increased after challenges of salinity, anesthesia, handling stress, and high living density (Barton et al. 1985, Krieger et al. 1989, Vijayan et al. 1989, Avella et al. 1991, Fevolden et al. 1991, Barton and Eitzow 1992, Belloso et al. 1996, Tort et al. 1996). Furthermore, plasma cortisol levels increased significantly after both stress and cadmium or copper exposure in rainbow trout (Gill et al. 1993, Tort et al. 1996, Dethloff et al. 1999). Therefore, cortisol levels have been considered an indicator of a primary stress response. To sum up, both MT and cortisol contents are indicators for monitoring the impacts of heavy metals (Dunn et al. 1987, Belloso et al. 1996, Hamza-Chaffai et al. 1997).

In mammals, mutation and deletion analyses have revealed the presence of short DNA segments in the MT gene promoter (MRE, metal regulatory element; GRE, glucocorticoid-responsive element), which is essential for metal-induced and glucocorticoid-responsive MT transcription (Carter et al. 1984, Karin et al. 1984, Stuart et al. 1984). Certain heavy metals and corticosterone have been identified as factors responsible for the induction of hepatic MT in mice and rats (Quaife et al. 1986, Hidalgo et al. 1994, Sato et al. 1996). Pretreatment with progesterone, a precursor for the major glucocorticoids, followed by cadmium exposure caused a marked 16-fold induction in MT synthesis in rat liver cells (Shimada et al. 1997). However, there were inconsistent results in *in vivo* systems. Shiraishi et al. (1993) did not find an effect of progesterone pretreatment on the cadmium-induced increase in hepatic or renal MT, or hepatic or testicular MT mRNA levels in the rat. These results indicate that control by glucocorticoid of MT expression and their relationships with waterborne cadmium are not yet clear *in vivo*.

Fish are not only a major ecosystem component but also an important food resource; therefore it is important to study the physiological mechanisms in response to the stressor, waterborne cadmium. In teleosts, induction of MT synthesis in response to heavy metals has also been reported (Olsson and Haux 1985, Hamilton and Mehrle 1986). Pretreatment with cortisol was found to enhance the induction of MT in primary culture of rainbow trout hepatocytes (Hyllner et al. 1989). However, there were no data examining the involvement of glucocorticoids in cadmium-induced MT expression in in vivo systems. The purpose of the present work was to study this situation in fish.

Based on previous studies (as described above) for *in vitro* systems, we propose a hypothesis for fish to demonstrate the involvement of glucocorticoid in MT expression and metal detoxification in in vivo systems. Pretreatment with cortisol and progesterone may induce the expression of MT and consequently enhance the tolerance to cadmium toxicity. Tilapia (*Oreochromis mossambicus*), a model species for fish physiological and toxicological studies, was used to test this hypothesis. Effects of treatments of progesterone and cortisol on MT protein expression were examined in tilapia, and mortalities were compared after the fish were exposed to waterborne cadmium.

## MATERIALS AND METHODS

### Fish

Mature adult tilapia (*Oreochromis mossambicus*) from the Tainan Branch of the Taiwan Fisheries Research Institute were reared in 182-L glass aquaria using plastic chips for substrate. 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. Larvae from the same brood were incubated under the same conditions as above. Two different sizes of tilapia were used in the present study; adult tilapia at 4-5 cm in total length and 5.0-6.0 g in body weight were used in experiment 1, while larval tilapia at 0.5-0.7 cm in total length and 0.012-0.017 g in body weight were used for experiment 2. Experiment 1 was conducted from Nov. to the following Jan., while experiment 2 was conducted from May to July. Water temperature was maintained at 26-28 ˚C throughout the experiments.

### Artificial feed containing glucocorticoids

Cortisol (hydrocortisone) and progesterone (Sigma, St. Louis, Mo, USA) were mixed with artificial dried feed (Tung-Pao, Tainan, Taiwan), which was stored at -20 ˚C until administration. Four doses of 0 (control), 50 (low dose), 125 (medium dose) and 250

mg/kg body weight (high dose) of both cortisol and progesterone were used in the following experiments.

**Measurement of MT content**

Fifteen to 20 larvae as a pooled sample or livers of adult fish were collected for MT ELISA (enzyme-linked immunosorbent assay). Fish were anesthetized with MS222 after sampling. Soluble extracts of larvae and liver were prepared by homogenizing the entire larvae or tissue with homogenization buffer (10 mM Tris-HCl, with 5 mM 2-mercaptoethanol, pH 7.0) in a 1:2.5-3.0 (w/v) mixture using a plastic 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 centrifuged again at 12 000 x*g* for 40 min at 4 ˚C; the final supernatants were subjected to MT ELISA.

**ELISA for MT**

ELISA for MT was as described by Wu et al. (2000). Briefly, a synthetic peptide from the N-terminal of tilapia MT (Wu et al. 1999) was coated in a microtiter plate (96 wells). Rabbit anti-tilapia MT IgG (Wu et al. 2000; diluted 1: 2000) and tissue extract or standard solution were mixed and incubated for 1.5 h at 37 ˚C. After washing 3 times, HRP (peroxidase-labeled goat anti-rabbit IgG, diluted 1:4000) was added as the secondary antibody. After 3 washes, ABTS peroxidase substrate (KPL, Gaitherburg, Md, USA) was added for color development, and results were measured at 405 nm with an automatic micro titer plate ELISA reader (Dynex MRX, Chantilly, Va, USA). The displacement curve for the serial dilutions of sample extracts was parallel to that of the MT standard. The line regression coefficient determined using Microsoft Excel 97 SR-1 (1997; Microsoft, USA) for the logarithms of 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.

***Experiment 1***

Adult tilapia were fed artificial feed mixed with cortisol or progesterone at doses of 0 (control), 50, 125, and 250 mg/kg of body weight at a rate of about 20% of body weight per day for 10 d. At the end of the rearing experiment, fish were treated with 300 µg/l $Cd^{2+}$ ($CdCl_2$) FW for 48 h. At the end of the exposure experiment, mortality was examined, and livers of fish were collected for the measurement of MT content.

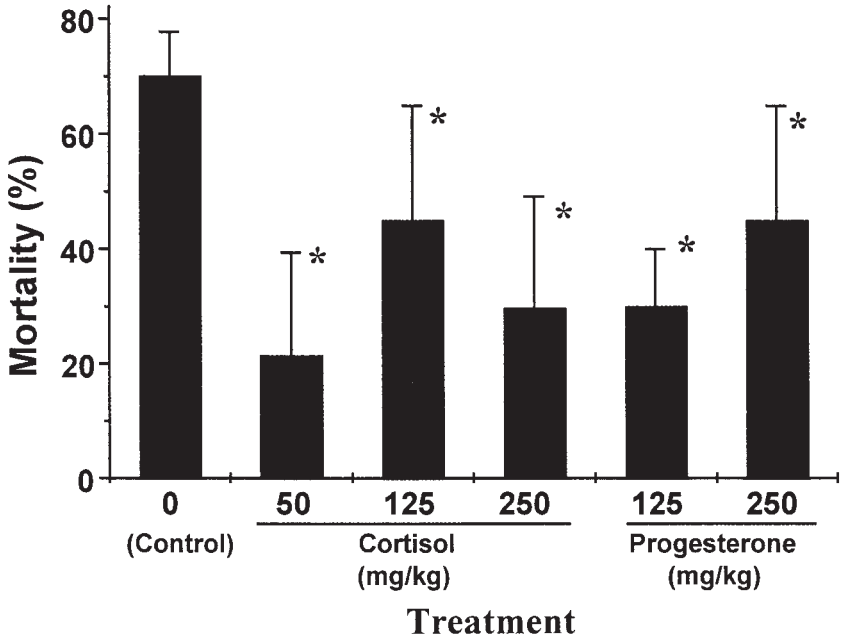


**Fig. 1.** Changes in mortality of tilapia adults after $Cd^{2+}$ challenge (300 µg/l for 48 h). Tilapia were pretreated with 50, 125, or 250 mg/kg of cortisol or progesterone for 10 d and then were exposed to 300 µg/l $Cd^{2+}$ for 48 h. Data were compared with one-way ANOVA using Dunnett's test analysis. *: Significant difference between the control and treatment groups at $p < 0.05$.

### *Experiment 2*

Tilapia larvae were administered cortisol or progesterone and exposed to waterborne $Cd^{2+}$ as described in experiment 1. At the end of the exposure experiment, mortality was examined, and larvae were collected for the measurement of whole-body MT content.

## RESULTS

Adult tilapia, administered cortisol or progesterone via feeding, showed better survival than did the control group upon $Cd^{2+}$ exposure (Fig. 1). Compared with the control, MT contents in the high- and medium-dose progesterone groups were 3.8-5.2 fold higher; moreover more MT (1.3-2.7 fold) was expressed in the progesterone groups upon $Cd^{2+}$ exposure (Table 1). Only cortisol treatment did not induce a significant increase in MT expression, but high-dose cortisol showed an additional effect (about 2.2 fold) of stimulating MT expression upon $Cd^{2+}$ exposure. Regardless of dose, both progesterone and cortisol pretreatments significantly diminished the mortality in tilapia after exposure to waterborne $Cd^{2+}$ (Fig. 1).

Neither progesterone nor cortisol pretreatment showed a significant effect on whole-body MT levels in tilapia larvae. Upon $Cd^{2+}$ exposure, some larvae increased their MT levels, but steroids showed no additional effect on the stimulation of MT in larvae (Table 2). Similarly, neither progesterone nor cortisol pretreatment enhanced the survival of larvae exposed to waterborne $Cd^{2+}$ (Fig. 2).

## DISCUSSION

The present results support our hypothesis that cortisol and progesterone are involved in the $Cd^{2+}$ detoxification mechanism via regulation of MT expression.

Mammalian MT genes have been revealed to contain both a metal-responsive element and a glucocorticoid-responsive element within the 5'-flanking regions of MT genes (Stuart et al. 1985), and MT expression was found to be regulated by metals, glucocorticoid hormones, and lipopolysaccharide-induced cytokines (Kelly et al. 1997). However, very little is known about the regulatory elements flanking MT genes in non-mammalian vertebrates, such as fish. Molecular approaches were used to analyze the cDNA clones which encode MTs for rainbow trout, pike, tilapia, and stone loach, and the deduced primary structures of the proteins were demonstrated to be highly homologous (Bonham et al. 1987, Kille et al. 1993, Chan 1994, Olsson et al. 1995). Only very few studies have discussed the hormone control of MT synthesis in fish. In the primary culture of rainbow trout hepatocytes, an in vitro system, Hyllner et al. (1989) determined MT by differential pulse polarography, and found that MT was induced about a 350% increase by treatments with cortisol and zinc. The present study using ELISA to detect MT levels in tilapia fish, an *in vivo* system has demonstrated the induction of MT by progesterone, cortisol, and $Cd^{2+}$. These findings provide some clues to the presence of metal- and glucocorticoid-responsive elements in fish MT genes. However, further molecular evidence is needed.

**Table 1.** Effects of exogenous steroids for 10 d on MT protein expression (ng/mg protein) in the liver of adult tilapia exposed to Cd for 48 h ($n$ = 5-8)

| | | Environmental Cd (μg/l) | |
|---|---|---|---|
| | Steroid dose (mg/kg) | 0 | 300 |
| | 0 | 328 ± 88 [a] | 547 ± 109 [a]* |
| Cortisol | 50 | 210 ± 43 [a] | 585 ± 250 [a]* |
| | 125 | 141 ± 34 [a] | – |
| | 250 | 378 ± 20 [a] | 1175 ± 204 [b]* |
| Progesterone | 125 | 1710 ± 609 [b] | 2226 ± 226 [c] |
| | 250 | 1300 ± 200 [b] | 3295 ± 212 [c]* |

[a,b,c] One-way ANOVA (Turkey's pairwise comparisons) analysis was run among different doses of steroids at the same Cd treatment level; different superscripts indicate a significant difference ($p < 0.05$).

* Significantly different (Student $t$-test, $p < 0.05$) from the 0 μg/l cadmium group for each concentration of steroids.

Medium and high doses of progesterone induced increases in liver MT, while cortisol did not unless a fish was exposed to waterborne $Cd^{2+}$, suggesting that progesterone is more potent than cortisol in stimulating liver MT in tilapia. Progesterone is the precursor in the biogenesis of corticosterone, estradiol, aldosterone, and cortisol, and the major glucocorticoid hormones are cortisol and corticosterone. Progesterone may induce the expression of MT via the pathways of both cortisol and corticosterone. Although data concerning the responses of MT to steroids other than cortisol and progesterone are unavailable in the present study, it is reasonable to consider the possibility that MT might show different responses to these steroids. In rainbow trout (*Oncorhynchus mykiss*), injection of estradiol-17ß in combination with cadmium resulted in inhibition of the transcription and translation of MT (Olsson et al. 1995). In cultured glial cells, metals plus corticosterone caused a significant increase in MT-I levels (Hidalgo et al. 1994).

In the present study, both progesterone and cortisol showed additional effects on the stimulation of liver MT expression in tilapia upon $Cd^{2+}$ exposure. Additional interactions between glucocorticoids and metals affecting MT expression have been reported in different species. In TRL-1215 rat liver cells, the effect of progesterone pretreatment (0, 1, and 10 µM) on cytotoxicity (percent of control) and MT content was not dose dependent after cells were exposed 10 µM of $Cd^{2+}$. However, progesterone (100 µM) pretreatment alone increased MT levels 2.4 fold, while $Cd^{2+}$ (10 µM) alone resulted in a 7-fold increase over the control. The combination of $Cd^{2+}$ exposure and progesterone pretreatment caused a marked, 16-fold induction in MT synthesis (Shimada et al. 1997). Hepatic MT levels increased more in rats treated with interleukin-6 and zinc plus dexamethasone than in rats treated with interleukin-6 and zinc alone (Sato et al. 1996). In the present study, both progesterone and cortisol showed additional effects on the stimulation of liver MT expression in tilapia and also enhanced fish survival upon $Cd^{2+}$ exposure. This suggests that these steroids probably enhance the resistance to waterborne $Cd^{2+}$ in fish by regulating the expression of MT, which is critical to the mechanism for metal detoxification as reported in other animals (Shimada et al. 1997, Hemandez et al. 2000).

In the present study, the effects of cortisol and progesterone on liver MT contents and on fish mortality upon $Cd^{2+}$ challenge were all positive but not dose dependent. Several possible reasons were considered. (1) Effects of steroid treatment via feeding may not follow the concentrations in feed because of variable uptake efficiencies among fish. (2) The range of steroid levels (50-250 mg/kg) may be too narrow to reveal a dose-dependent effect. (3) In addition to the induction of MT, some other

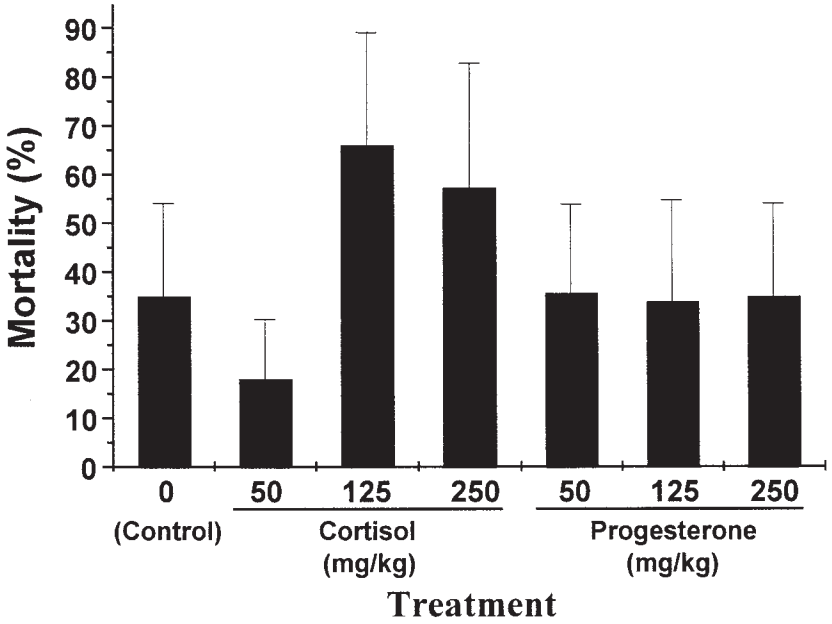


**Fig. 2.** Changes in mortality of tilapia larvae after $Cd^{2+}$ challenge (300 µg/l for 48 h). Tilapia larvae were pretreated with 50, 125, or 250 mg/kg of cortisol or progesterone for 10 d and then were exposed to 300 µg/l $Cd^{2+}$ for 48 h. No significant difference was found between the control and treatment groups.

mechanisms (see below) may also contribute to fish tolerance to $Cd^{2+}$ toxicity. It was noted that cortisol pretreatment alone did not stimulate MT synthesis, but diminished fish mortality during subsequent exposure to $Cd^{2+}$ in the present study. This is probably because of the effect of cortisol on $Cd^{2+}$ uptake in fish. Hypocalcaemia after $Cd^{2+}$ exposure is an important symptom of $Cd^{2+}$ toxicity, because $Cd^{2+}$ competes with $Ca^{2+}$ for the pathways of $Ca^{2+}$ uptake (Heath 1995, Hwang et al. 1995, Cheng et al. 1997 1998, Hwang and Yang 1997). In a transgenic strain of worm (*Caenorhabditis elegans*) carrying a stress-inducible *lacZ* reporter gene, the transgene response to $Cd^{2+}$ was strongly inhibited by $Ca^{2+}$ ions, and $Ca^{2+}$ reduced the net accumulation of $Cd^{2+}$ (Guven et al. 1995). Freshwater trout injected with cortisol showed increased $Ca^{2+}$ uptake, activity of $Ca^{2+}$-ATPase, and activation of chloride cell in gills (Flik and Perry 1989). Taking all these findings together into account, exogenous cortisol in doses, which can not stimulate MT synthesis may increase $Ca^{2+}$ uptake in tilapia, and consequently improve the survival upon subsequent exposure to $Cd^{2+}$.

In the present study, neither progesterone nor cortisol pretreatment caused a significant effect on whole-body MT content or survival in tilapia larvae following $Cd^{2+}$ exposure. In our previous studies (Hwang and Wu 1993, Lin et al. 1999), administration of cortisol reduced the cumulative mortality and diminished the degree of increase in body $Na^{+}$ content in tilapia larvae after transfer to sea water. However, cortisol did not cause a significant effect on the ouabain binding of yolk-sac epithelia of larvae even 12 h after the transfer, which differs from what has been reported for adult fish (Lin et al. 1999). Taking all these into consideration, two possibilities are proposed. (1) Cortisol may be involved in some physiological mechanisms (such as osmo- and ion- regulation) via other pathways different from those in adult fish. (2) Pathways for cortisol control in some mechanisms (such as metal detoxification) may be under or poorly developed in larval stages. These remain to be studied further. However, the present results on fish larvae provide additional data to support our previous hypothesis. In the presence of adverse environmental factors, developing fish larvae may respond via other pathways or mechanisms which differ from those in adults, probably because of different physiological demands during development (Hwang et al. 1996, Hwang and Young 1997, Cheng et al. 1998, Lin et al. 2001).

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**Table 2.** Effects of exogenous steroids for 10 d on MT protein expression (ng/mg protein) in larval tilapia exposed to Cd for 48 h ($n$ = 5-8)

| | | Environmental Cd (μg/l) | |
|---|---|---|---|
| | Steroid dose (mg/kg) | 0 | 300 |
| | 0 | 204±50 | 303±73 [a*] |
| Cortisol | 50 | 110±30 [a] | 340±98 [a*] |
| | 125 | 123±17 [a] | 255±50 [a*] |
| | 250 | 272 | 165±15 [a] |
| Progesterone | 50 | 285±60 [a] | 349.9±134.9 [a] |
| | 125 | 223±39 [a] | 410±58 [a*] |
| | 250 | 158±1 [a] | 370±70 [a*] |

[a,b,c] One-way ANOVA (Turkey's pairwise comparisons) analysis was run among different doses of steroids at the same Cd treatment level; different superscripts indicate a significant difference ($p < 0.05$).
* Significantly different (Student $t$-test, $p < 0.05$) from the 0 μg/L Cd group for each concentration of steroids.

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# 助孕酮和皮質醇對吳郭魚 (*Oreochromis mossambicus*) 金屬硫蛋白表現及鎘耐性之作用

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本研究之目的為驗證假說：糖皮質激素會誘發吳郭魚金屬硫蛋白表現進而增加對水中鎘之耐性。探討皮質醇與黃體酮前處理對吳郭魚 (*Oreochromis mossambicus*) 金屬硫蛋白表現及對鎘耐性之影響。吳郭魚成魚（全長4-5 cm、體重5.0-6.0 g）或仔魚（全長0.5-0.7 cm、體重0.012-0.017 g），投餵以0（對照組）、50（低劑量）、125（中劑量）和 250 mg/kg（高劑量）皮質醇與黃體酮10天。投餵實驗後，偵測成魚肝臟或仔魚全魚之金屬硫蛋白含量及鎘處理後的死亡率。結果顯示投餵皮質醇與黃體酮可以增加吳郭魚成魚在鎘水中之活存。中劑量及高劑量黃體酮具有提高吳郭魚成魚金屬硫蛋白表現之作用，而皮質醇則作用不明。浸泡鎘後，高劑量皮質醇及中、高劑量的黃體酮都顯著地增加金屬硫蛋白之產生。然而在仔魚有不同之結果，皮質醇與黃體酮前處理對仔魚鎘耐性或金屬硫蛋白誘導均無明顯之作用。我們推論：類固醇激具有參與誘導吳郭魚成魚金屬硫蛋白合成與重金屬解毒之機制，但是此機制在仔魚尚不明瞭。

**關鍵詞**：醣皮質固醇，金屬硫蛋白，鎘，耐受力，吳郭魚。

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