

## Effects of cadmium, 17 $\beta$ -estradiol and their interaction in the male Chinese loach (*Misgurnus anguillicaudatus*)

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To assess the interaction effect between cadmium (Cd) and 17 $\beta$ -estradiol (E2), male Chinese loaches (*Misgurnus anguillicaudatus*) were exposed to E2 (1  $\mu$ g/L) and Cd (50 or 500  $\mu$ g/L) alone and in combination using a semi-static waterborne exposure system. The effects of E2 on the accumulation and distribution of Cd, as well as the effects of Cd on vitellogenin (Vtg) synthesis induced by E2, were investigated. Cd mainly accumulated in the kidneys, liver, intestines, and gills, with little amounts in muscles, bones, and gonads. Co-exposure with E2 did not change the main targets for Cd. E2 could induce Vtg synthesis in male Chinese loaches, and co-exposure with 50 or 500  $\mu$ g/L Cd could inhibit the Vtg induced by 1  $\mu$ g/L E2. Compared with the results reported in the literature, it can be concluded that factors such as fish species, Cd dosage, and manner of exposure might make contributions to the interaction between Cd and E2. Our results also suggested that male Chinese loaches are susceptible to Cd, and can be recommended as a potential sentinel species to study the ecotoxicology of heavy metals.

**accumulation, cadmium, Chinese loach, combination effects, 17 $\beta$ -estradiol, vitellogenin**

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As one of the most toxic heavy metals, cadmium (Cd) is widely distributed in the environment due to natural and anthropogenic activities. The concentration of Cd in the water system ranges from 1 to 400  $\mu$ g/L in contaminated sites [1,2]. Cd has the potential to accumulate selectively within organisms [3–5]. The excretion rate of Cd is very low, with a biological half-life of 10–30 years in the kidney of humans [6,7]. Cd is also well-known as an endocrine-disrupting chemical (EDC). Exposure of different types of organisms to Cd can cause reproductive and developmental disorders [1,2,8–11]. In addition, Cd can mimic the effects of estradiol in an estrogen-responsive breast cancer cell line (MCF-7) and lead to activation of estrogen receptors [7,12,13].

Some studies demonstrated that bioaccumulation of Cd

could be affected by 17 $\beta$ -estradiol (E2). For example, Valencia et al. [14] showed that Cd accumulation in tissues was redirected from bone and liver to the gills, gut, and muscle upon exposure to E2 in the rainbow trout. Combined treatment with E2 and Cd could cause inhibition of the transcription and translation of metallothionein (MT) mRNA in the liver [15]. In contrast, E2 could induce a higher and fast uptake of Cd in the liver, together with a major induction of MT synthesis in the liver and kidney of rats [16].

Abnormal levels of vitellogenin (Vtg) in male or juvenile fish have been widely used as a biomarker to demonstrate the effects of estrogens or estrogen mimics in the aquatic environment [17,18]. Olsson et al. [15] found that injection of E2 and Cd in combination could inhibit the induction of Vtg in the liver of the rainbow trout, which might be attributed to the preferential binding of Cd to non-MT proteins in the liver of E2-treated rainbow trout. Hwang et al.

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[19] demonstrated that  $10^{-9}$  to  $10^{-7}$  mol/L Cd had no effect on Vtg production in hepatocytes of the rainbow trout, whereas  $10^{-6}$  mol/L Cd could markedly inhibit Vtg synthesis. One possible reason for this phenomenon was that the concentration of Cd exceeded the ability of hepatocytes to synthesize MTs. Cd is also known to accumulate in the liver and disrupt its normal function. This might result in a decrease in the clearance of corticosteroids from the blood, and then increase the circulating concentration of corticosteroids [1], and finally lead to the enhancement of Vtg levels. Sharma and Patiño [11] investigated the effects of Cd, E2, and their interaction on gonadal condition and metamorphosis of male and female specimens of the African clawed frog, *Xenopus laevis*. Their results showed that Cd (10 µg/L), E2 (1 µg/L) and their combination delayed metamorphosis in both sexes but more significantly in males. Lee et al. [20] investigated the estrogenic effects of Cd alone and in combination with E2 on the hepatocytes of the male Bullfrog (*Rana catesbeiana*). They found that Vtg levels induced by E2 (100 nmol/L) and CdCl<sub>2</sub> (100 nmol/L) in combination were significantly higher than those induced by E2 (100 nmol/L) alone.

The results of the above-mentioned studies suggest that complex modes of action between Cd and E2 might exist. Various factors, including fish species, dosages of E2 and Cd, as well as manner and time of exposure, might make contributions. The Chinese loach (*Misgurnus anguillicaudatus*) is a freshwater fish found throughout Asia that lives in the bottom of water, and sometimes in the sediment [21]. Therefore, Chinese loaches are exposed to EDCs more frequently than many other fish species. Studies have demonstrated that the Chinese loach could be a potential sentinel species to study the estrogenic effects of EDCs [22].

In the present study, the male Chinese loach was chosen as the experimental fish to study the effect of E2 on the accumulation and distribution of Cd. The effect of Cd on Vtg synthesis induced by E2 was investigated using a semi-static waterborne exposure system.

## 1 Materials and methods

### 1.1 Apparatus

A Hitachi Z-5700 atomic absorption spectrometer (AAS) (Hitachi High-Technologies Corporation, Japan) equipped with Zeeman background correction and a Cd hollow cathode lamp as the radiation source was used for the determination of Cd in water and tissues.

### 1.2 Reagents

A stock solution of E2 (98% purity, Sigma-Aldrich, USA) was dissolved in methanol. A stock solution of Cd was prepared with CdCl<sub>2</sub>·2.5H<sub>2</sub>O (Tianjin Chemical Reagent Company, China) and ultrapure water (EASY pure LF). Working

solutions of E2 and Cd were diluted from stock solutions with ultrapure water. All solutions were sealed and stored at 4°C.

### 1.3 Fish

Mature, healthy male Chinese loaches (mean weight,  $10.29 \pm 1.50$  g; mean length,  $14.20 \pm 0.76$  cm) were used. Prior to exposure, loaches were acclimatized for  $\geq 2$  weeks in stainless-steel tanks provided with dechlorinated tap water. Loaches were maintained under the natural length of the day. Throughout the experiments, water quality was monitored by detecting pH (6.9–7.9), dissolved oxygen (5–7 mg/L), and temperature (22.5–25.5°C).

### 1.4 Experimental design

At the beginning of experiments, 12 fish were randomly selected to 12-L glass tanks with working volumes of 5 L. Several separate experiments were conducted at the simultaneously.

In the first experiment, male Chinese loaches were exposed to 1 µg/L E2 for 42 d to investigate the vitellogenic effect of E2.

In the second experiment, male Chinese loaches were exposed to gradient doses of Cd at nominal concentrations of 50 and 500 µg/L for 42 d to investigate the accumulation and distribution of Cd.

In the third experiment, male Chinese loaches were exposed to E2 and Cd simultaneously at nominal concentrations of 1 + 50 µg/L and 1 + 500 µg/L for 42 d to investigate the effect of Cd on Vtg production induced by E2, and the effect of E2 on the distribution of Cd.

In the fourth experiment, male Chinese loaches were exposed to 50 and 500 µg/L Cd for 21 d followed by exposure to 1 µg/L E2 for another 21 d to investigate the pretreatment of Cd on the synthesis of Vtg induced by E2 and the elimination of Cd from the tissues.

The solvent-control and the control tanks received 0.001% methanol and dechlorinated tap water, respectively.

All concentrations were tested in at least two aquaria. Throughout the experimental period, water was changed daily, and the fish were fed every other day.

### 1.5 Collection of plasma and tissues

Fish were randomly sampled on day 7, 14, 21, 28, 35, and 42 of the entire exposure. Before sampling, fish were anesthetized with quinoline sulfate (40 mg/L). Blood samples were then taken from the caudal vessels using heparinized syringes. These samples were transferred to 1.5 mL centrifuge tubes in the presence of aprotinin (2.5 TIU, Roche, Germany). After centrifugation (3000 r/min, 4°C, 30 min), plasma samples were divided into aliquots and stored at -20°C for Vtg analysis. The liver, kidneys, intestines, gills,

muscles, bones, and gonads were dissected on day 42, weighed and stored at  $-20^{\circ}\text{C}$  for Cd analyses.

### 1.6 Determination of Cd concentrations in exposed water and tissues

Before the measurement of Cd concentrations in tissues, the latter were digested with nitric acid and hydrogen peroxide according to the method described by Liang et al. [23] with minor modifications. Briefly, 2 mL of concentrated nitric acid was added to the weighed tissues in a polytetrafluoroethylene (PTFE) digestion container. Tissues were left to pre-digest overnight at room temperature. Addition of 1 mL of 30% hydrogen peroxide followed. Thereafter, the containers were placed in stainless-steel bombs, which were sealed with a screw closure, and then placed in an oven. The temperature of the oven was kept at  $180^{\circ}\text{C}$  for 6 h. After cooling, the PTFE digestion containers were taken out, and the solutions within them heated to let nitric acid evaporate. The residues were transferred to a 15 mL polyethylene terephthalate (PET) bottle with 2% nitric acid for analyses using AAS. Aquatic Cd concentrations in various exposure tanks were directly monitored without pretreatment using AAS.

### 1.7 Determination of the level of Vtg in plasma

Plasma Vtg concentrations were determined with a competitive enzyme-linked immunosorbent assay (ELISA) as described previously [21] with some modifications. Briefly, a 96-well plate was coated with 250 ng/mL purified Vtg overnight. This plate was blocked with 1% bovine serum albumin (BSA). Samples and standard Vtg solutions incubated with the primary antiserum (final dilution, 1:64000) overnight at  $4^{\circ}\text{C}$  were transferred to the coated plate. The plate was incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (final dilution, 1:64000; Jackson, USA). The enzyme substrate solution (0.05 mol/L phosphate citrate buffer, 0.4 mg/mL *o*-phenylenediamine, and 0.16%  $\text{H}_2\text{O}_2$ ) was added; after 30 min, the reaction was stopped by the addition of 2 mol/L HCl. The absorbance was measured at 490 nm using a Microtiter Plate Reader (DNA Expert; TECAN, Austria). ELISA data were processed using a four-parameter Boltzmann equation:

$$y = a + \frac{b}{1 + \exp\left(\frac{x-c}{d}\right)}, \quad (1)$$

where  $y$  represents the percentage binding of sample or standard relative to analyte-free wells ( $\text{Bi}/\text{B}_0$ ) and  $x$  represents the log dose.

### 1.8 Statistical analysis

Values are mean  $\pm$  S.D. Significant differences were described

using one-way analysis of variance (ANOVA).  $P < 0.05$  was considered significant.

## 2 Results and discussion

### 2.1 Nominal and actual concentrations of E2 and Cd in exposed water

E2 concentrations in the control and solvent control groups could not be detected. Actual E2 concentrations in the  $1 \mu\text{g/L}$  E2-exposed group ranged from 1.06 to  $0.70 \mu\text{g/L}$  within 24 h (mean,  $0.93 \pm 0.13 \mu\text{g/L}$ ).

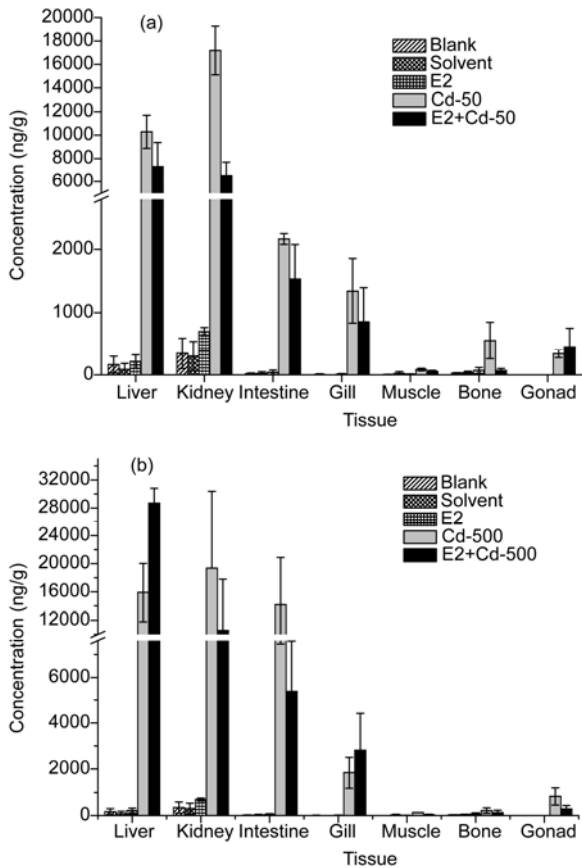
Cd concentrations in the control, solvent control, and E2-exposed groups were below the limit of detection of AAS. Actual Cd concentrations in the  $50 \mu\text{g/L}$  Cd- and  $500 \mu\text{g/L}$  Cd-exposed groups were  $40.55 \pm 4.03$  and  $484.50 \pm 54.45 \mu\text{g/L}$ , respectively, which were maintained at the levels of 81% and 97% of the nominal concentrations, respectively.

All the results confirmed that the semi-static systems could provide relatively stable exposure levels of E2 and Cd. This permitted convenient evaluation of the effects of Cd in male Chinese loaches using nominal values.

### 2.2 Accumulation and distribution of Cd in tissue

Cd concentrations in the liver, kidneys, intestines, gills, muscles, bones, and gonads of male Chinese loaches after 42-day exposure to different concentrations of Cd were measured (Figure 1). Cd could accumulate in the tissues of male loaches, and Cd contents in the exposed groups were higher than those in the control groups; moreover, Cd accumulation was dose-dependent. Cd concentrations in the liver, kidneys, intestines, and gills were higher than those in muscles, bones, and gonads. This indicated that the liver, kidneys, intestines, and gills were the main targets for Cd accumulation in the Chinese loach. Studies carried out with other experimental animals have shown that Cd is preferentially accumulated in the kidneys, liver, intestines, and gills [4,24–26]. One route of Cd incorporation into the biological systems of fish was movement into the gills. Moreover, the liver, kidneys, and intestinal tissues play an important part in the detoxification of the heavy metal Cd.

Some studies demonstrated that the bioaccumulation of Cd could be affected by E2. For example, after exposure of the rainbow trout to E2 and Cd in combination, Cd was re-directed from bones and the liver to the gills, gut, and muscles [14]. However, our results indicated that co-treatment with E2 did not change the main targets for Cd accumulation in male Chinese loaches: the liver, kidneys, intestines and gills were still the main targets. In addition, compared with single exposure to Cd, co-exposure with E2 could decrease the accumulation of Cd in tissues except for the liver and intestines in the exposed group of  $1 \mu\text{g/L}$  E2 +  $500 \mu\text{g/L}$  Cd. Some discrepancies in the Cd concentrations between single Cd and E2+Cd exposed groups were significantly



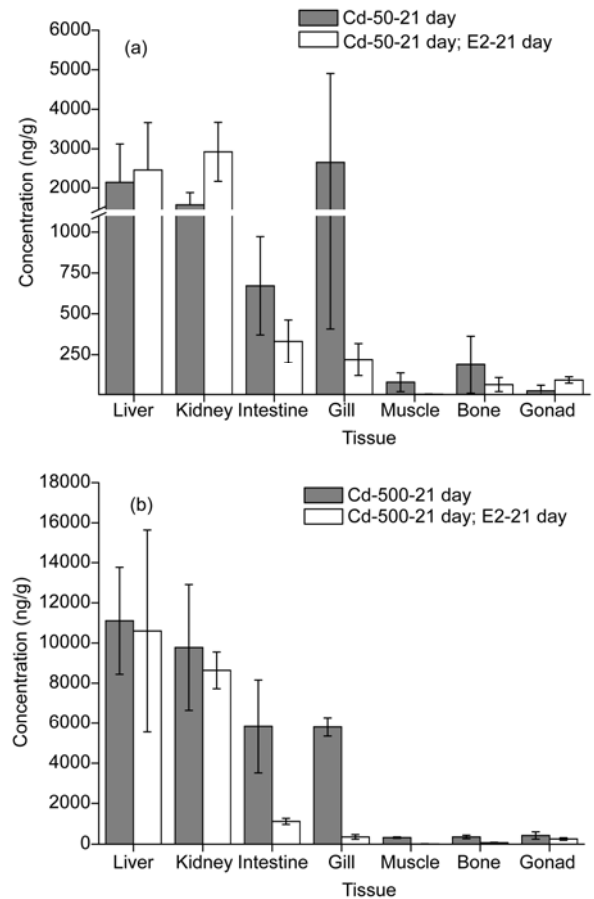
**Figure 1** Cd concentrations in liver, kidney, intestine, gill, muscle, bone, and gonad tissues in male Chinese loaches after exposure to 50 µg/L Cd and 1 µg/L E2 + 50 µg/L Cd (a), 500 µg/L Cd and 1 µg/L E2 + 500 µg/L Cd (b). The tissues were taken on day 42. Values are means ± S.D.

different. For example, Cd concentration in the kidney exposed to 1 µg/L E2 + 50 µg/L Cd was significantly lower than that in the 50 µg/L Cd group ( $P < 0.05$ ).

### 2.3 Cd elimination from tissues

After exposure to 50 and 500 µg/L Cd for 21 d, male Chinese loaches were exposed to 1 µg/L E2 for another 21 d, and the changes in Cd concentrations accumulated in tissues were detected (Figure 2). After 21-d exposure to E2, the concentrations of Cd in the intestine, gill, muscle, and bone tissues decreased. However, the concentrations of Cd in liver, kidney, and gonad tissues were maintained at levels equal to or even higher than those exposed to 50 µg/L and 500 Cd for 21 d.

Our results might suggest that, during the depuration period, the fast decrease in Cd concentrations in the gills and intestines might be caused by surface adsorption, or Cd accumulated in the intestines, gills, muscles, and bones might be transferred to the liver and kidneys; the slow elimination might be caused by the high affinity of Cd bound to ligands in the liver and kidneys [4,27]. Similar results on the elimination of Cd from tissues have been obtained in other



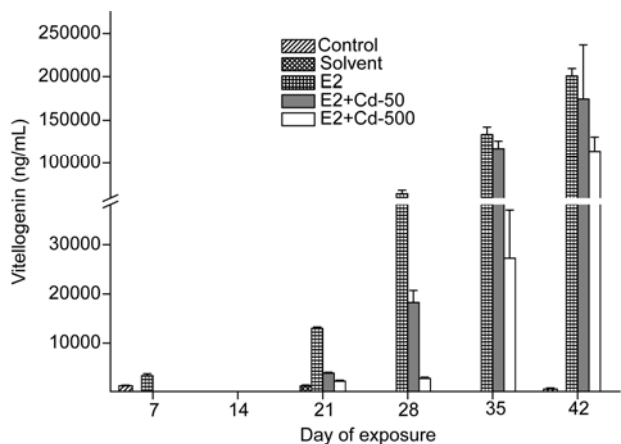
**Figure 2** Elimination of Cd from tissues after exposure to 50 µg/L (a) and 500 µg/L (b) Cd followed by exposure to 1 µg/L E2. Values are means ± S.D.

animals. For example, Cd concentrations in the kidneys and liver of the rainbow trout and whitefish remained constant after a depuration time [28], and no loss of Cd was found in the kidneys and liver of carp during a 42-d depuration period [27]. However, different elimination mechanisms of Cd might exist in different species. For example, the accumulated Cd in the intestine, liver, and gill tissues of rockfish decreased during 30-d depuration, but not in kidneys and muscles [4].

### 2.4 Determination of Vtg concentrations in plasma

Vtg concentrations in the control and solvent-control groups ranged from not detected (N.D.) to  $1.41 \pm 0.13$  µg/mL. No Vtg could be obviously induced in male Chinese loaches exposed to gradient doses of Cd, and plasma Vtg contents ranged from N.D. to  $1.62 \pm 0.41$  µg/mL.

Vtg could be induced in male Chinese loaches by 1 µg/L E2 and, during a 42-d exposure period, Vtg contents ranged from N.D. to  $200.66 \pm 8.73$  µg/mL (on day 42) (Figure 3). Vtg induction by E2 showed a time-related increase. This confirmed the suitability of male Chinese loaches as an experiment model fish to study the estrogenic effects of EDCs,

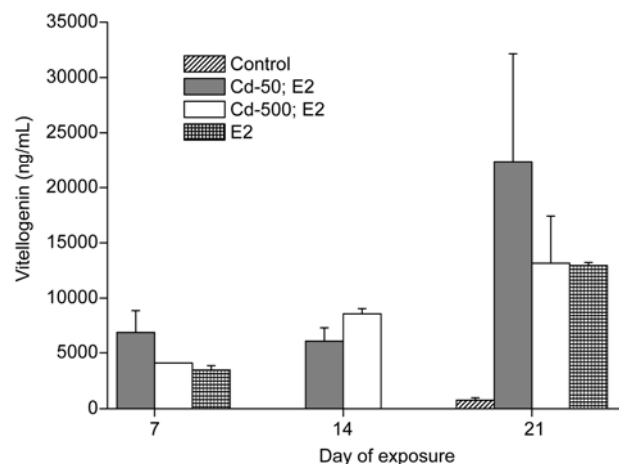


**Figure 3** Plasma Vtg concentrations in male Chinese loaches exposed to binary mixtures of E2 and Cd at the nominal concentrations of 1 + 50 µg/L and 1 + 500 µg/L for 42 d. Plasma samples were collected on days 7, 14, 21, 28, 35, and 42. Values are means ± S.D.

as proved in the previous study [22].

In the exposed groups of binary mixtures of E2 and Cd, Vtg levels increased from N.D. to  $174.61 \pm 62.22$  µg/mL (1 µg/L E2 + 50 µg/L Cd for 42 d), and Vtg concentrations also showed time-dependent features. It can be seen from Figure 3 that from 21 d onwards, Vtg was significantly induced in E2 + Cd exposed groups, and that Vtg contents in the co-exposure groups were relatively lower than those in the E2 group. Order of Vtg contents was: 1 µg/L E2 > 1 µg/L E2 + 50 µg/L Cd > 1 µg/L E2 + 500 µg/L Cd. These results might indicate that Cd can inhibit Vtg induced by E2, and that the inhibiting effect was dose-dependent. This phenomenon was in accordance with the results of Olsson et al. [15] and Hwang et al. [19], and their results indicated that Cd could significantly inhibit Vtg synthesis. The reason might be that Cd can bind to the estrogen receptor in the liver of male Chinese loaches, and that the interaction of Cd with the estrogen receptor inhibited E2 binding [7], finally leading to a decrease in Vtg level.

To confirm the inhibitory effect caused by Cd, male Chinese loaches were pretreated with 50 and 500 µg/L Cd for 21 d, and then exposed to 1 µg/L E2 for another 21 d. However, different from the results of co-exposure, plasma Vtg contents in Cd-pretreated male Chinese loaches were equal to or a little higher than those exposed to E2 for the same time (Figure 4). For example, after exposure to E2 for 21 d, Vtg contents in 50 µg/L Cd- and 500 µg/L Cd- pretreated groups were  $22.37 \pm 9.78$  and  $13.16 \pm 4.26$  µg/mL, respectively, whereas male Chinese loaches in the E2 group for 21 days possessed a mean Vtg level of  $12.95 \pm 0.28$  µg/mL. The phenomenon might show that pretreatment with Cd did not affect the binding of E2 to the estrogen receptor of male Chinese loaches. Our results were not consistent with those of Le Guével et al. [29], it was proved in their study that only E2-activated estrogen receptors might



**Figure 4** Vtg contents in male Chinese loaches pretreated with 50 µg/L and 500 µg/L Cd followed by 1 µg/L E2. Sampling points were counted from the addition of E2. Values are means ± S.D.

be affected by Cd in the rainbow trout. The reasons for the discrepancy should be studied further.

### 3 Conclusions

Effects of Cd, E2 and their interaction in male Chinese loaches were investigated. The results indicated that the kidneys, liver, intestines, and gills were the main targets for Cd accumulation. Addition of 1 µg/L E2 did not change the main target for Cd accumulation; 50 and 500 µg/L Cd could inhibit the Vtg synthesis induced by 1 µg/L E2. The results might suggest that complex modes of action might exist between Cd and E2 in male Chinese loaches. Various factors that might make a contribution (e.g., fish species, Cd dosage, manner of exposure) need further study for a more comprehensive explanation.

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