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Dietary Cadmium and Benzo(a)pyrene Increased Intestinal Metallothionein Expression in the Fish *Fundulus heteroclitus*

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Expression in the Fish *Fundulus heteroclitus*

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Abstract

Fish were individually fed food pellets containing cadmium, benzo(a)pyrene, or a combination of the two, then analyzed for metallothionein mRNA expression in the intestine, liver, and gill using real-time RT-qPCR. An initial experiment using only cadmium showed that ingestion of pellets varied in individual fish, and estimates of cadmium dose from the numbers of ingested pellets indicated considerable individual variability in cadmium dose. Induction of intestinal metallothionein mRNA was apparent, however, and a linear dose-response relationship was observed for metallothionein expression and cadmium dose in the intestine, but not the other organs, which showed no induction. In a second experiment, the entire daily cadmium dose was provided in a single contaminated pellet that was consumed by all treated fish, effectively eliminating the effect of variable ingestion rates on dose, and the interaction between cadmium and benzo(a)pyrene was also investigated. The intestine was again the primary organ for metallothionein induction by cadmium. When benzo(a)pyrene was administered together with cadmium, induction of metallothionein was potentiated by the presence of benzo(a)pyrene, with the main effect seen in the intestine, where already high levels of induction by cadmium alone increased by 1.74-fold when benzo(a)pyrene was present.

Keywords: Metallothionein; Metals; Hydrocarbons; Metal-organic interactions; mRNA; Fish

1. Introduction

Organs at the environmental-organism interface, which are involved in uptake processes, are important in the absorption of metals from the environment and especially useful in analyzing events associated with dietary exposure (Harrison et al., 1989; Thomann et al., 1997). In higher animals, dietary cadmium absorption is characterized by high levels of cadmium in the intestinal mucosa and its slow transfer to other internal organs (Elsenhans et al., 1997). About 60% of cadmium in the mucosa of the small intestine is bound to the metal-binding protein metallothionein shortly after dietary exposure (Lehman et al., 1986). Metallothionein is involved in intestinal cadmium absorption through its induction and binding of cadmium. Subsequent interorgan cadmium transport out of the intestine is, in part, associated with serosal release as cadmium-metallothionein complexes (Zalups et al., 2003). For dietary exposure to cadmium in fish such as salmon and rainbow trout, the intestine is also the site of absorption and the portal for redistribution via the systemic circulation, resulting in both cadmium accumulation and toxicity in other organs (Baldisserotto et al., 2005; Baldisserotto et al., 2006; Berntssen et al., 2001a; Berntssen et al., 2001b; Berntssen et al., 2000; Berntssen et al., 2003; Franklin et al., 2005; Lundebye et al., 1999; Ng et al., 2008; Thomann et al., 1997). Although the proportion of cadmium that crosses the gut for transport other organs is relatively low (Franklin et al., 2005; Ng et al., 2008), the dietary exposure route is considered significant in the movement of cadmium through food chains (Dallinger et al., 1987; Harrison et al., 1989; Wallace et al., 2003). While it is known that metallothionein is induced in fish intestines in response to dietary cadmium (Berntssen et al., 2001a; Chowdhury et al., 2005) and that, as noted above, the intestine is the site of absorption of dietary cadmium, there is still little information on the response of fish intestinal metallothionein to dietary cadmium exposure. It is reported that intestinal

metallothionein is induced in response to water-borne cadmium in the killifish *Fundulus heteroclitus*, a ubiquitous inhabitant of estuarine environments along the North American Atlantic coastline (Van Cleef-Toedt et al., 2001). Yet similar induction of killifish intestinal metallothionein in response to dietary cadmium has yet to be reported. Moreover, because exposures in the natural environment are likely to be to mixtures of substances that include hydrocarbons as well as metals (Rana, 2006), the potential modulation of cadmium-induced metallothionein induction by polycyclic aromatic hydrocarbons such as benzo(a)pyrene, an environmental mutagen, is also of interest. Our objective was to determine the effect of dietary exposure to cadmium and co-exposure to benzo(a)pyrene on metallothionein induction in *F. heteroclitus*, focusing on expression of the metallothionein mRNA in the intestine, liver, and gill.

2. Materials and methods

2.1. Fish

Individuals of *Fundulus heteroclitus* were collected from reference sites in Chesapeake Bay (e.g., Van Veld et al., 1997; Vogelbein et al., 1990) for the experiments described below. They were maintained in aquaria containing 120 L recirculating synthetic seawater (Instant Ocean, Aquarium Systems, Inc., in charcoal-filtered, deionized water) at 20 psu and either 20 or 25° C, depending on the ambient temperature at the time of collection. Aquaria were outfitted with Skilter Power Filters (Aquatic Ecosystems, Inc., Apopka, FL). Fish were initially fed a commercial flake food (Aquatox Food, Aquatic Ecosystems, Inc., Apopka, FL), supplemented by pellets prepared from Gel Fish Food (Aquatic Ecosystems, Inc., Apopka, FL) (see below for

food preparation). Fish were trained to eat food pellets described below during the maintenance period.

2.2. Preparation of food pellets

Gel Fish Food powder (45 g) was added to heated water (48 ml) according to the manufacturer's instructions. Pellets made from this unamended mixture were fed to the fish during the maintenance phase in aquaria. Control pellets prepared for the exposures to cadmium or benzo(a)pyrene received appropriate volumes of the carrier solvents, added to the Gel Fish Powder slurry before allowing the mixture to gel. The resultant slurry was poured between two glass electrophoresis plates separated by 1.5 mm spacers and allowed to gel at 4°C. Once solidified, 3 mm diameter discs weighing approximately 8.5 to 10 mg wet weight were cut from the gel sheets using punches from an immunodiffusion punch set (Gelman Sciences Inc [now Pall Life Sciences, Inc.]), Ann Arbor, MI).

To make cadmium-contaminated food for the first, preliminary, experiment, 72 µl of a 5 M stock solution of CdCl₂ in 0.01 N nitric acid was added during preparation of the Gel Fish Food. A comparable volume of 0.01 N nitric acid, which did not measurably alter pH, was added to the control food. The cadmium content was 5.7 µg cadmium per pellet, equivalent to 1.2 µg cadmium per mg dry weight. Cadmium-exposed fish were fed only the cadmium-laden pellets. Control pellets contained the dilute HNO₃ solvent carrier only, and averaged 5.9×10^{-4} µg cadmium per pellet, equivalent to 1×10^{-4} µg cadmium per mg dry pellet weight.

For the second follow-on experiment, the preparation of contaminated pellets was modified to minimize the effect of individual variability in pellet consumption on dose observed in the first experiment (see below). For this experiment, a single pellet contained the entire daily dose of cadmium, benzo(a)pyrene, or cadmium plus benzo(a)pyrene. To make the cadmium-laden pellets, 720 μ l of a 5 M CdCl_2 in 0.01 N HNO_3 plus 0.9 ml cod liver oil was added to the Gel Fish Food slurry to provide 36.1 μ g cadmium per pellet, equivalent to 9.0 μ g cadmium per mg dry weight pellet. To make benzo(a)pyrene laden pellets, 0.9 ml of 10.35 mg per ml benzo(a)pyrene in cod liver oil plus 720 μ l of 0.01N HNO_3 was added to the Gel Fish Food slurry to provide a dose of 0.83 μ g benzo(a)pyrene per pellet at a concentration of 0.21 μ g benzo(a)pyrene per mg dry pellet weight. The combined cadmium-benzo(a)pyrene laden pellets contained similar amounts of cadmium and benzo(a)pyrene. All exposure pellets contained the same carrier solvent background of 0.01 N HNO_3 plus cod liver oil. Control pellets contained similar volumes of the carrier solvents 0.01 N HNO_3 and cod liver oil, but no cadmium or benzo(a)pyrene.

2.3. Experiments

The fish used in the two experiments were obtained in different collections separated by time and seasons (September versus January). For experiments, fish (mean weight 8.3 g in the first experiment and 7.9 g in the second experiment) were transferred individually from maintenance tanks to separate glass containers (one fish per 17 cm height, 15 cm diameter container) containing 1.5 liters Instant Ocean at 20 psu and 25° C in the first experiment and 20° C in the second experiment. Aeration was supplied to each container by slow bubbling through a Pasteur pipette. Water was replaced every other day. Fish were maintained on a light:dark cycle of 12 h

light:12 h dark. Fish were acclimated to the experimental containers for three days prior to the exposure to dietary cadmium. During this period, fish were fed control pellets administered one-at-a-time by hand. The acclimation period was followed by dietary exposure during a seven-day experimental period.

In the first experiment, fish were fed either cadmium-laden or control pellets by hand once a day to either satiation or up to a limit of 15 pellets per fish over the course of the seven day experiment. Fish were not fed on the seventh day, when they were sacrificed for harvest of organs. Cadmium-exposed fish were fed only cadmium-laden pellets, and control fish were fed only control pellets. Each ingested pellet was swallowed whole, immediately after administration. Uneaten pellets were immediately removed to minimize their contact time with the water and leaching of contaminants to the external medium. None of the fish consumed the maximum number of pellets allotted for this experiment, and there was individual variability in the total number of pellets consumed. The average daily consumption was about nine pellets per day. The associated individual variability in the cumulative cadmium dose enabled us to analyze the responses of individuals as a function of dose.

In order to better control the dose in the second experiment, the dosing protocol was modified to insure that each fish received the same total amount of cadmium and benzo(a)pyrene over the course of the experiment. To do this, fish in the second experiment were provided a single experimental pellet containing the daily dose of cadmium, benzo(a)pyrene, cadmium plus benzo(a)pyrene combination, or control treatments, followed by the remainder of the daily ration of up to nine control pellets each day for seven days, roughly similar to the average daily consumption of nine pellets per day in the first experiment. Fish were sacrificed on the eighth

day. All fish in this experiment consumed the daily dose of treatment pellets and, with a few exceptions, also consumed the total control pellet ration provided immediately after the treatment pellet was eaten.

At the end of exposures, the fish were immobilized using a combination of concentrated sodium bicarbonate (Noga, 2000) in an ice-cold Instant Ocean slurry. The intestine, liver, and gill were excised, weighed, and frozen in liquid nitrogen. Samples were stored at -80°C until analyzed for metallothionein mRNA levels.

2.4. RNA isolation

Total RNA for the first experiment was extracted from frozen tissue using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH) per manufacturer's instructions, of which 10 μg was treated with DNase using the Promega RQ1 DNase 1 kit and protocols (Fitchburg, WI). Immediately following DNase treatment, the RNA was purified using the Qiagen RNeasy kit (Qiagen, Valencia, CA) following manufacturer's instructions. RNA was eluted in 30 μl of DEPC-treated H_2O . Subsequent RNA extractions used the RNeasy MiniKit (Qiagen, Valencia, CA), followed by treatment with DNase (RNase-Free DNase kit, Qiagen, Valencia, CA).

2.5. Identification of metallothionein and 18S RNA sequences in *Fundulus heteroclitus*

Metallothionein and 18S RNA sequences were amplified by reverse transcriptase polymerase chain reaction (RT-PCR) using consensus fish metallothionein primers, gel purified by agarose gel electrophoresis, then sent to the University of Florida's Interdisciplinary Center for

Biotechnology Research (UFICBR) for sequence verification. This laboratory used ABI Prism BigDye Terminator cycle sequencing protocols (part number 4336776) developed by Applied Biosystems (Perkin-Elmer Corp., Foster City, CA). Sequences for forward and reverse primers were provided to UFICBR for sequencing the sense and antisense strands. The amplified sequences served as templates for design of real-time PCR primers (Invitrogen's Lux™ Designer software).

2.6. Real-time qPCR

Real-time PCR reactions used cDNA libraries as template for amplification of metallothionein and 18S RNA sequences. For construction of cDNA libraries, 1 µg DNase-treated total RNA was hybridized with 500 ng of random hexamers at 70°C for 5 minutes. For reverse transcription, one µl of this mixture and a cocktail containing 4 µl of 5X buffer, 2 µl of 10 mM stock of dNTPs, 0.5 µl of 40U per µl stock of RNase inhibitor, and 1µl of 200 U per µl stock of M-MLV RT enzyme was incubated at 37°C for 60 minutes, 95°C for 5 minutes, then placed on ice for 5 minutes.

Real-time PCR reactions contained 23.8 µl of autoclaved Nanopure water (Barnstead), 25 µl Platinum Quantitative PCR Supermix-UDG (Invitrogen, Carlsbad, Ca), 0.1 µl appropriate Lux™ Fluorogenic Primers ((Invitrogen, Carlsbad, Ca; stock concentration 100 µM), and 1 µl of the cDNA library. Temperature programming followed the manufacturer's recommendations for Lux™ Fluorogenic Primers and Platinum Quantitative PCR Supermix-UDG: 50°C for 2 min; 95°C for 2 min; and 45 cycles of 95°C, 15 sec; 55°C, 30 sec; and 72°C, 30 sec. Reactions were

conducted in an MJResearch (now Bio-Rad Laboratories) DNA Engine Opticon 2. Each sample was analyzed in replicates of two using the LuxTM Fluorogenic Primers derived from sequences confirmed as those for *Fundulus heteroclitus* metallothionein and 18S RNA. The threshold cycle (C_t) for each amplification was used to calculate the $2^{-\Delta\Delta C_t}$ for relative expression of metallothionein mRNA (Livak et al., 2001). $\Delta\Delta C_t$ is the C_t of metallothionein mRNA target minus the C_t of the endogenous 18S RNA and the C_t of the 18S RNA of the plate calibrator sample that was added to every plate. To validate the assumption of similar amplification efficiencies for the target and reference specified by the $2^{-\Delta\Delta C_t}$ method (Livak et al., 2001), dilutions were amplified for the genes of interest. The slopes for plots of C_t 's versus log dilution for both metallothionein and the 18S mRNA were found not to be significantly different from zero in all cases.

2.7. Statistical analysis

The data were analyzed by analysis of variance with *post hoc* analysis using the Tukey HSD *post hoc* test, using the JMP software packageTM (SAS Institute, Cary, NC). Linear regression was conducted with the JMP software packageTM and Kaleidagraph (Synergy Software; Reading, PA).

3. Results

Initial efforts confirmed the identities of target sequences as those for metallothionein and 18S RNA. Amplified sequences for intestinal, liver, and gill metallothionein exhibited 95% or greater identity with *Fundulus heteroclitus* metallothionein (NCBI accession numbers: CN968347 and AB426465). That for the putative 18S RNA aligned with 98% identity with the

F. heteroclitus 18S RNA sequence (NCBI accession number: M91180). These findings confirmed the identity of the metallothionein and 18S RNA sequences for our experimental population of *F. heteroclitus*, and these amplified sequences served as templates for design of LuxTM Fluorogenic Primers for real-time PCR.

Representative real-time amplification profiles and C_t 's for intestinal, liver, and gill metallothionein from the first experiment are shown in Figure 1. The $2^{-\Delta\Delta C_t}$ values were calculated from the C_t 's as previously described. In controls of both experiments, respective levels in the gill, liver, and intestine were low and roughly equivalent, with those of gills consistently lower than in the liver and intestine (Tables 1 and 2).

In the first experiment, significant induction of metallothionein by cadmium was seen only in the intestine, which exhibited a 10.7-fold average increase in metallothionein mRNA in cadmium-exposed fish in comparison with controls; metallothionein was not induced in liver and gill (Table 1). The central tendencies represented by the means of Table 1 masked the effect of individual variability in food consumption noted in the Materials and Methods section and the resultant individual variability in cadmium dose over the course of exposure [average cumulative dose of cadmium-exposed fish over six days: 45.9 ± 7.2 (S.E.) μg cadmium/g fish]. There was a dose-dependent linearity between cadmium dose and induction of intestinal metallothionein when all samples, including controls and those exposed to cadmium, were plotted (Fig. 2). In comparison, there was no measurable effect on metallothionein mRNA levels in liver and gills as the cumulative cadmium dose increased.

In the second experiment, the individual variability in dose noted previously was controlled by loading the entire daily cadmium dose for each fish into a single pellet. All fish ate the daily exposure pellet, and, unlike the situation in the first experiment, all cadmium- and benzo(a)pyrene-exposed fish received the same cumulative dose (32.0 μg cadmium per g fish and/or 0.74 μg benzo(a)pyrene per g fish) over the course of the seven day exposure period. The cumulative cadmium dose per g fish in this experiment was not significantly different from those of the first experiment (Student's t-test; $p>0.05$). Induction in the intestine by cadmium alone resulted in a 20-fold increase over control levels (Table 2), whereas metallothionein mRNA levels in the gill and liver of cadmium-exposed fish were not significantly different from those of controls (Table 2). This pattern of organ-specific induction of metallothionein by dietary cadmium was similar to that described above for the first experiment, although the magnitude of induction in the intestine was lower in the first experiment. Differences in collection history and seasons are possible contributing factors. Benzo(a)pyrene alone did not induce metallothionein in the second experiment, although it appears that benzo(a)pyrene potentiated induction of intestinal metallothionein mRNA by cadmium, causing an approximate 1.74-fold increase over that by cadmium alone.

4.0. Discussion

Our findings showed that intestinal metallothionein was induced to relatively high levels in the intestine and that intestinal metallothionein induction was dependent on the ingested cadmium dose. Metallothionein in liver and gill, on the other hand, was not significantly induced by dietary cadmium. In feeding studies such as this, individual variability in ingestion can affect both the dose to individuals and the dose-response relationship, revealed by linear regression

analysis of intestinal metallothionein mRNA levels versus cadmium dose in the current study. It is not uncommon in studies on dietary exposure of fish to toxic substances to maintain multiple individuals in single containers and to feed fish by broadcast feeding of contaminated food to all individuals in a container. Although central tendencies in measured responses can be estimated in such cases, they can also be obscured by individual differences due to individual variability in ingestion of contaminated food. Closely monitoring and controlling ingestion of contaminated food by individual fish allowed us to minimize one component of the variance associated with ingested dose.

The lack of induction of hepatic and gill metallothionein in our study is believed to be due to slow transport of cadmium across the intestine to other organs and negligible waterborne cadmium exposure over the course of our experiments. This is consistent with studies on rainbow trout in which 93% or greater of the cadmium administered via a dietary path fails to cross the gut wall (Chowdhury et al., 2004a; Franklin et al., 2005; Ng et al., 2008). Induction of metallothionein in organs such as kidney, liver, and gill would be expected to occur following protracted exposure and export of cadmium from the intestine (Berntssen et al., 2001a; Chowdhury et al., 2005). Thus, after dietary cadmium exposure of one month and longer, cadmium accumulation in the liver, gill, and kidney of rainbow trout and Atlantic salmon is elevated, but generally remains lower than those detected in intestinal tissues (Berntssen et al., 2001a; Chowdhury et al., 2005; Szebedinszky et al., 2001). It was suggested from experiments on rainbow trout that the gut wall serves as a barrier that reduces cadmium transfer to internal tissues (Franklin et al., 2005). The low efficiency of gastrointestinal transfer of cadmium, whether associated with prey (0.9-6.4%) (Ng et al., 2008) or with cadmium-spiked commercial food (<0.1 %) (Franklin et al., 2005) is comparable to that reported for higher animals of 3-6%

(ATSDR, 1999). It is therefore not unexpected that the short-term cellular response in *Fundulus heteroclitus* would be heightened in the intestine, which is exposed directly during dietary exposures, in comparison with other organs that depend on interorgan transport from the intestine for cadmium exposure.

Induction of metallothionein, enhanced cadmium binding capacity in intestinal cells, and gradual transfer of cadmium-metallothionein complexes across the serosal membrane to other organs are proposed as mechanisms to protect against the rapid accumulation of cadmium by internal organs (Elsenhans et al., 1997). Our finding that metallothionein mRNA in the intestine is most responsive to dietary cadmium in comparison with other organs extends, to the gene expression level, observations that the intestine had the highest levels of the metallothionein protein during dietary exposure of fish to cadmium (Berntssen et al., 2001a; Chowdhury et al., 2005). Retention of dietary cadmium in rainbow trout is highest in the gut and lower in other organs (Chowdhury et al., 2004b; Franklin et al., 2005; Ng et al., 2008), a pattern consistent with our observations for metallothionein mRNA induction in *Fundulus heteroclitus*. The quantifiable relationship between intestinal metallothionein expression and exposure to dietary cadmium in *F. heteroclitus* may reflect a protective function for intestinal metallothionein. However, this induction may not be without its own contribution to the toxicity of cadmium, since cadmium transported out of the intestine as cadmium-metallothionein can be filtered and absorbed by the kidney, a target of cadmium toxicity (Zalups et al., 2003).

While the mechanism for potentiation of cadmium-induced metallothionein expression by benzo(a)pyrene is not known, our observations are consistent with an earlier report showing that hepatic metallothionein induction in *F. heteroclitus* is enhanced after intraperitoneal injection of

both cadmium and benzo(a)pyrene, in comparison with injection with cadmium alone (van den Hurk et al., 2000). In addition, the levels of both hepatic cadmium and metallothionein are also elevated in the Antarctic fish *Trematomus bernacchii* injected with a combination of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and cadmium, when compared with induction by cadmium alone (Regoli et al., 2005). Although the bioaccumulation pathway for benzo(a)pyrene and cadmium in our study differed from that of these two studies, i.e., dietary to intestine versus intraperitoneal injection to liver, the cellular mechanisms for regulating metallothionein gene expression may be similar. It is interesting to note that, in higher animals, the signal transduction pathways for benzo(a)pyrene and cadmium converge in the regulatory sequences in the metallothionein gene. The upstream stimulatory factor one (USF1) is involved in regulation of the metallothionein gene in response to cadmium (Andrews, 2000; Daniels et al., 2003; Datta et al., 1997), and there is overlap of the sequence for the xenobiotic response element with that of the USF1-binding site in the metallothionein I promoter (Takahashi et al., 1997). This suggests that the potentiation by benzo(a)pyrene may lie in interactions at the level of transcriptional regulation of the metallothionein gene.

At the broader level, our finding that benzo(a)pyrene potentiated intestinal metallothionein induction by cadmium is of particular interest due to the fact that environments contaminated by cadmium are often elevated in other substances such as polycyclic aromatic hydrocarbons. Based on our findings, fish in such environments could display levels of metallothionein expression that are higher than would be expected from cadmium exposure alone. However, the toxicological significance of the increased induction is not clear-cut. For example, increased intestinal metallothionein levels may further protect the intestine against cadmium toxicity and facilitate retention of cadmium in the intestine. On the other hand, increased induction could also enhance

metallothionein's role as the vehicle for the interorgan transport of cadmium from the intestine as the cadmium-metallothionein complex. How potentiation of metallothionein expression by polycyclic aromatic hydrocarbons such as benzo(a)pyrene affects the toxicodynamics and toxicity of cadmium and, perhaps, other metals remains to be determined.

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List of Figures:

Figure 1. Representative real-time RT-PCR amplification profiles for intestinal, liver, and gill metallothionein mRNAs in *Fundulus heteroclitus* exposed to dietary cadmium. The arrows point to representative threshold cycles (C_t 's) of each tissue group on the X-axis.

Figure 2. Dose-response relationship of ingested cadmium and levels of metallothionein mRNA in *Fundulus heteroclitus* exposed to dietary cadmium. Levels of metallothionein mRNA were determined by real-time qPCR and expressed as $2^{-\Delta\Delta C_t}$ (Livak et al., 2001). R^2 and significance of regression coefficients: $R^2 = 0.640$, $p < 0.006$ for intestine; $R^2 = 0.001$, $p < 0.98$ for liver; and $R^2 = 0.011$, $p < 0.79$ for gill.

Table 1. Comparison of metallothionein mRNA levels in intestine, liver, and gill tissues in control fish and fish exposed to dietary cadmium in the first experiment. Samples were analyzed by real-time PCR, and values are expressed as $2^{-\Delta\Delta C_t}$ (mean $\pm$ 1 S.E., n=3 for controls and n=7 or 8 for cadmium treated).

Treatment*	Organ*		
	Gill	Liver	Intestine
Control	0.108 $\pm$ 0.051	0.923 $\pm$ 0.044	0.611 $\pm$ 0.243
Cadmium	0.176 $\pm$ 0.055	1.151 $\pm$ 0.314	6.510 $\pm$ 1.000**
Fold increase	1.6	1.2	10.7

*Analysis of variance showed that differences due to the Treatment, Organ, and the Organ x Treatment interaction were significant ($p < 0.002$). The Tukey HSD test showed that the Control treatment was significantly different from that of Cadmium. For the effect of Organ, Intestine was significantly different from Liver and Gill, which were not significantly different from each other ($p < 0.05$).

** The Tukey HSD test also showed that the Cadmium x Intestine interaction was significantly different from all other combinations of Treatment x Organ, which were not significantly different from each other.

Table 2. Comparison of metallothionein mRNA levels in intestine, liver, and gill tissues in control fish and fish exposed to dietary cadmium, benzo(a)pyrene, or a combination of cadmium and benzo(a)pyrene in the second experiment. Samples were analyzed by real-time PCR, and values are expressed as $2^{-\Delta\Delta C_t}$ (mean $\pm$ 1 S.E., n=10)

Treatment*	Organ*		
	Gill	Liver	Intestine
Control	0.54 $\pm$ 0.17	0.81 $\pm$ 0.25	1.23 $\pm$ 0.39
Cadmium	1.24 $\pm$ 0.39	2.32 $\pm$ 0.73	24.52 $\pm$ 7.75**
Benzo(a)pyrene	0.63 $\pm$ 0.20	1.27 $\pm$ 0.40	3.27 $\pm$ 1.06
Combination	1.92 $\pm$ 0.60	4.84 $\pm$ 1.53	42.70 $\pm$ 12.80***

*Analysis of variance showed that differences due to the Treatment, Organ, and the Organ x Treatment interaction were significant ($p < 0.001$). The Tukey HSD test showed that the Control treatment was significantly different from that of Cadmium and the cadmium and benzo(a)pyrene Combination ($p < 0.05$). For the effect of Organ, Intestine was significantly different from Liver and Gill, which were not significantly different from each other ($p < 0.05$).

,* The Tukey HSD test also showed that Cadmium x Intestine interaction was significantly different from that of Combination x Intestine, and both of these combinations were significantly different from all other combinations of Treatment x Organ, which were not significantly different from each other.

Fig. 1 Roesijadi et al.

Fig. 2 Roesijadi et al.