



HAL
open science

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

G. Roesijadi, S. Rezvankhah, A. Perez-Matus, A. Mitelberg, K. Torruellas,
P.A. van Veld

► **To cite this version:**

G. Roesijadi, S. Rezvankhah, A. Perez-Matus, A. Mitelberg, K. Torruellas, et al.. Dietary Cadmium and Benzo(a)pyrene Increased Intestinal Metallothionein Expression in the Fish. *Marine Environmental Research*, 2008, 67 (1), pp.25. 10.1016/j.marenvres.2008.10.002 . hal-00563060

HAL Id: hal-00563060

<https://hal.science/hal-00563060>

Submitted on 4 Feb 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

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

G. Roesijadi, S. Rezvankhah, A. Perez-Matus, A. Mitelberg, K. Torruellas, P.A. Van Veld

PII: S0141-1136(08)00218-3

DOI: [10.1016/j.marenvres.2008.10.002](https://doi.org/10.1016/j.marenvres.2008.10.002)

Reference: MERE 3294

To appear in: *Marine Environmental Research*

Received Date: 8 August 2008

Revised Date: 6 October 2008

Accepted Date: 7 October 2008

Please cite this article as: Roesijadi, G., Rezvankhah, S., Perez-Matus, A., Mitelberg, A., Torruellas, K., Van Veld, P.A., Dietary Cadmium and Benzo(a)pyrene Increased Intestinal Metallothionein Expression in the Fish *Fundulus heteroclitus*, *Marine Environmental Research* (2008), doi: [10.1016/j.marenvres.2008.10.002](https://doi.org/10.1016/j.marenvres.2008.10.002)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



1 Running Head: Metallothionein and dietary cadmium in fish

2 Title of article: Dietary Cadmium and Benzo(a)pyrene Increased Intestinal Metallothionein

3 Expression in the Fish *Fundulus heteroclitus*

4

5 Author's complete names: G. Roesijadi^{a*}, S. Rezvankhah^a, A. Perez-Matus^a, A. Mitelberg, K.

6 Torruellas^a, P. A. Van Veld^b

7

8 Institutional address:

9 ^aFlorida Atlantic University, Department of Biological Sciences, 777 Glades Road, Boca Raton,

10 FL 33431

11 ^bVirginia Institute of Marine Science, Gloucester Point, VA

12

13 *Corresponding author.

14 Tel.: 360-582-253; fax: 360-681-4559

15 E-mail address: g.roesijadi@pnl.gov

16

17 ¹Current Address: Pacific Northwest National Laboratory, Environment and Energy Directorate,

18 Marine Science Division, 1529 West Sequim Bay Road, Sequim, WA 98382

19 **Abstract**

20

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

36

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

38 1. Introduction

39

40 Organs at the environmental-organism interface, which are involved in uptake processes, are
41 important in the absorption of metals from the environment and especially useful in analyzing
42 events associated with dietary exposure (Harrison et al., 1989; Thomann et al., 1997). In higher
43 animals, dietary cadmium absorption is characterized by high levels of cadmium in the intestinal
44 mucosa and its slow transfer to other internal organs (Elsenhans et al., 1997). About 60% of
45 cadmium in the mucosa of the small intestine is bound to the metal-binding protein
46 metallothionein shortly after dietary exposure (Lehman et al., 1986). Metallothionein is involved
47 in intestinal cadmium absorption through its induction and binding of cadmium. Subsequent
48 interorgan cadmium transport out of the intestine is, in part, associated with serosal release as
49 cadmium-metallothionein complexes (Zalups et al., 2003). For dietary exposure to cadmium in
50 fish such as salmon and rainbow trout, the intestine is also the site of absorption and the portal
51 for redistribution via the systemic circulation, resulting in both cadmium accumulation and
52 toxicity in other organs (Baldisserotto et al., 2005; Baldisserotto et al., 2006; Berntssen et al.,
53 2001a; Berntssen et al., 2001b; Berntssen et al., 2000; Berntssen et al., 2003; Franklin et al.,
54 2005; Lundebye et al., 1999; Ng et al., 2008; Thomann et al., 1997). Although the proportion of
55 cadmium that crosses the gut for transport other organs is relatively low (Franklin et al., 2005;
56 Ng et al., 2008), the dietary exposure route is considered significant in the movement of
57 cadmium through food chains (Dallinger et al., 1987; Harrison et al., 1989; Wallace et al., 2003).
58 While it is known that metallothionein is induced in fish intestines in response to dietary
59 cadmium (Berntssen et al., 2001a; Chowdhury et al., 2005) and that, as noted above, the intestine
60 is the site of absorption of dietary cadmium, there is still little information on the response of fish
61 intestinal metallothionein to dietary cadmium exposure. It is reported that intestinal

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

72

73 **2. Materials and methods**

74

75 *2.1. Fish*

76

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

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

87

88 *2.2. Preparation of food pellets*

89

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

99

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

107

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

122

123 *2.3. Experiments*

124

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

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

136

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

148

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

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

159
160 At the end of exposures, the fish were immobilized using a combination of concentrated
161 sodium bicarbonate (Noga, 2000) in an ice-cold Instant Ocean slurry. The intestine, liver, and
162 gill were excised, weighed, and frozen in liquid nitrogen. Samples were stored at -80°C until
163 analyzed for metallothionein mRNA levels.

164

165 *2.4. RNA isolation*

166

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

174

175 *2.5. Identification of metallothionein and 18S RNA sequences in Fundulus heteroclitus*

176

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

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

186

187 *2.6. Real-time qPCR*

188

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

196

197 Real-time PCR reactions contained 23.8 µl of autoclaved Nanopure water (Barnstead), 25 µl
198 Platinum Quantitative PCR Supermix-UDG (Invitrogen, Carlsbad, Ca), 0.1 µl appropriate
199 Lux™ Fluorogenic Primers ((Invitrogen, Carlsbad, Ca; stock concentration 100 µM), and 1 µl of
200 the cDNA library. Temperature programming followed the manufacturer's recommendations for
201 Lux™ Fluorogenic Primers and Platinum Quantitative PCR Supermix-UDG: 50°C for 2 min;
202 95°C for 2 min; and 45 cycles of 95°C, 15 sec; 55°C, 30 sec; and 72°C, 30 sec. Reactions were

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

214

215 2.7. Statistical analysis

216

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

220

221 3. Results

222

223 Initial efforts confirmed the identities of target sequences as those for metallothionein and
224 18S RNA. Amplified sequences for intestinal, liver, and gill metallothionein exhibited 95% or
225 greater identity with *Fundulus heteroclitus* metallothionein (NCBI accession numbers:
226 CN968347 and AB426465). That for the putative 18S RNA aligned with 98% identity with the

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

231

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

237

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

249

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

266

267 **4.0. Discussion**

268

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

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

282

283 The lack of induction of hepatic and gill metallothionein in our study is believed to be due to
284 slow transport of cadmium across the intestine to other organs and negligible waterborne
285 cadmium exposure over the course of our experiments. This is consistent with studies on
286 rainbow trout in which 93% or greater of the cadmium administered via a dietary path fails to
287 cross the gut wall (Chowdhury et al., 2004a; Franklin et al., 2005; Ng et al., 2008). Induction of
288 metallothionein in organs such as kidney, liver, and gill would be expected to occur following
289 protracted exposure and export of cadmium from the intestine (Berntssen et al., 2001a;
290 Chowdhury et al., 2005). Thus, after dietary cadmium exposure of one month and longer,
291 cadmium accumulation in the liver, gill, and kidney of rainbow trout and Atlantic salmon is
292 elevated, but generally remains lower than those detected in intestinal tissues (Berntssen et al.,
293 2001a; Chowdhury et al., 2005; Szebedinszky et al., 2001). It was suggested from experiments
294 on rainbow trout that the gut wall serves as a barrier that reduces cadmium transfer to internal
295 tissues (Franklin et al., 2005). The low efficiency of gastrointestinal transfer of cadmium,
296 whether associated with prey (0.9-6.4%) (Ng et al., 2008) or with cadmium-spiked commercial
297 food (<0.1 %) (Franklin et al., 2005) is comparable to that reported for higher animals of 3-6%

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

302
303 Induction of metallothionein, enhanced cadmium binding capacity in intestinal cells, and
304 gradual transfer of cadmium-metallothionein complexes across the serosal membrane to other
305 organs are proposed as mechanisms to protect against the rapid accumulation of cadmium by
306 internal organs (Elsenhans et al., 1997). Our finding that metallothionein mRNA in the intestine
307 is most responsive to dietary cadmium in comparison with other organs extends, to the gene
308 expression level, observations that the intestine had the highest levels of the metallothionein
309 protein during dietary exposure of fish to cadmium (Berntssen et al., 2001a; Chowdhury et al.,
310 2005). Retention of dietary cadmium in rainbow trout is highest in the gut and lower in other
311 organs (Chowdhury et al., 2004b; Franklin et al., 2005; Ng et al., 2008), a pattern consistent with
312 our observations for metallothionein mRNA induction in *Fundulus heteroclitus*. The quantifiable
313 relationship between intestinal metallothionein expression and exposure to dietary cadmium in *F.*
314 *heteroclitus* may reflect a protective function for intestinal metallothionein. However, this
315 induction may not be without its own contribution to the toxicity of cadmium, since cadmium
316 transported out of the intestine as cadmium-metallothionein can be filtered and absorbed by the
317 kidney, a target of cadmium toxicity (Zalups et al., 2003).

318
319 While the mechanism for potentiation of cadmium-induced metallothionein expression by
320 benzo(a)pyrene is not known, our observations are consistent with an earlier report showing that
321 hepatic metallothionein induction in *F. heteroclitus* is enhanced after intraperitoneal injection of

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

337
338 At the broader level, our finding that benzo(a)pyrene potentiated intestinal metallothionein
339 induction by cadmium is of particular interest due to the fact that environments contaminated by
340 cadmium are often elevated in other substances such as polycyclic aromatic hydrocarbons. Based
341 on our findings, fish in such environments could display levels of metallothionein expression that
342 are higher than would be expected from cadmium exposure alone. However, the toxicological
343 significance of the increased induction is not clear-cut. For example, increased intestinal
344 metallothionein levels may further protect the intestine against cadmium toxicity and facilitate
345 retention of cadmium in the intestine. On the other hand, increased induction could also enhance

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

350

351 **Acknowledgements**

352

353 This study was supported in part by the Chesapeake Ecotox Research Project through an award
354 from Maryland Sea Grant to the University of Maryland Center for Environmental Science.

355

356 **References**

357

358 Andrews, G.K., 2000. Regulation of metallothionein gene expression by oxidative stress and
359 metal ions. *Biochem. Pharmacol.* 59, 95-104.

360 ATSDR, 1999. Toxicological profile for cadmium. Agency for Toxic Substances and Disease
361 Registry, Atlanta, GA, U.S.A.

362 Baldisserotto, B., Chowdhury, M.J., Wood, C.A., 2005. Effects of dietary calcium and cadmium
363 on cadmium accumulation, calcium and cadmium uptake from the water, and their
364 interactions in juvenile rainbow trout. *Aquatic Toxicology* 72, 99-117.

365 Baldisserotto, B., Chowdhury, M.J., Wood, C.M., 2006. *In vitro* analysis of intestinal absorption
366 of cadmium and calcium in rainbow trout fed with calcium- and cadmium-supplemented
367 diets. *Journal of Fish Biology* 69, 658-667.

- 368 Berntssen, M.H.G., Lundebye, A.K., Hamre, K., 2000. Tissue lipid peroxidative responses in
369 Atlantic salmon (*Salmo salar* L.) parr fed high levels of dietary copper and cadmium.
370 Fish Physiology and Biochemistry 23, 35-48.
- 371 Berntssen, M.H.G., Aspholm, O.O., Hylland, K., Bonga, S.E.W., Lundebye, A.K., 2001a. Tissue
372 metallothionein, apoptosis and cell proliferation responses in Atlantic salmon (*Salmo*
373 *salar* L.) parr fed elevated dietary cadmium. Comp. Biochem. Physiol. C-Toxicol.
374 Pharmacol. 128, 299-310.
- 375 Berntssen, M.H.G., Lundebye, A.K., 2001b. Energetics in Atlantic salmon (*Salmo salar* L.) parr
376 fed elevated dietary cadmium. Comp. Biochem. Physiol. C-Toxicol. Pharmacol. 128,
377 311-323.
- 378 Berntssen, M.H.G., Waagbo, R., Toften, H., Lundebye, A.K., 2003. Effects of dietary cadmium
379 on calcium homeostasis, Ca mobilization and bone deformities in Atlantic salmon (*Salmo*
380 *salar* L.) parr. Aquaculture Nutrition 9, 175-183.
- 381 Chowdhury, M.J., McDonald, D.G., Wood, C.M., 2004a. Gastrointestinal uptake and fate of
382 cadmium in rainbow trout acclimated to sublethal dietary cadmium. Aquatic Toxicology
383 69, 149-163.
- 384 Chowdhury, M.J., Pane, E.F., Wood, C.M., 2004b. Physiological effects of dietary cadmium
385 acclimation and waterborne cadmium challenge in rainbow trout: respiratory,
386 ionoregulatory, and stress parameters. Comp. Biochem. Physiol. C-Toxicol. Pharmacol.
387 139, 163-173.
- 388 Chowdhury, M.J., Baldisserotto, B., Wood, C.M., 2005. Tissue-specific cadmium and
389 metallothionein levels in rainbow trout chronically acclimated to waterborne or dietary
390 cadmium. Arch. Environ. Contam. Toxicol. 48, 381-390.

- 391 Dallinger, R., Prosi, F., Segner, H., Back, H., 1987. Contaminated food and intake of heavy
392 metals by fish: a review and a proposal for further research. *Oecologia* 73, 91-98.
- 393 Daniels, P.J., Andrews, G.K., 2003. Dynamics of the metal-dependent transcription factor
394 complex *in vivo* at the mouse metallothionein-I promoter. *Nucleic Acids Research* 31,
395 6710-6721.
- 396 Datta, P.K., Jacob, S.T., 1997. Activation of the metallothionein-I gene promoter in response to
397 cadmium and USF *in vitro*. *Biochem. Biophys. Res. Commun.* 230, 159-163.
- 398 Elsenhans, B., Strugala, G.J., Schafer, S.G., 1997. Small-intestinal absorption of cadmium and
399 the significance of mucosal metallothionein. *Human & Experimental Toxicology* 16,
400 429-434.
- 401 Franklin, N.M., Glover, C.N., Nicol, J.A., Wood, C.M., 2005. Calcium/cadmium interactions at
402 uptake surfaces in rainbow trout: Waterborne versus dietary routes of exposure. *Environ.*
403 *Toxicol. Chem.* 24, 2954-2964.
- 404 Harrison, S.E., Klaverkamp, J.F., 1989. Uptake, elimination and tissue distribution of dietary and
405 aqueous cadmium by rainbow trout (*Salmo gairdneri* Richardson) and lake whitefish
406 (*Coregonus clupeaformis* Mitchill). *Environ. Toxicol. Chem.* 8, 87-97.
- 407 Lehman, L.D., Klaassen, C.D., 1986. Dosage-dependent disposition of cadmium administered
408 orally to rats. *Toxicol. Appl. Pharmacol.* 84, 159-167.
- 409 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time
410 quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25, 402-408.
- 411 Lundebye, A.K., Berntssen, M.H.G., Bonga, S.E.W., Maage, A., 1999. Biochemical and
412 physiological responses in Atlantic salmon (*Salmo salar*) following dietary exposure to
413 copper and cadmium. *Marine Pollution Bulletin* 39, 137-144.

- 414 Ng, T.Y.T., Wood, C.A., 2008. Trophic transfer and dietary toxicity of Cd from the oligochaete
415 to the rainbow trout. *Aquatic Toxicology* 87, 47-59.
- 416 Noga, E.J., 2000. *Fish Disease-Diagnose and Treatment*. Iowa State University Press, Ames,
417 Iowa, USA, 367 pp.
- 418 Rana, S.V.S., 2006. *Environmental Pollution: Health and Toxicology*. Alpha Science
419 International, Ltd, Oxford, UK, 250 pp.
- 420 Regoli, F., Nigro, M., Benedetti, M., Gorbi, S., Pretti, C., Gervasi, P.G., Fattorini, D., 2005.
421 Interactions between metabolism of trace metals and xenobiotic agonists of the aryl
422 hydrocarbon receptor in the antarctic fish *Trematomus bernacchii* environmental
423 perspectives. *Environ. Toxicol. Chem.* 24, 1475-1482.
- 424 Szebedinszky, C., McGeer, J.C., McDonald, D.G., Wood, C.M., 2001. Effects of chronic Cd
425 exposure via the diet or water on internal organ-specific distribution and subsequent gill
426 Cd uptake kinetics in juvenile rainbow trout (*Oncorhynchus mykiss*). *Environ. Toxicol.*
427 *Chem.* 20, 597-607.
- 428 Takahashi, Y., Nakayama, K., Itoh, S., FujiiKuriyama, Y., Kamataki, T., 1997. Inhibition of the
429 transcription of CYP1A1 gene by the upstream stimulatory factor 1 in rabbits -
430 Competitive binding of USF1 with AhR center dot Arnt complex. *J. Biol. Chem.* 272,
431 30025-30031.
- 432 Thomann, R.V., Shkreli, F., Harrison, S., 1997. A pharmacokinetic model of cadmium in
433 rainbow trout. *Environ. Toxicol. Chem.* 16, 2268-2274.
- 434 Van Cleef-Toedt, K.A., Kaplan, L.A.E., Crivello, J.F., 2001. Killifish metallothionein messenger
435 RNA expression following temperature perturbation and cadmium exposure. *Cell Stress*
436 & Chaperones 6, 351-359.

- 437 van den Hurk, P., Faisal, M., Roberts, M.H., 2000. Interactive effects of cadmium and
438 benzo[a]pyrene on metallothionein induction in mummichog (*Fundulus heteroclitus*).
439 Mar. Environ. Res. 50, 83-87.
- 440 Van Veld, P.A., Vogelbein, W.K., Cochran, M.K., Goksoyr, A., Stegeman, J.J., 1997. Route-
441 specific cellular expression of cytochrome P4501A (CYP1A) in fish (*Fundulus*
442 *heteroclitus*) following exposure to aqueous and dietary benzo[a]pyrene. Toxicol. Appl.
443 Pharmacol. 142, 348-359.
- 444 Vogelbein, W.K., Fournie, J.W., VanVeld, P.A., Huggett, R.J., 1990. Hepatic neoplasms in the
445 mummichog *Fundulus heteroclitus* from a creosote-contaminated site. Cancer Research
446 50, 5978-5986.
- 447 Wallace, W.G., Luoma, S.N., 2003. Subcellular compartmentalization of Cd and Zn in two
448 bivalves. II. Significance of trophically available metal (TAM). Marine Ecology-Progress
449 Series 257, 125-137.
- 450 Zalups, R.K., Ahmad, S., 2003. Molecular handling of cadmium in transporting epithelia.
451 Toxicology and Applied Pharmacology 186, 163-188.
452
453

454 List of Figures:

455

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

459

460 Figure 2. Dose-response relationship of ingested cadmium and levels of metallothionein mRNA
461 in *Fundulus heteroclitus* exposed to dietary cadmium. Levels of metallothionein mRNA were
462 determined by real-time qPCR and expressed as $2^{-\Delta\Delta C_t}$ (Livak et al., 2001). R^2 and significance of
463 regression coefficients: $R^2 = 0.640$, $p < 0.006$ for intestine; $R^2 = 0.001$, $p < 0.98$ for liver; and $R^2 =$
464 0.011 , $p < 0.79$ for gill.

465 Table 1. Comparison of metallothionein mRNA levels in intestine, liver, and gill tissues in
 466 control fish and fish exposed to dietary cadmium in the first experiment. Samples were
 467 analyzed by real-time PCR, and values are expressed as $2^{-\Delta\Delta Ct}$ (mean \pm 1 S.E., n=3 for
 468 controls and n=7 or 8 for cadmium treated).
 469

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

470
 471 *Analysis of variance showed that differences due to the Treatment, Organ, and the
 472 Organ x Treatment interaction were significant ($p < 0.002$). The Tukey HSD test showed
 473 that the Control treatment was significantly different from that of Cadmium. For the
 474 effect of Organ, Intestine was significantly different from Liver and Gill, which were not
 475 significantly different from each other ($p < 0.05$).

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

479

480

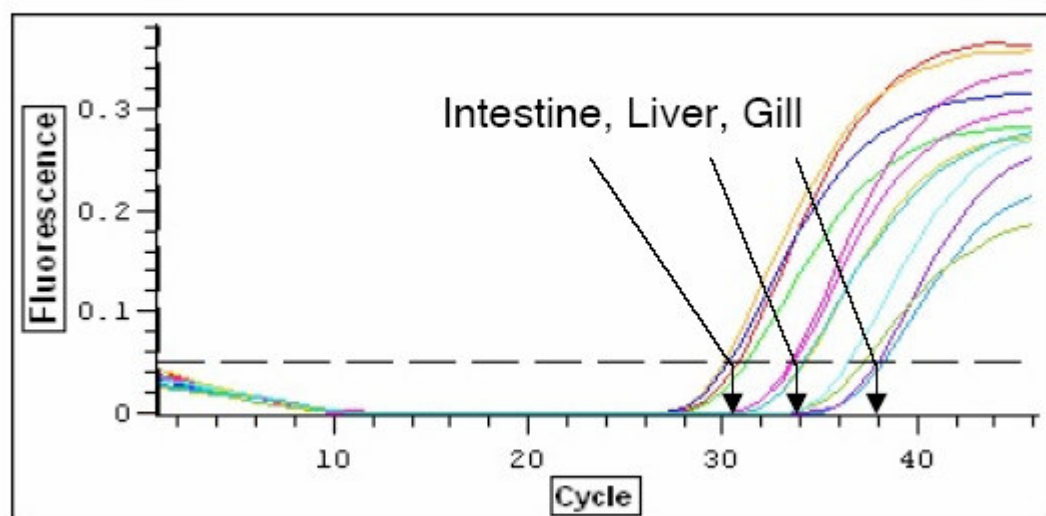
481 Table 2. Comparison of metallothionein mRNA levels in intestine, liver, and gill tissues
 482 in control fish and fish exposed to dietary cadmium, benzo(a)pyrene, or a
 483 combination of cadmium and benzo(a)pyrene in the second experiment. Samples
 484 were analyzed by real-time PCR, and values are expressed as $2^{-\Delta\Delta Ct}$ (mean \pm 1
 485 S.E., n=10)
 486

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***

487
 488 *Analysis of variance showed that differences due to the Treatment, Organ, and the
 489 Organ x Treatment interaction were significant ($p < 0.001$). The Tukey HSD test showed
 490 that the Control treatment was significantly different from that of Cadmium and the
 491 cadmium and benzo(a)pyrene Combination ($p < 0.05$). For the effect of Organ, Intestine
 492 was significantly different from Liver and Gill, which were not significantly different from
 493 each other ($p < 0.05$).

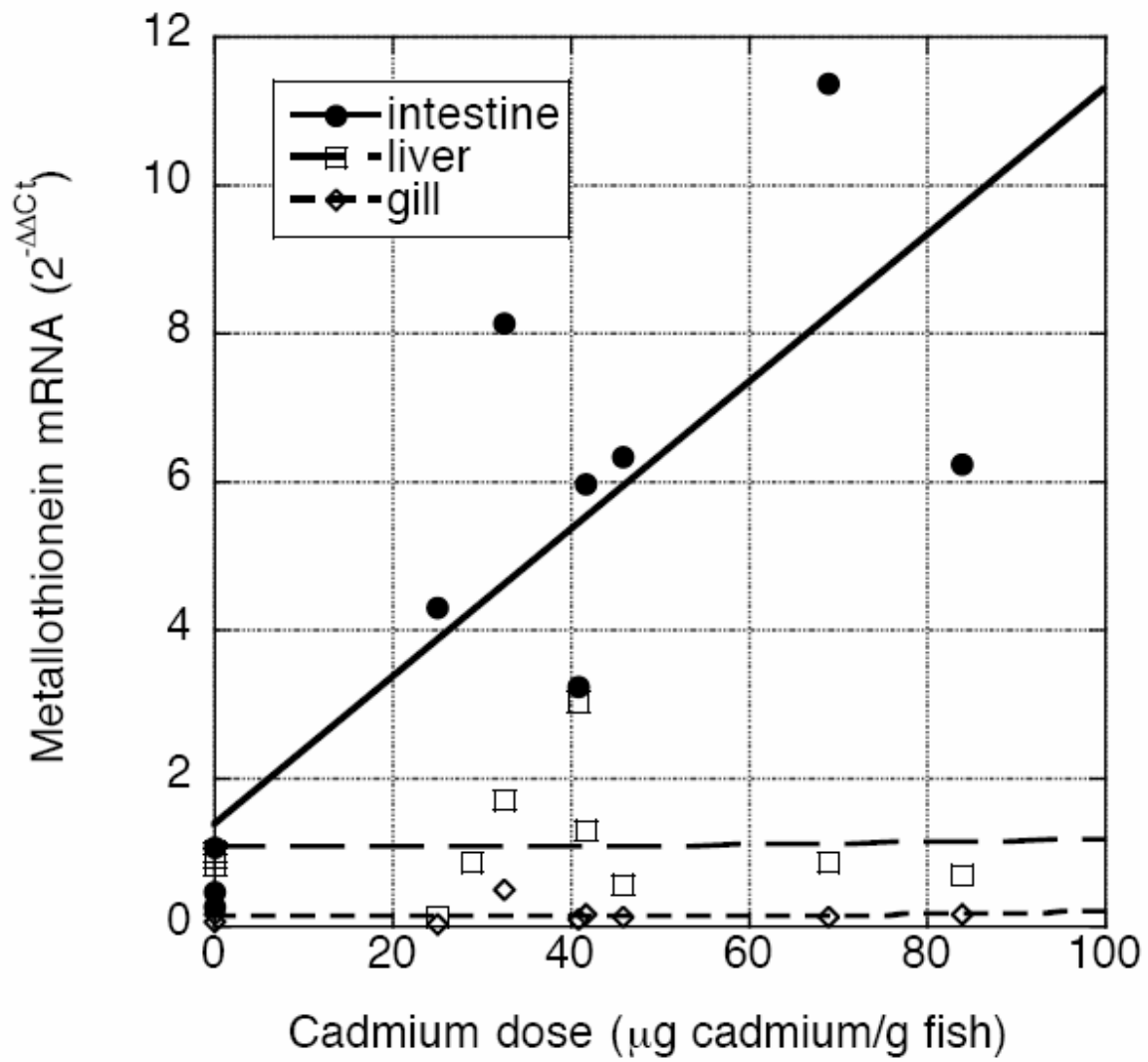
494 **,** The Tukey HSD test also showed that Cadmium x Intestine interaction was
 495 significantly different from that of Combination x Intestine, and both of these
 496 combinations were significantly different from all other combinations of Treatment x
 497 Organ, which were not significantly different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

Fig. 1 Roesijadi et al.



498

Fig. 2 Roesijadi et al.

499
500

AC