

Natural Arsenic Contaminated Diets Perturb Reproduction in Fish

DAVID BOYLE,[†] KEVIN V. BRIX,[‡]
HEIDI AMLUND,[§]
ANNE-KATRINE LUNDEBYE,[§]
CHRISTER HOGSTRAND,[†] AND
NIC R. BURY^{*†}

King's College London, Nutritional Sciences Division,
Franklin-Wilkins Building, 150 Stamford Street, London,
SE1 9NH, United Kingdom, EcoTox, Jacksonville, Florida, and
National Institute of Nutrition and Seafood Research (NIFES),
P.O. Box 2029 Nordnes, 5817 Bergen, Norway

Received January 29, 2008. Revised manuscript received
April 28, 2008. Accepted April 28, 2008.

The toxicological effect of natural diets elevated in metals on reproduction in fish is poorly understood. The reproductive output of zebrafish fed the polychaete *Nereis diversicolor* collected from a metal-impacted estuary, Restronguet Creek, Cornwall, UK, was compared to fish fed *N. diversicolor* collected from a nonmetal impacted estuary, Blackwater, Essex, UK. Fish fed the metal laden *N. diversicolor* for 68 days showed reduced reproductive output, characterized by reduced cumulative egg production (47%), cumulative number of spawns (30%), as well as reduced average number of eggs produced per spawn and % hatch rate. The mRNA transcript levels of the egg-yolk protein vitellogenin was also reduced 1.5 fold in the livers of female fish fed metal-laden *N. diversicolor*. No difference was seen between the lipid, protein, or moisture content of the two diets and no difference in growth was seen between the two fish populations. The Restronguet Creek polychaetes have elevated arsenic, cadmium, copper, zinc, lead, and silver body burdens, but the only element found to accumulate in the tissues of zebrafish fed this diet was As. The As in these *N. diversicolor* was found to be predominantly potentially toxic inorganic As species, 58% of total As content, which is unusual for aquatic organisms where arsenic is typically biotransformed into less toxic organoarsenical compounds. These results demonstrate that reproduction in fish is a sensitive target of exposure to a natural diet contaminated with As and this exposure route could be of significance to the health of fish populations.

Introduction

Aquatic invertebrates that inhabit metal-impacted environments may contain elevated metal body burdens (1), and thus potentially contribute significantly to total metal exposure, accumulation, and chronic toxicity in aquatic predators (2, 3). In fish chronic toxicity tests growth is often used as a toxicity end point to assess the consequences of prolonged exposure to elevated dietary metals, and exposure to natural metal-laden invertebrates has been shown to

reduce growth rates in salmonid populations (4). The possibility that chronic exposure to metals can adversely affect reproduction in fish has often been overlooked, despite growing evidence in the invertebrate, mammalian, and avian literature that ingested metals can contribute to reproductive disorders (5–8).

Field observations are suggestive of a link between metal exposure and reproductive impairment in feral fish populations. Yellow perch (*Perca flavescens*) from metal-impacted Canadian lakes exhibited dose-dependent decreases in plasma sex hormone concentrations and gonadosomatic index (GSI) along a metal contamination gradient (9). Elevated intestinal metal concentrations in these fish suggest an important role of dietary metal exposure and indicate that reproduction may be an ecologically relevant target of dietary metals in impacted areas (10).

Pharmacokinetic evidence suggests that dietary metal exposure accentuates metal accumulation in the liver (11), an estrogen-responsive organ central to the reproductive axis of fish and the site of protein synthesis for developing oocytes (12). Laboratory studies have shown that vitellogenin (Vtg) synthesis, an egg-yolk protein, is reduced in rainbow trout injected with high doses of Cd (13), as well as in rainbow trout hepatocyte cultures incubated with either Cd or Al (14). In a recombinant yeast system expressing rainbow trout estrogen receptor, Le Guével et al. (15) demonstrated a Cd-induced inhibition of estradiol-stimulated gene expression via inhibition of the interaction between the estrogen receptor (ER) and its response element.

In vivo studies of dietary metal-induced reproductive impairment in fish are scarce and where present often employ single metal exposures that utilize artificial diets spiked with metals, and/or elevated metal concentrations unreflective of metal concentrations of prey items in the field. For example, a reduction in the gonadosomatic index of female Prussian carp (*Carassius auratus gibelio* B.) fed a Cd-spiked artificial diet (10 mg g⁻¹) for 3 years was strongly associated with elevated hepatic Cd (16), and Hatakeyama and Yasuno (17) found that cumulative numbers of fry produced by guppy (*Poecilia reticulata*) fed Cd-exposed midge larvae (210 μg Cd g⁻¹ d⁻¹) for 2 months decreased to 80% of the controls. Thus, there is a growing body of evidence that dietary metal exposure can negatively affect reproduction in fish. Considering that current metal regulations in aquatic environments are based solely on waterborne metal concentrations, this effect may be of ecotoxicological significance if proved to occur at environmentally relevant concentrations.

Polymetallic mining in the watershed of the Carnon river, Cornwall, has led to elevated sediment As, Cu, and Zn concentrations in Restronguet Creek, an intertidal zone, that are among the highest recorded for an estuary in the UK (18). *Nereis diversicolor*, a polychaete worm, resident in Restronguet Creek has previously been shown to be tolerant to both Cu and Zn and are atypically strong net accumulators of Cu (19). *N. diversicolor* is a common prey item of wading birds and benthic feeding fish and Rainbow et al. (20) have previously demonstrated trophic transfer of Cu from *N. diversicolor* to an invertebrate predator, *N. virens*, during a 50-day dietary exposure. The aim of the present study was to assess the possible effects of a natural metal-contaminated diet of *N. diversicolor* collected from a metal-impacted environment on a fish model, zebrafish (*Danio rerio*) using reproduction as a toxicity end point—an approach which presents the metal in ecologically relevant forms.

* Corresponding author e-mail: nic.bury@kcl.ac.uk; tel: (+44) 2078484091; fax: (+44) 2078484500.

[†] King's College London.

[‡] EcoTox.

[§] National Institute of Nutrition and Seafood Research (NIFES).

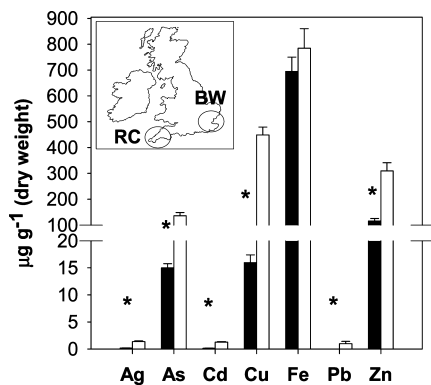


FIGURE 1. Metal content of *Nereis diversicolor* collected from Blackwater estuary (black bars) and Restronguet Creek (white bars). Values represent mean \pm SEM, $N = 13$, asterisk indicates significant difference between the polychaetes from the two sites (Student's t test, $p < 0.05$).

Materials and Methods

Experimental Fish. Zebrafish (*Danio rerio*) (0.30 ± 0.08 g) were obtained from Neil Hardy Aquatica Ltd. (Carshalton, Surrey). The fish were acclimated to reverse osmosis water supplemented with sea salts (60 mg Tropic Marin (TM) L⁻¹ ddH₂O) with the addition of 200 μ M CaCl₂, (termed synthetic water throughout the remainder of the article) under a photoperiod regime of 12 h light, 12 h dark, and reared at 29 ± 1 °C. After two weeks, 15 male and 15 female fish were randomly assigned to each of four aquarium tanks containing 40 L of continuously aerated and filtered synthetic water. Twenty L of synthetic water was renewed every 2 days.

Exposure Diets. *N. diversicolor* were collected from intertidal mudflats in an unimpacted area, Blackwater estuary, Essex, (N 051 44 08, E 000 41 34), termed a reference diet, and a metal-impacted estuary, Restronguet Creek (N 050 12 36, W 005 05 41). The polychaetes were depurated for 72 h in artificial seawater (TM) at a salinity of 15‰ at 15 °C. After depuration, *N. diversicolor* were rinsed with deionized water to remove external salt and stored at -80 °C until required. A subsample ($N = 13$) from each site, including animals from each sampling date, were depurated, the head was removed, and they were weighed and then dried to constant weight at 80 °C to assess moisture content and for metal analysis.

Feeding Regime. Zebrafish were fed twice a day at a rate of 3% wet weight d⁻¹ (adjusted for *N. diversicolor* moisture content and following removal of head). The jaws of *N. diversicolor* contain high concentrations of metals unlikely to be bioavailable to predators. A subsample of the fish from each tank was weighed fortnightly and the quantity of ration was adjusted accordingly. *N. diversicolor* total metal content and the average daily dietary metal exposure are presented in Figure 1 and Table 1, respectively. Following the final spawn (68 d) zebrafish were not fed for 2d to allow evacuation of the gut prior to dissection of tissues for metal analysis, and measurements of liver metallothionein-2 (MT-2) and vitellogenin (Vtg) by quantitative RT-PCR.

Lipid and Protein Analysis. Total lipid content was determined on pooled samples. Briefly, 1 g (wet weight) of polychaete composed of approximately 5 individuals was homogenized with a Dounce homogenizer in 20 mL of chloroform/methanol (1:1 v/v). Fifteen ml of the supernatant was removed and made up to 25 mL with chloroform/methanol. Two mL of the supernatant was methylated under acidic condition with a known amount of internal standard (50 μ g/mL; 15:0, pentadecanoic acid). The resulting fatty acid methyl esters were analyzed by gas liquid chromatography using a Hewlett-Packard 6890 series and data were collected and assessed using Hewlett-Packard Chem Station software.

Protein analysis was conducted on individual worms using a Biorad protein assay, following manufacturers instructions and with bovine serum albumin (BSA) as standards.

Metal and Arsenic Analysis. Following an overdose of anesthetic (benzocaine), gill, intestine, ovary, and remaining carcass (including liver) were excised and weighed. Zebrafish tissue samples and whole body *N. diversicolor* were digested overnight in 2 mL of 6 N Ultrapur HNO₃ (Merck, Poole, UK) and made up to a known volume with MilliQ Ultrapure water. Samples were analyzed in triplicate for Ag, As, Cd, Cu, Fe, and Zn using an inductively coupled plasma mass spectrometer (ICP-MS, PlasmaQuad PQ2 Turbo; Thermo Elemental, Winsford, UK) and/or atomic absorption spectrometer (AAS, SpectrAA-50; Varian, Walton on Thames, UK). Water samples from exposure tanks were acidified (0.1%, 6 N Ultrapur HNO₃) and analyzed as for tissues.

Arsenic Speciation Analysis in *N. diversicolor*. *N. diversicolor* samples were freeze-dried and sent to the National Institute of Nutrition and Seafood Research (NIFES, Bergen, Norway) where As species were determined by anion and cation exchange HPLC-ICPMS (high-performance liquid chromatography-ICPMS; Agilent HPLC 1100, Waldborn, Germany; Agilent ICPMS 7500ce, Yokogawa Analytical Systems, Tokyo, Japan) following microwave-assisted alkaline-alcoholic dissolution for inorganic As and aqueous methanol (50%) extraction by agitation for organic As analysis, respectively (21, 22).

Reproductive End Points. Reproduction was assessed on days 42, 50, 59, 64, and 68 of the experimental period. Approximately 2 h after the second feed, 18 zebrafish (9 male and 9 female) from each of the four exposure tanks (2 Restronguet Creek, 2 Blackwater Estuary) were selected at random and placed into nine (1 male and 1 female) 2 L marble bottomed beakers filled with 1.5 L synthetic water. Isolating pairs of zebrafish reduces the effects of social hierarchy and mate choice on reproductive output and obtains a broader measure of reproductive fitness in individuals from experimental populations (23). Prior to feeding the following day, the zebrafish were returned to their respective tanks and embryos were collected. The number of spawning pairs and numbers of embryos produced was recorded. Fifty embryos per spawning pair were removed to Petri dishes with 0.0002% methylene blue, an antifungal agent, in 50 mL of synthetic water and incubated at 30 ± 1 °C until hatching (hatching normally occurs between 48 and 72 h postfertilization).

Vitellogenin and Metallothionein-2 mRNA Transcript Levels in Livers of Female Fish. mRNA and cDNA synthesis, as well as PCR followed standard protocols (see Supporting Information). Zebrafish Vtg and MT-2 primer information can also be found in the Supporting Information.

Statistical Analysis. Metal levels in polychaetes from the two sites and tissue levels from zebrafish fed the two diets were compared using suitable parametric or nonparametric tests (Student's t test or Mann-Whitney U test). The difference in cumulative number of spawns and cumulative number of eggs produced per treatment was compared using ANCOVA with time as the covariate. The average number of eggs per spawning pair and % hatch of eggs for each treatment at each time point were assessed by two-way ANOVA, on untransformed or transformed data where appropriate. In addition a two-way ANOVA was used to assess the hepatic expression levels of MT-2 and Vtg to account for interassay variation on nontransformed data. All statistical tests were performed using SPSS version 15.0 and $p \leq 0.05$ was considered significant.

Results

Lipid, Water and Protein Content of Polychaetes. There was no difference in size, or moisture, lipid, and protein content between the two polychaetes populations (see

TABLE 1. Metal Concentrations ($\mu\text{g g}^{-1}$) of Male and Female Zebrafish Tissues^a Fed *Nereis diversicolor* Collected from Blackwater Estuary (BW) and Restronguet Creek (R)

		Ag		As		Cd		Cu		Fe		Zn	
		BW	R	BW	R	BW	R	BW	R	BW	R	BW	R
♀	G	0.38* (0.06)	0.16 (0.05)	0.01 (0.01)	1.32* (0.29)	0.37* (0.08)	0.13 (0.04)	1.33 (0.18)	1.25 (0.09)	92.3 (13.1)	105.4 (7.3)	32.8 (3.5)	25.6 (1.6)
	I	0.05 (0.02)	0.06 (0.02)	0.26 (0.14)	1.32* (0.29)	0.10 (0.01)	0.07 (0.02)	1.48 (0.44)	1.67 (0.36)	58.8 (7.1)	115.4 (20.4)	34.3 (12.2)	25.6 (1.6)
	C	0.02* (0.01)	0.01 (0.002)	0.39 (0.04)	0.51* (0.03)	0.02* (0.003)	0.01 (0)	0.85 (0.09)	1.08 (0.09)	10.8 (0.9)	13.1 (1.0)	24.0 (2.6)	28 (2.4)
	O	0.04 (0.01)	0.02 (0.01)	0.41 (0.15)	1.24* (0.36)	0.07* (0.01)	0.03 (0.01)	1.10 (0.06)	1.16 (0.06)	62.5 (11.8)	91.0 (23.8)	29.5 (2.2)	30.4 (2.5)
♂	G	0.19* (0.04)	0.09 (0.02)	0.38 (0.23)	1.87* (0.64)	0.28* (0.04)	0.12 (0.02)	2.40 (0.56)	1.43 (0.17)	128.4 (36.0)	121.9 (20.9)	63.9 (18.4)	30.6 (4.4)
	I	0.09 (0.01)	0.07 (0.02)	0.69 (0.32)	2.14* (0.26)	0.14 (0.03)	0.12 (0.02)	3.66 (0.95)	2.13 (0.45)	140.5 (20.2)	179.8 (40.1)	98.7* (29.5)	22.1 (3.1)
	C	0.02* (0.01)	0.003 (0.002)	0.27 (0.03)	0.41 (0.08)	0.02* (0.01)	0.006 (0.01)	0.90 (0.07)	0.91 (0.02)	28.6 (8.7)	15.2 (1.4)	25.7 (2.2)	24.3 (0.5)

^a G = gills; I = intestine; C = carcass; O = ovary. Values represent mean and 1 SEM (in parentheses), $N = 7$. * denotes significant difference between treatments (Student's t test or Mann-Whitney U-test; $p \geq 0.05$).

TABLE 2. Arsenic Speciation in *Nereis diversicolor* Homogenates^a

sample	total As	inorganic As	AB	TMAO	DMA	TETRA
Restronguet Creek	136.5 ± 14.5	80.6 ± 14.9 (58 ± 8.4%)	1.1 ± 0.07 (0.8 ± 0.09%)	4.8 ± 1.0 (3.5 ± 0.9%)	0.45 ± 0.07 (0.3 ± 0.1%)	9.8 ± 1.7 (7.2 ± 1.2%)
Blackwater Estuary	15 ± 0.5	0.1 ± 0.05 (0.7 ± 0.3%)	5.4 ± 0.4 (35.7 ± 2.4%)	n.d.	n.d.	0.24 (0.04%)

^a All values are expressed as mg kg^{-1} dry weight and values represent mean ± SEM, $N = 3$. Values in parentheses are those measured concentrations expressed as a percentage of the total As. n.d. = not detected, AB = arsenobetaine, TMAO = trimethylarsine oxide, DMA = dimethylarsenate, TETRA = tetramethylarsonium.

Supporting Information Table S2). Metal analyses shows an elevation of total Ag, As, Cd, Cu, Zn, Fe, Pb, and Zn concentrations in polychaetes collected from Restronguet Creek (Figure 1).

Metal and Arsenic Analysis. There was found to be a significant increase in total water Cu (13.3 ± 2.2 , compared to $3.8 \pm 0.8 \mu\text{g L}^{-1}$), Zn (16.4 ± 3.5 , compared to $6.0 \pm 1.3 \mu\text{g L}^{-1}$) and As (11.8 ± 3.8 , compared to $2.5 \pm 0.6 \mu\text{g L}^{-1}$) concentrations in the exposure tanks of zebrafish fed *N. diversicolor* from Restronguet Creek presumably due to leaching from the diets.

Significant increases were observed in As metal burdens in gills and intestines of male and female fish and also the carcass and ovary of females fed a metal-contaminated *N. diversicolor* diet compared to zebrafish fed the reference diet (Table 1).

Female zebrafish fed the metal-contaminated diets also exhibited lower gill and carcass Ag burdens (Table 1), and lower gill, carcass, and ovary Cd burdens (Table 1). Male zebrafish fed the metal-rich diet also exhibited lower gill and carcass Ag burdens (Table 1), lower gill and carcass Cd concentrations (Table 1), and lower intestine zinc burdens (Table 1) compared to zebrafish fed the reference diet polychaetes. There was no significant difference in gill, intestine, carcass, or ovary Cu or Fe concentrations in males or females or zinc in tissues from female fish between fish fed *N. diversicolor* from Restronguet Creek and Blackwater estuary.

Arsenic Speciation. *N. diversicolor* collected from Restronguet Creek possessed the majority of their As body burden ($136.5 \mu\text{g g}^{-1}$) as inorganic As (43–72% As^{3+} and As^{5+} combined), with 2.3–5.3% being found as trimethylarsine oxide, 5.8–9.6% as tetramethylarsonium ion, 0.2–0.4% as dimethylarsinate, and 0.7–1% as arsenobetaine. In contrast,

N. diversicolor collected from Blackwater estuary possessed very little of their total As body burden ($15 \mu\text{g g}^{-1}$) as inorganic As (0–1.3% As^{3+} and As^{5+} combined), trimethylarsine oxide and dimethylarsinate were undetectable, tetramethylarsonium ion accounted for less than 1%, whereas 35.7% was in the form of arsenobetaine (Table 2). The reason for the summation of the % As species of the total As concentration not totaling 100% can be attributed to differences among the 3 separate analytical techniques for total As, inorganic As, and organic As measurements, and in the case of the Blackwater polychaetes, the organoarsenical species detected being extremely close to the detection limit.

Growth and Mortality. There was no significant difference between the growth rate of either male or female zebrafish fed either of the diets and no mortalities were observed.

Reproductive Output. The group of fish fed the metal laden *N. diversicolor* diet showed a significant reduction in cumulative number of eggs, a 47% reduction (Figure 2a; df 8, $p = 0.003$), and cumulative number of spawns, a 30% reduction (Figure 2b; df 8, $p = 0.021$), compared to zebrafish fed the reference diet. Two-way ANOVA analysis showed that there was a significant effect of time (Figure 3a; df 4, $p = 0.006$) and diet (Figure 3a; df 1, $p < 0.001$), but no interaction between these parameters (Figure 3a; df 4, $p = 0.297$) on the average number of eggs produced per spawn, as well as a significant effect of the metal laden diet (Figure 3b; df 1, $p = 0.038$), but not time (Figure 3b; df 4, $p = 0.367$) or an interaction of the two parameters (Figure 3b; df 4, $p = 0.587$), on reducing % hatch rate.

Vitellogenin and Metallothionein-2 Expression Analysis. In female zebrafish, a significant decrease was observed in hepatic transcript levels of Vtg ($p = 0.011$; 1.5 fold reduction) in zebrafish fed *N. diversicolor* from Restronguet Creek. Although there was a trend of increasing liver transcript levels

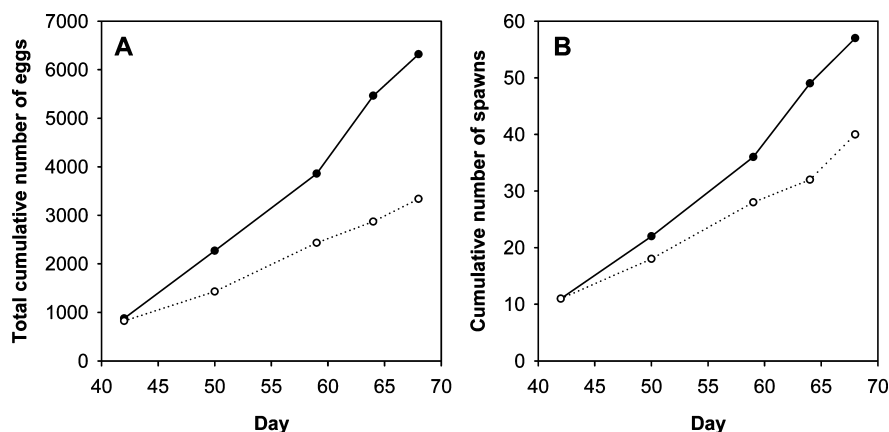


FIGURE 2. Cumulative number of eggs (A) and cumulative number of spawning events (B) for zebrafish breeding pairs fed *Nereis diversicolor* collected from Restronguet Creek (white circle) or Blackwater estuary (black circle) for 68 days. The fish fed the Restronguet Creek polychaetes show a significant decrease in both cumulative number of eggs and spawning events (ANCOVA, df 8 and $p = 0.003$; df 8 and $p = 0.021$, respectively).

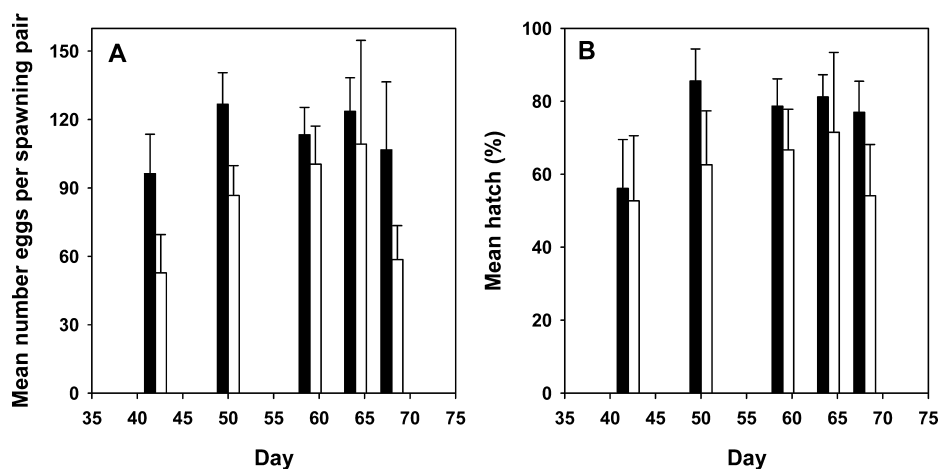


FIGURE 3. Mean number of eggs produced per spawn (A) and mean % hatch rate (B) for zebrafish breeding pairs on days 42, 50, 59, 64, and 68 during dietary exposure to *Nereis diversicolor* collected from Restronguet Creek (white bars) or Blackwater estuary (black bars). Values are mean \pm SEM, $N = 4-14$. There is a significant reduction in average number of eggs and % hatch rate in those fed the metal laden diets (two-way ANOVA; df 1, $p < 0.001$ and $p = 0.038$, respectively).

of MT-2 in the Restronguet Creek treatment, the difference was not statistically significant ($p = 0.08$) (Figure 4).

Discussion

Zebrafish fed a natural metal-contaminated diet exhibited significant reductions in fecundity and a lower average % hatch rate of the F_1 generation when compared to zebrafish fed an equivalent diet collected from an unimpacted site (Figures 2 and 3). The present study provides a plausible mechanistic link between a naturally occurring metal/metalloid-enriched prey item and reproductive impairment in fish. Previous studies have reported metal-induced reproductive impairment in feral fish populations, however the dietary metal contribution to these effects is unclear (9, 24). Tissue analysis of metal concentrations in zebrafish reveals that arsenic is the only metal that is significantly accumulated from *N. diversicolor* collected from Restronguet Creek. Arsenic has been found to be an endocrine-disrupting compound and reproductive toxicant in higher vertebrates (25). While we cannot completely dismiss the possible presence of other organic pollutants in *N. diversicolor* not measured in this study, our results suggest that the dietary As exposure was the plausible cause of the reduced reproductive output in zebrafish.

Arsenic toxicity is more often associated with the inorganic species, arsenite (As^{3+}) and arsenate (As^{5+}) (26). Many marine

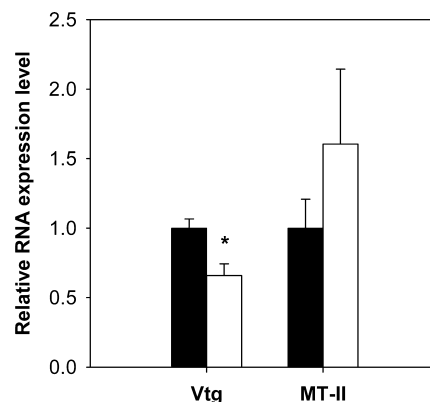


FIGURE 4. Vitellogenin (Vtg) and Metallothionein-2 (MT-II) transcript expression levels in the livers of zebrafish tissues fed *Nereis diversicolor* collected from Restronguet Creek (white bars) or Blackwater estuary (black bars). Values are normalized to 18s RNA expression levels and expressed as a ratio of the transcript levels measured in the Blackwater estuary treatment. Values are mean \pm SEM, $N = 9-10$, and asterisks indicate significance difference between treatments (two-factor ANOVA performed on untransformed data, $p = 0.011$).

organisms are thought to detoxify As via methylation to form less toxic organoarsenical compounds (26-28). However, this

detoxification pathway should not be considered ubiquitous because whole organism As speciation has only been carried out in a few species (27, 28). Out of six polychaete species reviewed by Waring and Mahrer (28) organoarsenicals were predominantly found in five. The exception being an estuarine polychaete, the lugworm *Arenicola marina* (29), which, following a laboratory seawater exposure trial to arsenate, was found to contain predominately inorganic As (58% As^{3+} and 16% As^{5+} of the total As body burden) (29). Geiszinger et al. (30) documented rapid biomethylation of arsenate to tetramethyl arsonium ion in *N. diversicolor* and *N. virens*. A similar profile of organoarsenicals was observed in *N. diversicolor* from Blackwater estuary where 31–39% of the total As body burden was found to be as arsenobetaine. However, in contrast, speciation of the 10-fold higher content of As in *N. diversicolor* from Restronguet Creek is markedly different and arsenobetaine is a minor species, accounting for <1% of total As. Inorganic arsenicals accounted for 59% of total As in this population (Table 2). Inorganic As as low as $1 \mu\text{g As}^{5+} \text{g}^{-1}$ in artificial diets has been shown to cause liver and gallbladder histopathology to lake whitefish (*Coregonus clupeaformis*) (31), and it is the presence of these inorganic As species in the diet that has the potential to increase the toxicological risk to organisms (26).

The reduction in average number of eggs (Figure 3a) produced by zebrafish fed *N. diversicolor* from Restronguet Creek compared to those fed *N. diversicolor* from Blackwater estuary suggests a reduction in vitellogenic follicles in female fish fed the high-metal diet. A reduction in follicle production may be due to the quality of the lipid content of the diet or adverse effects of metal exposure on general health (24). In our study, we found no difference in the lipid content between the two polychaete diets or indeed any difference in growth rate between the two fish populations suggesting a toxic action at the reproductive axis of females. In female oviparous vertebrates the liver is central to the reproductive axis and the site of synthesis for Vtg (12). Expression of Vtg is controlled by the steroid hormone estradiol, which binds to its receptor (estrogen receptor (ER)) in the cytoplasm of cells; this ligand–ER complex forms dimers and migrates to the nucleus where it interacts with the estrogen responsive elements (ERE) upstream of target genes (12). Female fish fed *N. diversicolor* from Restronguet Creek in the present study exhibited a significant reduction (1.5 fold) in hepatic Vtg transcript levels. This is consistent with the recent understanding that inorganic As may act as an endocrine disruptor (25, 32, 33). Inorganic As has been found to simultaneously interfere with a number of steroid receptor mediated pathways, including the estrogen receptor (25, 32, 33). Inorganic As ($74\text{--}3700 \mu\text{g kg}^{-1}$) inhibits estradiol induced vitellogenin synthesis in chick embryo livers and at lower noncytotoxic doses ($20\text{--}225 \mu\text{g L}^{-1}$) and As inhibits estradiol mediated gene expression in a human breast cancer (MCF-7) cell line (25).

The reduced fecundity, characterized by a 47% reduction in cumulative egg production during the experimental period, observed resulted from significant reductions in both the number of spawning events (Figure 2a) and average clutch size (Figure 3a). Thus, it is possible that in addition to direct effects on female fecundity, As may also interfere with spawning behavior either of the male or female fish. There is no evidence that arsenic exposure may affect behavior in fish, but dietary methyl mercury (34) and waterborne lead (35) have been found to alter spawning behavior in male fathead minnows.

The decreased hatch rate of oocytes produced by zebrafish fed *N. diversicolor* from Restronguet Creek could be due to lower male fertility and reduced fertilization and/or reduced embryo viability. Male fertility was not directly measured in the present study, but As^{5+} accumulation has been shown to inhibit spermatogenesis of Japanese eel (*Anguilla japonica*)

(36). Oocyte quality was probably affected in our study, because Vtg transcript levels were found to be reduced (Figure 2) and ovary arsenic content increased 3-fold. This is consistent with the observation of Shukla and Pandey (37), who reported a reduction in mature oocytes and an increase in atretic follicles in gonads of the freshwater fish, *Colisa fasciatus*, after exposure to waterborne arsenite oxide.

Rainbow et al. (20) have demonstrated trophic transfer of Cu from *N. diversicolor* from Restronguet Creek to a second nereid species, *N. virens*. This is despite *N. diversicolor* from Restronguet Creek partitioning greater proportions of accumulated metals in insoluble granules which are not readily assimilated by predators (19, 20). The zebrafish did not accumulate copper or zinc in their tissues when fed the Restronguet Creek *N. diversicolor*. This was unexpected because the copper and zinc concentrations in these worms are ~20-fold and ~3-fold, respectively, greater than those of the reference diet. Copper and zinc are essential micronutrients thus one can only assume that, in addition to a proportion of the metals being locked up in unavailable granules (19, 20), the fish is also able to adjust physiologically to restrict dietary metal uptake or increase excretion to maintain essential metal homeostasis (38).

The greater cadmium and silver in gill and carcass, and cadmium ovary burdens in fish fed *N. diversicolor* the reference diet were counter-intuitive, because this result does not relate to the dietary intake concentration (Supporting Information Table S1). It suggests that cadmium and silver within these metal-unimpacted polychaetes are more readily available. Mouneyrac et al. (19) have shown that *N. diversicolor* from Blackwater estuary are more tolerant to Ag and contain higher concentrations of potentially bioavailable cytosolic heat stable compounds, including metal binding proteins, than *N. diversicolor* from Restronguet Creek. In addition, metal–metal interactions at shared sites of uptake (39) in the intestine of zebrafish may also influence metal bioavailability especially in those fish fed metal-rich Restronguet Creek polychaetes. The toxicological significance of this increase in silver and cadmium body burden is unclear, but the reproductive output of zebrafish fed Blackwater worms is on par with those reported in other studies (40).

The elevated mean waterborne As, Cu, and Zn concentrations in the exposure tanks of fish fed *N. diversicolor* from Restronguet Creek are likely to be due to leaching from the experimental diets. The contribution of waterborne As to elevated tissue arsenic concentrations cannot therefore be totally ruled out. However, arsenic concentrations are an order of magnitude below that reported to induce toxicity or accumulation in freshwater fish (41, 42). For example, Rankin and Dixon (41) found no effect on growth in rainbow trout exposed to $0.76 \text{ mg As}^{3+} \text{L}^{-1}$ (a 76-fold higher concentration than that measured in the present study) for 121 days and McGeachy and Dixon (42) found no effects on hepatosomatic index, growth rate, and whole body As concentration at an exposure concentration 150-fold greater, $1.5 \text{ mg As}^{5+} \text{L}^{-1}$.

Our results are the first to show that a natural metal-contaminated diet can have profound effects on reproduction in fish. The data also suggest that dietary As is the causative agent. The *N. diversicolor* population collected from the metal-impacted estuary retains As in the inorganic form suggesting that it may be the presence of these more toxic forms of the metalloid that is of health risk to predators. The prevalence of potentially toxic inorganic As species (26–28) within macroinvertebrate populations needs to be established. Other polychaetes like the lugworm *Arenicola marina* (29) have been shown to retain inorganic As species. The uncertainty over the distribution of inorganic arsenic in natural diets, coupled with the recent observations that exceptionally low levels of inorganic arsenic disrupts steroid receptor function (25, 32, 33), as well as dietary arsenic

exposure being linked to reduced growth rate in salmonid fish (3) and impaired reproductive in fish in the present study means that the risk of dietary arsenic exposure to wildlife health may currently be underestimated.

Acknowledgments

This work was funded by a Fisheries Society of the British Isles Postgraduate Fellowship awarded to D.B.

Supporting Information Available

Information on daily metal dose the fish received, the nutritional status of the two polychaete populations, as well as detailed methods for the PCR experiment. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Literature Cited

- Rainbow, P. S. Trace metal concentrations in aquatic invertebrates: why and so what. *Environ. Pollut.* **2002**, *120*, 497–507.
- Farag, A. M.; Boese, C. J.; Woodward, D.; F; Bergman, H. L. Physiological changes and tissue metal accumulation in rainbow trout exposed to foodborne and waterborne metals. *Environ. Toxicol. Chem.* **1994**, *13*, 2021–2029.
- Hansen, J. A.; Lipton, J.; Welsh, P. G.; Cacula, D.; MacConnell, B. Reduced growth of rainbow trout (*Oncorhynchus mykiss*) fed a live invertebrate diet pre-exposed to metal-contaminated sediments. *Environ. Toxicol. Chem.* **2004**, *23*, 1902–1911.
- Woodward, D. F.; Brumbaugh, W. G.; Delonay, A. J.; Little, E. E.; Smith, C. E. Effects on rainbow-trout fry of a metals-contaminated diet of benthic invertebrates from the Clark Fork River, Montana. *Trans. Am. Fish. Soc.* **1994**, *123*, 51–62.
- Johnson, M. D.; Kenney, N.; Stoica, A.; Hilakivi-Clarke, L.; Singh, B.; Chepko, G.; Clarke, R.; Sholler, P. F.; Lirio, A. A.; Foss, C.; Reiter, R.; Trock, B.; Paik, S.; Martin, M. B. Cadmium mimics the *in vivo* effects of estrogen in the uterus and mammary gland. *Nat. Med.* **2003**, *9*, 1081–1084.
- Larison, J. R.; Likens, G. E.; Fitzpatrick, J. W.; Crock, J. G. Cadmium toxicity among wildlife in the Colorado Rocky Mountains. *Nature* **2000**, *406*, 181–183.
- Hook, S. E.; Fisher, N. S. Reproductive toxicity of metals in calanoid copepods. *Mar. Biol.* **2001**, *138*, 1131–1140.
- Hornberger, M. I.; Luoma, S. N.; Cain, D. J.; Parchaso, F.; Brown, C. L.; Bouse, R. M.; Wellise, C.; Thompson, J. K. Linkage of bioaccumulation and biological effects to changes in pollutant loads in south San Francisco Bay. *Environ. Sci. Technol.* **2000**, *34*, 2401–2409.
- Levesque, H. M.; Dorval, J.; Hontela, A.; Van Der Kraak, G. J.; Campbell, P. G. Hormonal, morphological, and physiological responses of yellow perch (*Perca flavescens*) to chronic environmental metal exposures. *J. Toxicol. Environ. Health A* **2003**, *66*, 657–676.
- Giguere, A.; Campbell, P. G. C.; Hare, L.; McDonald, D. G.; Rasmussen, J. B. Influence of lake chemistry and fish age on cadmium, copper, and zinc concentrations in various organs of indigenous yellow perch (*Perca flavescens*). *Can. J. Fish. Aquat. Sci.* **2004**, *61*, 1702–1716.
- Harrison, S. E.; Klaverkamp, J. F. Uptake, elimination and tissue distribution of dietary and aqueous cadmium by rainbow trout (*Salmo gairdneri* Richardson) and the lake whitefish (*Coregonus clupeaformis* Mitchell). *Environ. Toxicol. Chem.* **1989**, *8*, 87–97.
- Arukwe, A.; Goksøyr, A. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. *Comp. Hepatol.* **2003**, *6*, 4.
- Olsson, P. E.; Kling, P.; Petterson, C.; Silversand, C. Interaction of cadmium and oestradiol-17 beta on metallothionein and vitellogenin synthesis in rainbow trout *Oncorhynchus mykiss*. *Biochem. J.* **1995**, *307*, 197–203.
- Hwang, U. G.; Kagawa, N.; Mugiya, Y. Aluminium and cadmium inhibit vitellogenin and its mRNA induction by estradiol-17 beta in the primary culture of hepatocytes in the rainbow trout *Oncorhynchus mykiss*. *Gen. Comp. Endocrinol.* **2000**, *119*, 69–76.
- Le Guével, R. L.; Petit, F. G.; Goff, P. L.; Métivier, R.; Valotaire, Y.; Pakdel, F. Inhibition of rainbow trout (*Oncorhynchus mykiss*) estrogen receptor activity by cadmium. *Biol. Reprod.* **2000**, *63*, 259–266.
- Szczerbik, P.; Mikolajczyk, T.; Sokolowska-Mikolajczyk, A.; Socha, M.; Chyb, J.; Epler, P. Influence of long-term exposure to dietary cadmium on growth, maturation and reproduction of goldfish (subspecies: Prussian carp *Carassius auratus gibelio* B.). *Aquat. Toxicol.* **2006**, *77*, 126–135.
- Hatakeyama, S.; Yasuno, M. Chronic Effects of Cd on the Reproduction of the Guppy (*Poecilia-Reticulata*) through Cd-Accumulated Midge Larvae (*Chironomus-Yoshimatsui*). *Ecotoxicol. Environ. Saf.* **1987**, *14*, 191–207.
- Bryan, G. W.; Gibbs, P. E.; Hummerstone, L. G.; Burt, G. R. Copper, zinc and organotin as long-term factors governing the distributions of organisms in the Fal estuary in southwest England. *Estuaries* **1987**, *10*, 208–219.
- Mouneyrac, C.; Mastain, O.; Amiard, J. C.; Amiard-Triquet, C.; Beaunier, P.; Jeantet, A. Y.; Smith, B. D.; Rainbow, P. S. Trace-metal detoxification and tolerance of the estuarine worm *Hediste diversicolor* chronically exposed in their environment. *Mar. Biol.* **2003**, *143*, 731–744.
- Rainbow, P. S.; Geffard, A.; Jeantet, A. Y.; Smith, B. D.; Amiard, J. C.; Amiard-Triquet, C. Enhanced food-chain transfer of copper from a diet of copper-tolerant estuarine worms. *Mar. Ecol.: Prog. Ser.* **2004**, *271*, 183–191.
- Amlund, H.; Francesconi, K. A.; Bethune, C.; Lundebye, A. K.; Berntssen, M. H. G. Accumulation and elimination of dietary arsenobetaine in two species of fish, Atlantic salmon (*Salmo salar* L.) and Atlantic cod (*Gadus morhua* L.). *Environ. Toxicol. Chem.* **2006**, *25*, 1787–1794.
- Sloth, J. J.; Julshamn, K.; Lundebye, A. K. Total arsenic and inorganic arsenic content in Norwegian fish feed products. *Aquacult. Nutr.* **2005**, *11*, 61–66.
- Spence, R.; Smith, C. Mating preference of female zebrafish, *Danio rerio*, in relation to male dominance. *Behav. Ecol.* **2006**, *17*, 779–783.
- Alquezar, R.; Markich, S. J.; Booth, D. J. Effects of metals on condition and reproductive output of the smooth toadfish in Sydney estuaries, south-eastern Australia. *Environ. Pollut.* **2006**, *142*, 116–122.
- Davey, J. C.; Bodwell, J. E.; Gosse, J. A.; Hamilton, J. W. Arsenic as an endocrine disruptor: effects of arsenic on estrogen-mediated gene expression in vivo and in cell culture. *Toxicol. Sci.* **2007**, *98*, 75–86.
- Neff, J. M. Ecotoxicology of arsenic in the marine environment. *Environ. Toxicol. Chem.* **1997**, *16*, 917–927.
- Fattorini, D.; Notti, A.; Halt, M. N.; Gambi, M. C.; Regoli, F. Levels and chemical speciation of arsenic in polychaetes: a review. *Mar. Ecol.* **2005**, *26*, 255–264.
- Waring, J.; Mahrer, W. Arsenic bioaccumulation and species in marine polychaete. *Appl. Organomet. Chem.* **2005**, *19*, 917–929.
- Geiszinger, A. E.; Goessler, W.; Francesconi, K. A. The marine polychaete *Arenicola marina*: its unusual arsenic compound pattern and its uptake of arsenate from seawater. *Mar. Environ. Res.* **2002**, *53*, 37–50.
- Geiszinger, A. E.; Goessler, W.; Francesconi, K. A. Biotransformation of arsenate to the tetramethylarsonium ion in the marine polychaetes *Nereis diversicolor* and *Nereis virens*. *Environ. Sci. Technol.* **2002**, *36*, 2905–2910.
- Pedlar, R. M.; Ptashynski, M. D.; Evans, R.; Klaverkamp, J. F. Toxicological effects of dietary arsenic exposure in lake whitefish (*Coregonus clupeaformis*). *Aquat. Toxicol.* **2002**, *57*, 167–189.
- Bodwell, J. E.; Gosse, J. A.; Nomikos, A. P.; Hamilton, J. W. Arsenic disruption of steroid receptor gene activation: complex dose-response effects are shared by several steroid receptors. *Chem. Res. Toxicol.* **2006**, *19*, 1619–1629.
- Bodwell, J. E.; Kingsley, L. A.; Hamilton, J. W. Arsenic at very low concentrations alters glucocorticoid receptor (GR)-mediated gene activation but not GR-mediated gene repression: complex dose-response effects are closely correlated with levels of activated GR and require a functional GR DNA binding domain. *Chem. Res. Toxicol.* **2004**, *17*, 1064–1076.
- Sandheinrich, M. B.; Miller, K. M. Effects of dietary methylmercury on reproductive behavior of fathead minnows (*Pimephales promelas*). *Environ. Toxicol. Chem.* **2006**, *25*, 3053–3057.
- Weber, D. N. Exposure to sublethal levels of waterborne lead alters reproductive behavior patterns in fathead minnows (*Pimephales promelas*). *Neurotoxicology* **1993**, *14*, 347–358.
- Yamaguchi, S.; Miura, C.; Ito, A.; Agusa, T.; Iwata, H.; Tanabe, S.; Tuyen, B. C.; Miura, T. Effects of lead, molybdenum, rubidium, arsenic and organochlorines on spermatogenesis in fish: Monitoring at Mekong Delta area and in vitro experiment. *Aquat. Toxicol.* **2007**, *83*, 43–51.
- Shukla, J. P.; Pandey, K. Impaired ovarian function in arsenic-treated freshwater fish *Colis fasciatus* (Bl and Sch). *Toxicol. Lett.* **1984**, *20*, 1–3.

- (38) Kamunde, C.; Grosell, M.; Higgs, D.; Wood, C. M. Copper metabolism in actively growing rainbow trout (*Oncorhynchus mykiss*): interactions between dietary and waterborne copper uptake. *J. Exp. Biol.* **2002**, *205*, 279–290.
- (39) Bury, N. R.; Walker, P. A.; Glover, C. N. Nutritive metal uptake in teleost fish. *J. Exp. Biol.* **2003**, *206*, 11–23.
- (40) Santos, E. M.; Workman, V. L.; Paull, G. C.; Filby, A. L.; Van Look, K. J. W.; Kille, P.; Tyler, C. R. Molecular basis of sex and reproductive status in breeding zebrafish. *Physiol. Genomics* **2007**, *30*, 111–122.
- (41) Rankin, M. G.; Dixon, D. G. Acute and chronic toxicity of waterborne arsenite to rainbow trout (*Oncorhynchus mykiss*). *Can. J. Fish. Aquat. Sci.* **1994**, *51*, 372–380.
- (42) McGeachy, S. M.; Dixon, D. G. Effect of temperature on the chronic toxicity of arsenate to rainbow trout (*Oncorhynchus mykiss*). *Can. J. Fish. Aquat. Sci.* **1990**, *47*, 2228–2234.

ES800230W