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# Reproductive impacts and physiological adaptations of zebrafish to elevated dietary nickel

Derek Alsop [a,*], Santosh P. Lall [b], Chris M. Wood [a]

[a] McMaster University, 1280 Main St. W., Hamilton, ON, Canada
[b] National Research Council of Canada, 1411 Oxford Street, Halifax, NS, Canada

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ABSTRACT

Nickel (Ni) concentrations in the environment can rise due to human industrial activities. The toxicity of waterborne Ni to aquatic animals has been examined in a number of previous studies; however, little is known about the impacts of elevated dietary Ni. In the present study, zebrafish were chronically fed diets containing two concentrations of Ni [3.7 (control) and 116 μg Ni/g diet]. Ni-exposed males, but not females, were significantly smaller (26%) compared to controls at 80 days. In addition, total egg production was decreased by 65% in the Ni treatment at 75–78 days of the experiment. Ni was ubiquitously distributed in control animals (similar to previous studies), and concentrations varied between tissues by 15-fold. Ni exposure resulted in modest but significant Ni accumulation in some tissues (increases were highest in brain, vertebrae and gut; 44%, 34% and 25%, respectively), an effect observed only at 80 days. The limited Ni accumulation may be due to (1) the lack of an acidified stomach in zebrafish and/or (2) the efficient upregulation of Ni transport and excretion mechanisms, as indicated by the 4.5-fold increase in waterborne $^{63}$Ni uptake by Ni-exposed fish. Eggs from Ni-exposed adults had Ni concentrations that were 5.2-fold higher than controls. However, by 4 days post fertilization, larvae had similar Ni concentrations as controls, demonstrating a capacity for rapid Ni depuration. Larvae from Ni-exposed adults were also more resistant to waterborne Ni (35% increase in the 96-h LC50 over controls). In conclusion, elevated dietary Ni significantly affected zebrafish reproduction despite only modest tissue Ni accumulation. There were also indications of adaptation, including increased Ni uptake rates and increased Ni tolerance of offspring from Ni-exposed adults. Ni concentrations were particularly elevated in the brain with exposure; possible relations to growth and reproductive impacts require further study.



## 1. Introduction

Nickel (Ni) is a Group III transition metal of commercial importance that is relatively abundant in the earth's crust (Cempel and Nikel, 2006; Reck et al., 2008). Ni is essential in plants and bacteria (Hänsch and Mendel, 2009; Higgins et al., 2012; Pyle and Couture, 2012); in fish, there is circumstantial evidence that this is also the case, although it has not been proven (Muyssen et al., 2004; Pyle and Couture, 2012). The mining and processing of Ni as well as other anthropogenic activities are primarily responsible for elevated Ni levels in the environment (Eisler, 1998; Cempel and Nikel, 2006; Reck et al., 2008; Pyle and Couture, 2012). Indeed, waters surrounding Ni mining areas have increased concentrations of Ni (Chau and Kulikovsky-Cordeiro, 1995; Eisler, 1998; Couture and Rajotte, 2003; Couture et al., 2008; Pierron et al., 2009; Pyle and Couture, 2012), and this is problematic due to the toxicity that elevated Ni concentrations present to many aquatic animals (Schubauer-Berigan et al., 1993). In rainbow trout, acute waterborne Ni toxicity was associated with disruption of gill respiratory function (Pane et al., 2004a), while $Na^+$ loss appeared to be the acute toxic mechanism in zebrafish (Alsop and Wood, 2011). A number of chronic effects of elevated waterborne Ni on fish have also been documented, including impacts on survival (Hunt et al., 2002; Deleebeeck et al., 2007), behavior (Giatina et al., 1982; Leonard et al., 2014), decreased swimming capacity (Pane et al., 2004b), delayed embryo hatching (Dave and Xiu, 1991) and histopathological changes in a number of organs (Athikesavan et al., 2006).

In contrast to waterborne Ni, there is far less known about the effects of elevated dietary Ni in fish. One study fed lake whitefish (*Coregonus clupeaformis*) Ni-supplemented diets (0, 10, 100 and 1000 μg Ni/g diet) for up to 104 days (Ptashynski and Klaverkamp, 2002; Ptashynski et al., 2002). Ni accumulation occurred in a variety of tissues including different parts of the gastrointestinal tract, kidney, scales and others (Ptashynski and Klaverkamp, 2002). Although growth and hematological parameters were not affected, histopathological impacts in kidney and liver were observed (Ptashynski et al., 2002). Ni transport in the gastrointestinal tract has been examined in rainbow trout, where

* Corresponding author. Tel.: +1 905 525 9140x20320; fax: +1 905 522 6066.
*E-mail addresses:* alsopde@mcmaster.ca, Derek.RXR@gmail.com (D. Alsop).

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one study infused a dose of radiolabeled $^{63}Ni$ into the stomach and followed the radiolabeled Ni over time (Chowdhury et al., 2008). After 24 h, 3.7% of the Ni had crossed the gut and entered the internal organs, while 2.3% was in the gut tissue itself (Chowdhury et al., 2008). Interestingly, fish pre-exposed to waterborne Ni for 45 days took up less Ni through the gut, indicating integrative homeostatic regulation (Chowdhury et al., 2008). Another study in trout determined that 50% of the Ni present in ingested food (normal commercial trout pellets containing 25 μg Ni/g diet) was absorbed across the gut over 72 h (Leonard et al., 2009). The stomach proved to be the main site of Ni uptake, while the anterior intestine was involved in Ni secretion (Leonard et al., 2009).

The objectives of the present study were to determine the bioaccumulation and effects of elevated dietary Ni in zebrafish, specifically examining a variety of reproductive and physiological end points. We fed zebrafish a control diet (3.7 μg Ni/g diet) or an elevated Ni diet (116 μg Ni/g diet). The exposure concentration was chosen based on the previous studies of Ptashynski et al. (2002) and Ptashynski and Klaverkamp (2002) as well as levels in plants and animals that are found in Ni-polluted environments (Eisler, 1998). Fish were fed the diets for 80 days, during which time growth, tissue Ni concentrations and reproductive capacity were monitored. After 80 days, endocrine status, metabolism and branchial Ni transport were tested. In addition, the offspring of the adults from the two treatments were examined: the Ni content of the embryos and larvae as well as the acute sensitivity of larvae to waterborne Ni. The latter tests were prompted by a report that the offspring of *Daphnia magna* chronically exposed to elevated waterborne Ni exhibited greater resistance to Ni challenge (Pane et al., 2004c).

## 2. Materials and methods

### 2.1. Animals and housing

Zebrafish (*Danio rerio*; 0.2 to 0.3 g) were purchased from a commercial supplier and held in six 40-L tanks (3 control and 3 Ni-exposed), each containing 40 fish. Each tank was equipped with aeration, a heater (set to 28 °C), and a recirculating charcoal filter, while photoperiod was maintained at 12 h light/12 h dark. Water was moderately hard, dechlorinated City of Hamilton tap water, from Lake Ontario (hardness = 141 mg $CaCO_3$/L, pH 7.8, $Na^+$ = 700 μM, $K^+$ = 38 μM, $Ca^{2+}$ = 1350 μM, $Mg^{2+}$ = 336 μM, $Cl^-$ = 950 μM, dissolved organic carbon = 3.0 mg/L, Ni = 1.7 μg/L). Fish were fed to satiation two times per day. Uneaten food and feces were siphoned out of the tank daily. In addition, every 2–3 days, 75% of the water was removed and replaced with fresh water. Water samples were taken periodically from all tanks to measure waterborne Ni concentrations, in order to determine if there was significant leaching of Ni from the diet, fish or feces. Over the course of the experiment, the Ni concentrations in the water from tanks of fish fed the control diet were 2.61 ± 0.20 μg Ni/L ($N$ = 18), while in the tanks of fish fed the elevated Ni diet, waterborne Ni concentrations were 5.27 ± 0.69 μg Ni/L ($N$ = 18). These waterborne Ni concentrations are well below the Canadian Water Quality Guidelines. At the water hardness of the present experiment (141 mg $CaCO_3$/L), the guideline limit would be 110 μg Ni/L (CCME, 2007).

### 2.2. Ni measurements in the diets and tissues

Two experimental diets containing different concentrations of $NiSO_4.6H_2O$ (Sigma-Aldrich, Oakville, ON, Canada) were formulated (Table 1). The basic feed formulation was based on previous zebrafish dietary studies (Karanth et al., 2009), and the National Research Council's nutrient requirement recommendations for warm-water fishes (NRC, 1993). Analyzed Ni concentrations in the formulated zebrafish diets were 3.66 ± 0.30 μg Ni/g diet ($N$ = 5) for the control treatment and 115.8 ± 11.3 μg Ni/g diet ($N$ = 5) for the elevated dietary Ni treatment (see below for Ni measurement methods).

Fish were sampled on days 0, 5, 20 and 80 of the exposure to determine fish weights and tissue Ni concentrations. Fish were not fed for 18 h prior to sampling. Fish were first terminally anesthetized with an overdose of MS-222 (neutralized; 0.25 g/L) (Sigma–Aldrich, St. Louis MO, USA), weighed and decapitated. Tissues (blood, brain, eyes, gills, G.I. tract, liver, ovaries, muscle, vertebrae and remaining carcass) were dissected, placed in pre-weighed tubes and weighed. Samples were then snap frozen in liquid nitrogen and stored at −70 °C.

For Ni concentration measurements, tissues were thawed and 10× volume of 33% trace metal grade $HNO_3$ (Sigma–Aldrich) was added to each diet or fish sample, which were then digested for 48 h at 60 °C. Samples were diluted approximately 5-fold (depending on the tissue) with nanopure water (Sybron/Barnstead 16508 megohm-cm), and Ni was measured by graphite furnace atomic absorbance spectroscopy (Spectra AA 220Z; Varian, Palo Alto, CA, USA). Ni recovery was 99.4 ± 1.8%, as determined with certified reference water for trace elements (TM-15; National Water Research Institute, Environment Canada, Burlington, ON, Canada). Ni concentrations were not corrected for recovery. A certified reference tissue material was not used. Blanks and the TM-15 reference standard were reanalyzed every 30 measurements.

### 2.3. Reproductive capacity

From 75 to 78 days of the exposure, reproductive performance was evaluated by collecting, counting, incubating and analyzing eggs for four consecutive days. Eggs were acquired with capturing trays placed in each tank just prior to the end of the light period. Trays consisted of a plastic container 7 cm in height by 30 cm in width by 45 cm in length. The container lid was replaced with plastic mesh that allowed the eggs to fall through and prevented fish from consuming them. Plastic plants were glued to the mesh. In the morning, 2 h after the commencement of the light period, the trays were removed from the tanks, and eggs were collected and sorted into the following: (1) groups of 25 eggs that were placed into tubes and snap frozen for Ni analysis, (2) eggs that were incubated in 120-mL beakers with 50 mL of water (50 eggs per beaker) at 28.5 °C. These embryos were raised to 4 days post fertilization (dpf)

**Table 1**
Composition of experimental diets containing different supplemental quantities of nickel.

| Ingredient | Weight (g) |
|---|---|
| Vitamin-free casein[a] | 330 |
| Wheat gluten meal[b] | 100 |
| Gelatin[a] | 40 |
| Corn oil[c] | 40 |
| Fish oil[d] | 40 |
| Corn starch (pre-gel)[e] | 330 |
| Celufil[a] | 81 |
| Vitamin mix[f] | 12 |
| Mineral mix[g] | 10 |
| Betaine[h] | 15 |
| L-Methionine[a] | 2 |
| $NiSO_4 \cdot 6H_2O$[i] | Control: 0.013<br>Ni-supplemented: 0.447 |
| Total | 1000 |

[a] US Biochemical (Cleveland, OH, USA).
[b] Dover Mills Ltd. (Halifax, NS, Canada).
[c] Corey Feed Mills Limited (Fredericton, NB, Canada).
[d] Obtained from the local market.
[e] National Starch and Chemical Co. (Bridgewater, NJ, USA).
[f] Vitamin added to supply the following (per kg diet): vitamin A, 8000 IU; vitamin $D_3$, 4000 IU; vitamin E, 300 IU; vitamin $K_3$, 40 mg; thiamine HCl, 50 mg; riboflavin, 70 mg; d-Ca pantothenate, 200 mg; biotin, 1.5 mg; folic acid, 20 mg; vitamin $B_{12}$, 0.15 mg; niacin, 300 mg; pyridoxine HCl, 20 mg; ascorbic acid, 300 mg; inositol, 400 mg; choline chloride, 2000 mg; butylated hydroxy toluene, 15 mg; butylated hydroxy anisole, 15 mg.
[g] Mineral added to supply the following (per kg diet): manganous sulphate (32.5% Mn), 40 mg; ferrous sulphate (20.1% Fe), 30 mg; copper sulphate (25.4% Cu), 5 mg; zinc sulphate (22.7% Zn), 75 mg; sodium selenite (45.6% Se), 1 mg; cobalt chloride (24.8% Co), 2.5 mg; sodium fluoride (42.5% F), 4 mg.
[h] Betaine anhydrous (96% feed grade) (Finnfeeds, Finland).
[i] Sigma-Aldrich (Oakville, ON, Canada).

when larvae were either sampled to determine Ni concentrations (10 larvae per pooled sample) or used in acute waterborne Ni challenges (see below). Lastly, (3) eggs were placed in neutral buffered formalin to be counted at a later time.

### 2.4. Larval (offspring) waterborne Ni tolerance

The acute waterborne Ni tolerance of offspring from the two treatments was evaluated with 96-h LC50 tests that began when larvae reached 4 dpf, which is 2 days post hatch (dph), in the same manner as previous studies (Alsop and Wood, 2011, 2013). Briefly, experiments were performed in polystyrene 6-well tissue culture plates (Falcon™) with 10 mL of dechlorinated Hamilton tap water and 10 larvae per well. Water was changed every 24 h. Tests used six Ni concentrations (as $NiSO_4.6H_2O$), including a control treatment.

### 2.5. Waterborne $^{63}$Ni uptake

At 82 days of exposure, the uptake rates of waterborne Ni were examined in the two treatments. The radiotracer $^{63}$Ni (PerkinElmer; Boston, MA) was used to quantify Ni uptake.

Flux experiments were carried out in 800-mL plastic containers, with aeration, 8 control or Ni-exposed fish per tank, and 2.5 μCi of $^{63}$Ni in 100 μL of nanopure water per tank. Measured concentrations of total waterborne Ni were 2.0 μg Ni/L and 3.2 μg Ni/L in the control and Ni treatments, respectively. These concentrations represent the averages of the waterborne Ni measured at the start and the end of the flux period. After 4 h, fish were terminally anesthetized in water containing 0.25 g/L MS-222 and 500 μg Ni/L (unlabeled) for 1 min to displace the $^{63}$Ni that was loosely bound to the external body surface. Individual fish were weighed and transferred to 15-mL tubes. Fish were digested in 10× volume of trace-metal grade $HNO_3$ (33%) and incubated at 60 °C for 48 h, with occasional shaking.

After digestion, samples were centrifuged for 5 min at 3500 *g*, and 1 mL of the supernatant was transferred to 20-mL glass scintillation vials with 5 mL of scintillation fluid (Ultima Gold; PerkinElmer, Waltham, MA, USA). Radioactivity (measured in β emissions) was determined with a Tri-Carb 2900TR Liquid Scintillation Analyzer (PerkinElmer). Waterborne $^{63}$Ni was measured by adding 3 mL of scintillation fluid (Opti-phase, PerkinElmer) to 1 mL water samples, with all values quench-corrected to a common counting efficiency. The internal (fish) specific activity was always less than 1% of the external (water) specific activity, eliminating the need to correct for backflux (Maetz, 1956).

The appearance of $^{63}$Ni from the water into the fish was calculated from the $^{63}$Ni activity of the whole-body and the specific activity of Ni in the water. The mean specific activity (SA) of Ni in the water over the experiment was calculated as

$$SA = \left({}^{cpm}/_{mL}\right)/[Ni] \quad (1)$$

where cpm is the beta counts per minute and [Ni] is the concentration of Ni (μg/mL). Total Ni appearance in the body was then calculated as

$$\text{Total Ni appearance} = \left({}^{cpm}/_{\text{body weight}}\right) \cdot \left({}^{1}/_{SA}\right) \quad (2)$$

where total Ni appearance was in μg Ni/g body, which was then divided by 4 h (the duration of the exposure) to yield a final uptake rate in μg Ni/g/h.

### 2.6. Ovarian follicle estradiol secretion

To test whether the effects of Ni on reproductive capacity were due to an impairment of ovarian estradiol ($E_2$) synthesis, an in vitro ovarian follicle assay was utilized to examine $E_2$ secretion on day 84 of the exposure.

Follicular steroid secretion was determined using methods similar to those from Alsop et al. (2009). Briefly, female zebrafish were anesthetized (0.15 g MS-222/L, neutralized), weighed and decapitated. Whole ovaries were removed and placed in Leibovitz-15 media (Gibco, Grand Island, NY, USA) at room temperature, which also contained actinomycin D, penicillin, streptomycin and amphotericin (Sigma-Aldrich). Follicles from three fish were separated and pooled for each N, and 50 stage 2 or 3 oocytes were distributed into wells of 24 well plates. To start the experiment, medium was removed and replaced by 0.6 mL of fresh medium with 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich). Half of the wells also contained human chorionic gonadotropin (hCG, 10 I.U./mL; Sigma-Aldrich). $E_2$ concentrations were measured using a validated ELISA kit (Cayman Chemicals, Ann Arbor, MI, USA). Samples were tested in duplicate.

### 2.7. Whole-body cortisol

The effects of elevated dietary Ni on basal- and stress induced-cortisol concentrations were examined on day 104 of the exposure. For basal whole-body cortisol determination, fish were netted on the first attempt and transferred to ice-cold water with 0.3 g MS-222/L (<5 s). Fish were then quickly blotted dry, weighed, snap frozen (<30 s) and stored at −70 °C until processing. Stress-induced cortisol concentrations were also determined. To activate the acute cortisol stress response, zebrafish were netted and subjected to 1 min air exposure, followed by a return to water for a 20-min holding period. Fish were then sacrificed and processed in the same manner as for the basal cortisol sampling above. Cortisol concentrations have been shown to peak around 20 min after an acute stress has been applied (Ramsay et al., 2009; Fuzzen et al., 2010).

Cortisol was extracted by partially thawing fish on ice and homogenizing in 5× volume of ice-cold nanopure water for 30 s with a rotor-stator homogenizer. Cortisol was extracted from 500 μL homogenate three times with 4 mL of diethyl ether. Each time, tubes were vortexed then centrifuged to accelerate separation of the ether phase. Tubes were placed in methanol with dry-ice to freeze the aqueous phase. The ether phases were poured into a new tube and evaporated by placing tubes in a 45 °C water bath for 1 h. The tubes were allowed to air-dry at room temperature for an additional 2 h before reconstitution in 0.4 mL of enzyme immunoassay (EIA) buffer, which was provided in the cortisol assay kit (see below). The tubes were kept at 4 °C for 12 h, with occasional vortexing, prior to use directly in the assay. Cortisol was quantified using a commercially available colorimetric 96-well EIA kit (Cayman Chemical). Samples were analyzed in duplicate, and these two measurements were averaged.

### 2.8. Metabolic rate

Routine metabolic rate of fish from each treatment was determined by closed-system respirometry on day 93 of the exposure. Respirometers consisted of 120-mL Erlenmeyer flasks sealed with a rubber bung, fitted with 3-way sampling/replacement ports, and held in a water bath at 28 °C. During the experiment, 3 fish were placed in each flask, with 6 flasks per treatment. The exact amount of water was measured for each individual flask. Fish were allowed to settle for 2 h with aeration, after which time, the air lines were removed and the bungs were inserted in the tops of the flasks and water samples (3 mL) were taken at 0 and 50 min for $PO_2$ measurements with a Clarke-type oxygen electrode (Cameron Instruments, Port Aransas, TX, USA) connected to an AM Systems Polarographic Amplifier (Model 1900, Carlsberg, WA, USA) digital dissolved oxygen meter. The electrode was maintained and calibrated at 28 °C.

The following formula was used to calculate the absolute $O_2$ consumption rate from $PO_2$ levels:

$$Mo_2 = \frac{(\Delta Po_2 \times \alpha o_2 \times v)}{(m \times t)} \quad (3)$$

where $\Delta Po_2$ (mmHg) is the measured change in $Po_2$ values between the beginning and end of each test period, $v$ is the volume (L) of water in each respirometer, $m$ (g) is the total mass of the 3 fish in the respirometer, $t$ is time (h) and $\alpha o_2$ (μmol/L/mm Hg) is the solubility constant for $O_2$ in water (Boutilier et al., 1984).

### 2.9. Statistics

Differences between control and Ni treatments for total eggs spawned, $^{63}$Ni uptake and $Mo_2$ were analyzed with a Student's $t$-test. Follicular $E_2$ secretions between control and Ni treatments were compared under both basal and hCG-stimulated conditions with a Student's $t$-test. The effects of hCG within a treatment were also tested with a Student's $t$-test. Similarly, whole-body cortisol concentrations in control and Ni treatments were compared in both unstressed and stressed fish with a Student's $t$-test. The effects of a stressor within a treatment were also tested with a Student's $t$-test. Differences in the Ni content of eggs and larvae between the control and Ni treatments were also examined with a Student's $t$-test as was the Ni content between eggs and larvae within a treatment. Differences were considered significant if $P < 0.05$.

Differences between the control and Ni treated fish body masses (within a sex) and tissue Ni concentrations were compared with a Student's $t$-tests at 5, 20 and 80 days of exposure. However, the Bonferroni correction was applied to avoid false-positive results due to multiple testing. In these cases, $\alpha$ was divided by 3 (three days with testing), and therefore differences were considered significant if $P \leq 0.0167$ ($\alpha = 0.5/3$).

The environmental toxicity data analysis software, Tox Calc™ package (Tidepool Scientific Software), was used to estimate the 96-h LC50s with 95% confidence intervals (CI). The values were calculated from the survivorship and measured Ni concentration data from all treatments. LC50 values were considered significantly different when the 95% CI did not overlap (Environment Canada, 2005).

## 3. Results

### 3.1. Survival and growth

There was low mortality during the course of the experiment; five (4%) and four (3%) fish died in the control and Ni-supplemented diets, respectively.

There was no effect of dietary Ni content on growth after 5 or 20 days of exposure. However, by 80 days of exposure, male fish fed the elevated Ni diet were 26% smaller than their control counterparts (control = 0.353 ± 0.033 g, Ni = 0.261 ± 0.017 g; $P = 0.013$) (Fig. 1A). There was no difference in the mass of female fish between treatments at any time during the experiment (at 80 days, control =0.420 ± 0.040 g, Ni = 0.441 ± 0.046 g; $P = 0.74$) (Fig. 1B).

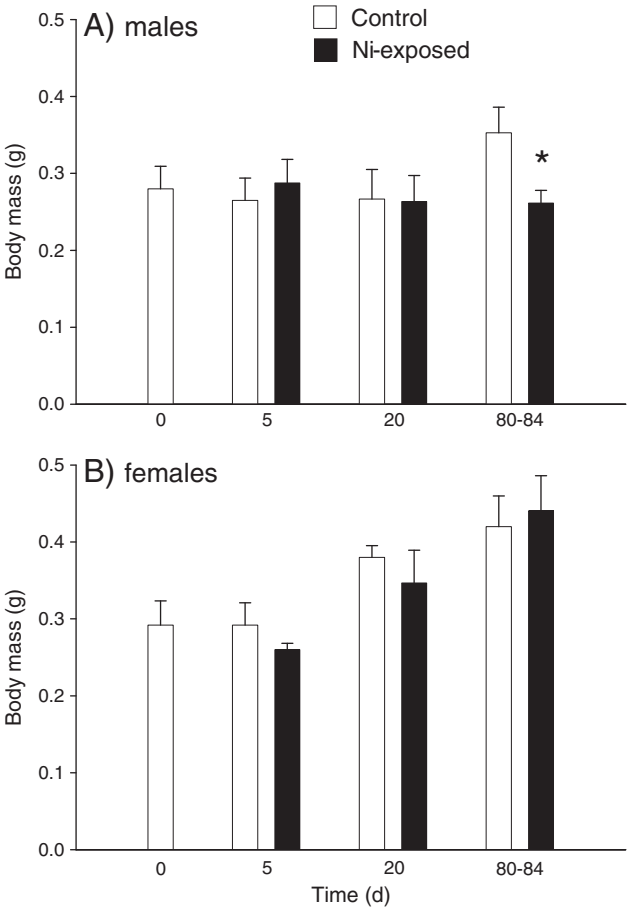


**Fig. 1.** Body weight of (A) male and (B) female zebrafish over the course of the experiment. Bars represent means ± SEM. $N = 4$–6 per sex in the control and nickel treatments at 0, 5 and 20 days, and $N = 22$–25 per sex at 80–84 days. Weights were measured on fish that were sampled for tissue Ni analysis on days 0, 5, 20 and 80, and also sampled on day 84 for other tests. Sex was verified by direct observation of the gonads. An asterisk (*) indicates a significant difference between the control and Ni treatments as determined with a Student's $t$-test, with the Bonferroni correction for multiple comparisons.

### 3.2. Metabolic rate

Routine metabolic rate was not significantly different between the control and dietary Ni treatments; oxygen consumption rates were 18.8 ± 1.9 μmol $O_2$/g/h and 19.4 ± 0.7 μmol $O_2$/g/h, respectively ($N =$ 6 pools of 3 fish; $P = 0.77$).

### 3.3. Tissue Ni accumulation

In tissues of fish fed the control diet, Ni concentrations varied by a factor of 14.9. The greatest amount of Ni was observed in the carcass (average over the experiment = 2.25 μg Ni/g wet weight), while the lowest was in the liver (0.151 μg Ni/g wet weight) (Table 2). Tissue Ni concentrations from highest to lowest levels in fish fed the control diet were as follows: carcass > vertebrae > ovary > gut > muscle > brain > eye > gill > blood > liver (Table 2).

Over the first 20 days of the experiment, there were no differences in tissue Ni concentrations between treatments, in any tissue (Table 2). Increases in Ni burdens with dietary Ni exposure were observed at 80 days, in a subset of tissues: gut (25% increase over controls; $P =$ 0.002), vertebrae (34%; $P = 0.003$), brain (44%; $P = 0.002$) and muscle (15%; $P = 0.002$) (Table 2). Similar to control tissues, Ni concentrations in tissues at 80 days of dietary Ni exposure varied by a factor of 16.4, and the order of highest to lowest levels was as follows: vertebrae > carcass > ovary > gut > muscle > brain > gill > eye > blood > liver (Table 2). There were no significant differences in tissue Ni concentrations between sexes.

### 3.4. Waterborne $^{63}$Ni uptake

After 82 days of exposure, fish fed the elevated Ni diet had a $^{63}$Ni uptake rate that was 4.5-fold higher than control fish (control = 0.041 ng Ni/g fish/h, Ni = 0.185 ng Ni/g fish/h; $P = 0.023$) (Fig. 2). These rates represent 0.0020% and 0.0088% of the total whole-body Ni that is turned-over per hour in the control and Ni treatments, respectively. At the end of the flux experiment, total Ni concentrations in the water were 2.0 μg Ni/L and 3.2 μg Ni/L in the control and Ni treatments, respectively.

**Table 2**
Tissue Ni concentrations over the course of the 80-day exposure. Values are in μg Ni/g tissue, wet weight. $N = 8$–10 at 0, 5, 20 days and $N = 12$–14 at 80 days, except ovary where $N = 3$–5. Values are means ± SEM. Values from the elevated Ni treatment with an asterisk (*) are significantly different than the control value at the same time point as determined with a Student's *t*-test, with the Bonferroni correction for multiple comparisons.

| | 0 days | 5 days | 20 days | 80 days |
|---|---|---|---|---|
| Gut—control | 1.00 ± 0.08 | 0.94 ± 0.06 | 0.90 ± 0.05 | 0.83 ± 0.05 |
| Gut—Ni | | 0.88 ± 0.07 | 0.93 ± 0.04 | 1.03 ± 0.04 * |
| Gill—control | 0.29 ± 0.04 | 0.30 ± 0.03 | 0.25 ± 0.03 | 0.35 ± 0.04 |
| Gill—Ni | | 0.35 ± 0.04 | 0.30 ± 0.02 | 0.38 ± 0.03 |
| Vertebrae—control | 1.81 ± 0.08 | 1.81 ± 0.12 | 1.78 ± 0.27 | 1.96 ± 0.16 |
| Vertebrae—Ni | | 3.40 ± 0.96 | 2.17 ± 0.07 | 2.62 ± 0.11 * |
| Ovary—control | 1.71 ± 0.09 | 1.63 ± 0.05 | 1.69 ± 0.22 | 1.63 ± 0.06 |
| Ovary—Ni | | 1.49 ± 0.09 | 1.80 ± 0.21 | 1.80 ± 0.23 |
| Liver—control | 0.14 ± 0.05 | 0.12 ± 0.06 | 0.20 ± 0.05 | 0.15 ± 0.03 |
| Liver—Ni | | 0.13 ± 0.04 | 0.24 ± 0.05 | 0.16 ± 0.03 |
| Brain—control | 0.42 ± 0.06 | 0.43 ± 0.03 | 0.45 ± 0.07 | 0.39 ± 0.05 |
| Brain—Ni | | 0.49 ± 0.03 | 0.45 ± 0.05 | 0.56 ± 0.02 * |
| Eye—control | 0.41 ± 0.01 | 0.31 ± 0.02 | 0.29 ± 0.02 | 0.26 ± 0.02 |
| Eye—Ni | | 0.34 ± 0.03 | 0.32 ± 0.02 | 0.22 ± 0.01 |
| Muscle—control | 0.79 ± 0.03 | 0.61 ± 0.03 | 0.60 ± 0.01 | 0.67 ± 0.02 |
| Muscle—Ni | | 0.66 ± 0.06 | 0.57 ± 0.02 | 0.76 ± 0.02 * |
| Blood—control | 0.15 ± 0.08 | 0.17 ± 0.02 | 0.14 ± 0.02 | 0.18 ± 0.01 |
| Blood—Ni | | 0.21 ± 0.02 | 0.15 ± 0.02 | 0.17 ± 0.01 |
| Carcass—control | 2.19 ± 0.19 | 2.33 ± 0.30 | 2.03 ± 0.21 | 2.44 ± 0.16 |
| Carcass—Ni | | 1.95 ± 0.12 | 2.32 ± 0.19 | 2.50 ± 0.30 |
| Total—control | 1.84 ± 0.23 | 2.01 ± 0.24 | 1.68 ± 0.25 | 2.06 ± 0.13 |
| Total—Ni | | 1.56 ± 0.20 | 1.96 ± 0.22 | 2.11 ± 0.22 |

### 3.5. Reproductive capacity

The control treatment spawned an average of 57.2 total eggs per female over 4 days. The elevated Ni treatment spawned 65% fewer eggs, or a total of 20.3 eggs per female over four days, a significant decrease ($P = 0.032$; Fig. 3).

### 3.6. Endocrinology—estradiol and cortisol

hCG increased $E_2$ secretion by 2.6-fold in the control treatment follicles ($P = 0.0003$) and 2.8-fold in dietary Ni treatment follicles ($P = 0.0165$) (Fig. 4A). Dietary Ni treatment had no effect on basal ($P = 0.97$) or hCG-stimulated ($P = 0.81$) $E_2$ secretion (Fig. 4A).

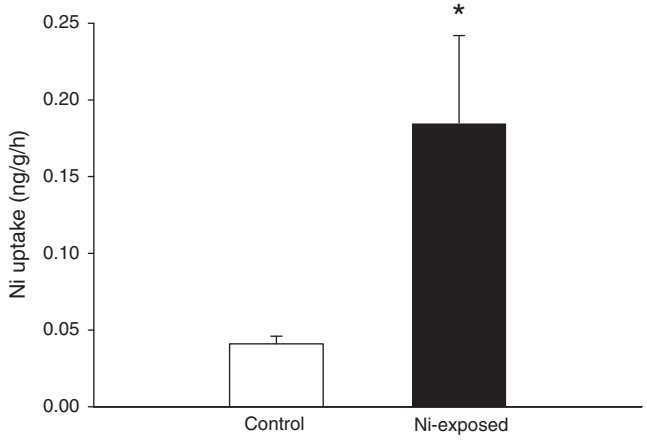


**Fig. 2.** Waterborne uptake of radiolabeled $^{63}$Ni over 4 h in zebrafish from the control and dietary Ni treatments at 82 days of exposure. Bars represent means ± SEM. $N = 8$. An asterisk (*) indicates a significant difference between treatments as determined with a Student's *t*-test ($P < 0.05$).

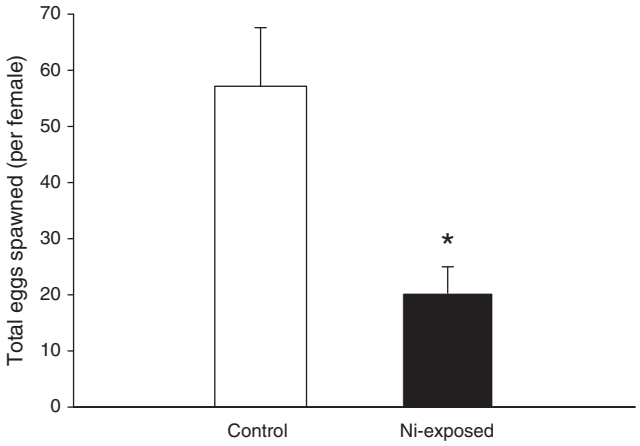


**Fig. 3.** Total eggs spawned per tank divided by the total number of females in the tank (verified through gonadal observation at the end of the experiment). Eggs were collected every day for 4 days starting on day 75 of the exposure. Bars represent means ± SEM. $N = 3$ (tanks). An asterisk (*) indicates a significant difference between treatments as determined with a Student's *t*-test ($P < 0.05$).

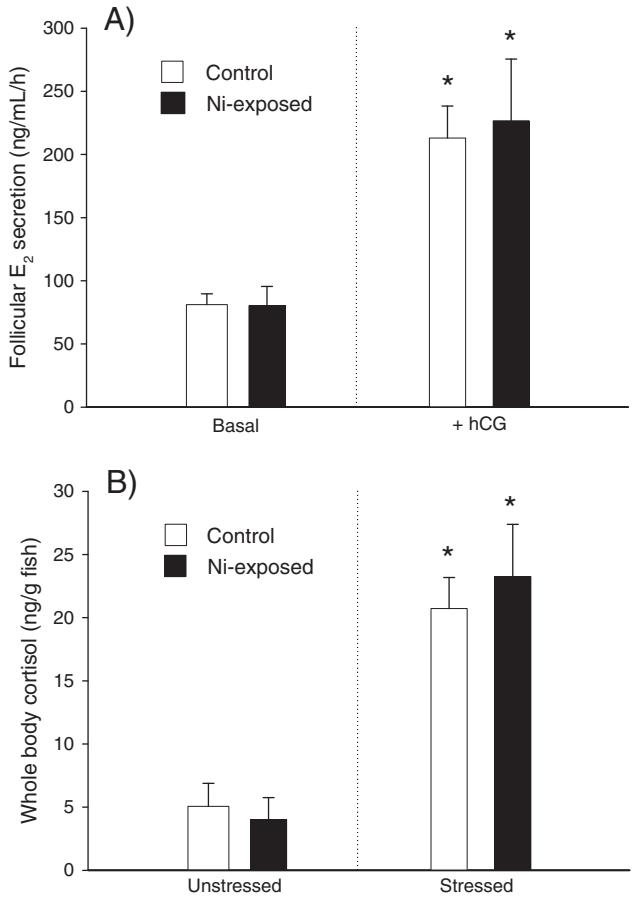


**Fig. 4.** (A) Estradiol ($E_2$) secretion by zebrafish ovarian follicles in vitro over 1.5 h from control and Ni-exposed females. Basal and human chorionic gonadotropin (hCG)-stimulated(10 I.U./mL) $E_2$ secretion rates were determined at 84 days of exposure. $N = 9$. (B) Basal (unstressed) and stress-induced whole-body cortisol concentrations in control and Ni-exposed zebrafish at 104 days of exposure. For the stress-induced cortisol measurement, fish were air-exposed for 1 min, then returned to water for a 20 min period. Fish were sampled after 20 min. In both graphs, bars represent means ± SEM ($N = 8$). There was no effect of Ni treatment compared to controls on $E_2$ or cortisol in basal/unstressed or + hGC/stressed conditions as determined by a Student's *t*-test ($P < 0.05$). Bars with an asterisk (*) are significantly different from the basal/unstressed condition within a treatment (Student's *t*-test; $P < 0.05$).

In addition, dietary Ni did not increase basal (unstressed) whole-body cortisol concentrations ($P = 0.70$) (Fig. 4B). While an acute stressor increased cortisol concentrations 4.1-fold in control animals ($P = 0.0003$) and 5.8-fold in dietary Ni treated animals ($P = 0.0008$), there was no difference between the stressed cortisol levels ($P = 0.61$) (Fig. 4B).

### 3.7. Egg and larval Ni and larval Ni tolerance

Eggs from Ni-exposed adults had 5.2-fold greater Ni concentrations compared to controls (control = 0.043 ng Ni/egg, Ni = 0.224 ng Ni/egg; $P = 0.001$) (Fig. 5A). However, by 4 dpf (2 dph), the Ni concentrations in larvae from the Ni treatment had dropped to levels observed in control larvae (control = 0.059 ng Ni/larva, Ni = 0.039 ng Ni/larva; $P = 0.31$) (Fig. 5A).

Control larvae had an LC50 of 24.3 mg Ni/L (95% CI = 23.5–25.1 mg/L), while larvae from Ni-exposed adults showed a significantly increased tolerance to waterborne Ni with an LC50 35% higher at 32.8 mg Ni/L (95% CI = 31.6–34.1 mg/L) (Fig. 5B).

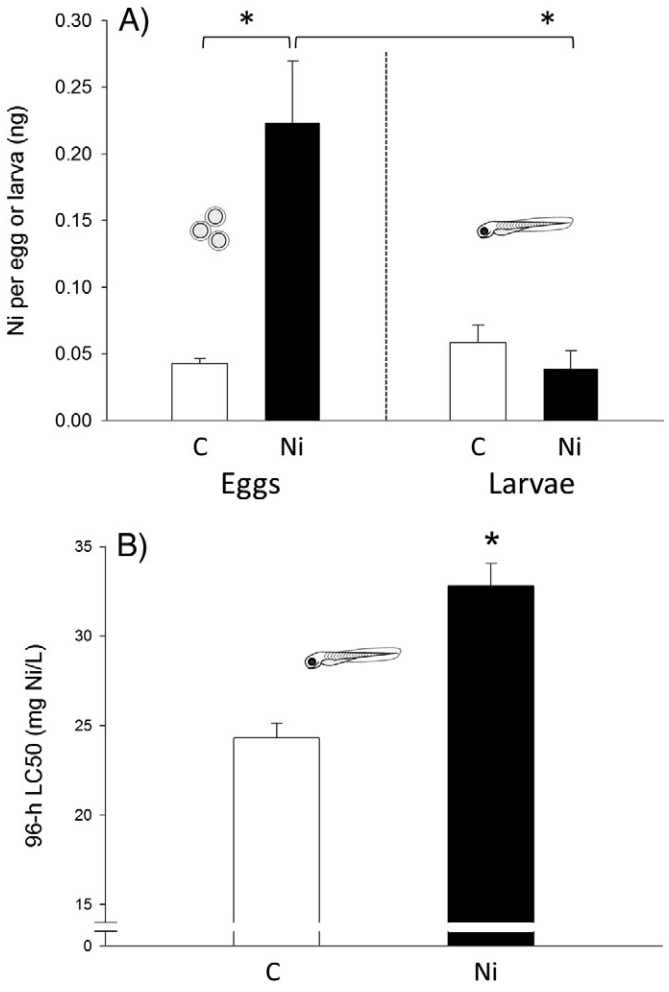


**Fig. 5.** (A) Ni content of eggs (2 hours post fertilization) and larvae (4 days post fertilization) from adults in the control and dietary Ni treatments. Eggs were collected between 75 and 78 days of the exposure, some of which were sampled for Ni measurements and some were incubated to the larval stage. $N = 14$ (Ni treatment) or 19 (control) pools of 25 eggs, and $N = 4$ pools of 10 larvae. Bars with an asterisk (*) are significantly different as determined by a Student's $t$-test ($P < 0.05$). (B) The 96-h Ni LC50s for 4 dpf larvae from adults in the control and dietary Ni treatments. An asterisk (*) indicates a significant difference as determined by the lack of overlap of the 95% CIs.

## 4. Discussion

An environmentally relevant 31-fold elevation of dietary Ni (above the control diet to 116 μg Ni/g) resulted in elevated Ni concentrations in only a few tissues, suggesting either low bioavailability through the diet and/or strong homeostatic regulation of this metal. While survival, metabolic rate, the corticosteroid stress response and ovarian estradiol secretion were not affected, chronic dietary Ni exposure nevertheless decreased the number of eggs spawned per female and the growth of male zebrafish, suggesting potential population-level impacts. There were also other physiological effects that were observed with Ni-exposure, such as increased branchial Ni uptake rates and increased Ni tolerance of the offspring.

### 4.1. Negative impacts of dietary Ni on growth and reproduction

Ni treatment decreased the growth of male zebrafish after 80 days of exposure. There was no effect on female body weight. Previous Ni studies generally did not observe effects on fish growth. For example, two of the few studies that tested the long term effects of dietary Ni were those of Ptashynski et al. (2002) and Ptashynski and Klaverkamp (2002), who fed diets containing 0, 10, 100 or 1000 μg Ni/g diet to lake whitefish and found no effects on growth rates over 104 days. An earlier, shorter study by the authors (Ptashynski et al., 2001) fed lake trout and whitefish diets with 0, 1000 and 10000 μg Ni/g over 18 days and observed lower weights in lake trout at the highest Ni concentration. However, this was likely due to the observation that at 10000 μg Ni/g diet, both species refused to consume the food that was offered after 3 to 5 meals (Ptashynski et al., 2001). In addition to the dietary studies, a number of waterborne Ni exposures have not observed a growth effect on fish (e.g., Brix et al., 2004; Deleebeeck et al., 2007; Chowdhury et al., 2008). However, previous studies did not report growth rates for each sex separately. The effect of dietary Ni on the growth of males does not appear to be due to a metabolic cost of Ni exposure since there was no difference in routine metabolic rate between treatments. In addition, Ni did not impact basal or stress-induced cortisol levels in either sex. Cortisol was examined due to previously reported studies on the effects of hypothalamic-pituitary-interrenal (HPI) stress axis activation on fish growth (Mommsen et al., 1999; Barton, 2002).

Dietary Ni also decreased the reproductive capacity of zebrafish; from 75 to 78 days of exposure, the number of eggs spawned per female was 65% lower in the Ni treatment. A previous study with fathead minnows (*Pimephales promelas*; another cyprinid) examined the effects of waterborne Ni on reproduction (Pickering, 1974). Chronic exposure to 0.73 and 1.6 mg Ni/L (2.7% and 5.9% of the 96-h LC50, respectively) prior to and throughout the spawning period reduced the average number of eggs collected per female by 69% and 98%, respectively (Pickering, 1974). The effects of Ni on reproduction do not appear to be due to changes in endocrine status; ovarian follicle $E_2$ secretion was presently not affected by dietary Ni treatment. In addition, there was no effect of Ni on basal or post-stress cortisol levels (activation of the HPI axis can inhibit the HP-gonadal axis at multiple sites; Wendelaar Bonga, 1997; Lethimonier et al., 2000; Alsop et al., 2009).

The effects of Ni on reproduction and growth of males may be due to Ni accumulation in the brain, which had the greatest increase in Ni concentrations of all tissues that were presently analyzed (Fig. 6). Previously, male and female fathead minnows exposed to another metal, lead (Pb), accumulated similar Pb concentrations in bone (brain Pb was not measured; Rademacher et al., 2005). However, effects on endocannabinoid (eCB) levels in the brain were only observed in males (Rademacher et al., 2005). eCBs are neuromodulatory lipids that control neurotransmitter release and play roles in appetite (Scherma et al., 2013) and reproduction (Coddington et al., 2007; Cottone et al., 2013). Given that Ni accumulation in the present study was highest in the brain and bone, effects of Ni on eCB levels could be a factor in the lower growth of males and impacts on reproduction.

The mechanism underlying this significant effect of Ni on reproductive capacity remains unknown. Although examining female-based end points is a logical start, a decrease in the number of eggs spawned may also be a male-related issue, especially given the effect of Ni on the growth of males. For example, the multipart courtship behaviors of zebrafish are primarily a male activity and constitute a mechanism to stimulate a female to release a clutch of eggs (Darrow and Harris, 2004). The female's role in courtship is more simply to accept or decline the male's behaviors (Darrow and Harris, 2004). Altered male courtship behaviors from pharmaceutical exposure have been shown to reduce the reproductive capacity of female zebrafish (Galus et al., 2014).

### 4.2. Ni accumulation

In control zebrafish, Ni was ubiquitously distributed in all tissues that were analyzed, similar to previous studies with other fish (e.g., Ptashynski and Klaverkamp, 2002; Chowdhury et al., 2008; Leonard et al., 2014). In zebrafish, concentrations varied 15-fold between tissues, where liver had the lowest Ni concentrations, and carcass the highest (likely due to the higher levels of Ni in the bone, kidney and skin/scales; e.g., Ptashynski and Klaverkamp, 2002). Ni is an enzyme cofactor in plants and bacteria (Hänsch and Mendel, 2009; Higgins et al., 2012; Pyle and Couture, 2012), and although there is some evidence it may be a micronutrient in animals, a specific role has not yet been identified (Pyle and Couture, 2012).

There were only modest increases in tissue Ni concentrations with elevated dietary Ni-exposure in zebrafish, and these were only observed in select tissues after 80 days. Surprisingly, Ni concentrations in the brain had the greatest increase with Ni exposure (44% increase), followed by vertebrae (34%), gut (25%) and muscle (15%) (Fig. 6). This magnitude and pattern of Ni accumulation is in stark contrast to the study on dietary Ni in lake whitefish (Ptashynski and Klaverkamp, 2002). Whitefish fed 110 μg Ni/g diet (similar to 116 μg Ni/g in the present study) experienced several-fold increases of Ni in most tissues including intestine, stomach, liver, kidney, skin, scales and gill (Ptashynski and Klaverkamp, 2002). Notably, zebrafish readily accumulate Ni during waterborne exposures (Searle, 1988), so this difference appears to be related to dietary Ni uptake. We propose two hypotheses to explain the lower Ni accumulation in zebrafish with dietary exposure. These two hypotheses are not mutually exclusive. First, less Ni accumulation in zebrafish compared to whitefish may be due to the lack of an acidified stomach in the cyprinid species. Approximately 15% of teleosts do not develop an acid-secreting portion of their digestive system (Kleinow and James, 2001; Wilson and Castro, 2011). Examples include fish from Cyprinidae (e.g., zebrafish, carp), Catostomidae (sucker) and Labridae (wrasse) among many others. Studies in rainbow trout have shown the stomach and pyloric caeca are the primary sections of the gut that facilitate Ni uptake; these two sections were responsible for 79% and 19% of the total Ni absorbed from the diet, respectively (Leonard et al., 2009). Second, the concentration of dietary Ni in the present study (116 μg/g diet) may not have exceeded a level that would initially overwhelm the zebrafish's Ni homeostatic mechanisms such as excretion by the kidneys (Pane et al., 2005). In contrast, whitefish fed 110 μg Ni/g diet showed Ni accumulation in all gut segments, kidney and scales after 10 days. By 31 days, the liver and gill also showed increased Ni burdens (Ptashynski and Klaverkamp, 2002). It appears zebrafish may be better able to transport and excrete Ni than whitefish. Fish can upregulate renal Ni clearance ratios and reduce membrane Ni permeability upon Ni exposure (Pane et al., 2005; Pane et al., 2006).

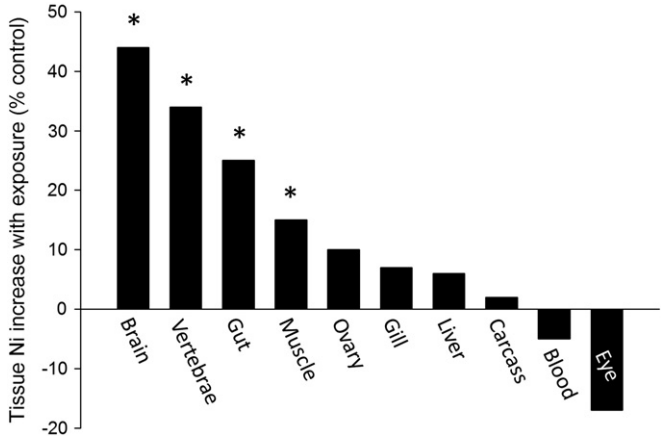


**Fig. 6.** The mean increase in tissue Ni concentrations of dietary Ni exposed fish as a percent (%) of the control concentration at 80 days of the experiment. Please see Table 2 for absolute Ni concentrations in tissues from control and Ni-exposed treatments. Bars with an asterisk (*) are significantly different than the control value as determined with a Student's *t*-test, with the Bonferroni correction for multiple comparisons.

In rainbow trout, pre-exposure to waterborne Ni decreased the uptake of Ni through the gut (Chowdhury et al., 2008), presumably a protective mechanism. However, we observed that after 80 days of dietary Ni exposure, there was a 4.5-fold increase in branchial $^{63}$Ni uptake from the water. This may be indicative of an increased numbers of Ni transporters in the gill that facilitate Ni depuration after a meal. The relatively slow rates of uptake, 0.002% (control) and 0.009% (Ni treatment) of the total whole-body Ni turned over per hour, suggest this Ni may be incorporated into tissues such as bone and scales (Ptashynski and Klaverkamp, 2002).

Waterborne Ni studies have found that plasma is the major sink for Ni accumulation in fish (Pane et al., 2004a, 2004b, 2005; Chowdhury et al., 2008). In the present study, the concentration of Ni in the blood was not elevated at any time point (Table 2; Fig. 6), suggesting zebrafish are able to quickly transport and excrete excess dietary Ni. Zebrafish can quickly lose Ni they have accumulated from a waterborne exposure; after a 7-day exposure, zebrafish were returned to control water where they proceeded to lose 45% of the accumulated whole-body Ni within 10 h (Searle, 1988). In the present study, fish were not fed for approximately 18 h prior to sampling, which may be enough time to excrete much of the Ni absorbed from the diet.

The greatest increase in Ni after 80 days of dietary exposure was in the brain (Fig. 6). Few studies have analyzed Ni accumulation in the brains of fish with Ni exposure. One study that utilized short-term (96 h), waterborne Ni exposures found no accumulation of Ni in the brains of rainbow trout or round gobies (Leonard et al., 2014). However, in mammals, Ni can change neuronal activity through effects on the *N*-methyl-D-aspartate receptor channels (Gavazzo et al., 2011) and is also a well-known selective blocker of T-type calcium channels (Lee et al., 1999; Kang et al., 2006; Nosal et al., 2013). Ni has also been shown to affect enzyme activities in the brain of rats, including $Na^+/K^+$ ATPase and acetylcholinesterase (Liapi et al., 2011). Given these effects of Ni on neuron function and the observed accumulation of Ni in the zebrafish brain, future Ni studies may consider monitoring concentrations of Ni in the brain and attempt to determine whether accumulation is responsible for the observed effects of Ni.

Although eggs from Ni-exposed adults possessed higher Ni concentrations than controls (see discussion below, Fig. 5A), whole ovary samples did not (Table 2). The reproductive trials occurred immediately before the 80-day sampling time point, and the ovulation and spawning off eggs may have decreased the ovarian Ni burden just prior to sampling.

### 4.3. Egg and larval Ni and offspring Ni tolerance

The eggs from adults in the dietary Ni treatment contained 5.2-fold more Ni than control eggs (at 2 hpf). However, by 4 dpf, Ni concentrations in the larvae had dropped to those observed in larvae from control adults. The ability to quickly depurate Ni was also observed in adult

zebrafish after waterborne Ni exposures (discussed above; Searle, 1988).

Larvae from Ni-exposed adults had a higher tolerance to waterborne Ni than their control counterparts (increased 96-h LC50). Although the maternally deposited Ni burden they originally possessed at the early embryo stage was depurated by 4 dpf, it may have been sufficient to stimulate Ni-acclimation of the embryo. In this respect, the zebrafish larvae exhibited some similarities to the offspring of maternal *Daphnia magna* that had been chronically exposed to elevated waterborne Ni (21 μg Ni/L) for 21 days (Pane et al., 2004c). These F1 generation daphnids exhibited greatly elevated whole-body Ni burdens and an 87% increase in 48-h LC50. In a previous study on cadmium (Cd), adult female zebrafish were exposed to three different waterborne Cd concentrations plus a control for 72 h (Wu et al., 2012). Fish were then bred with males and the offspring raised to 72 hpf. At this point, larvae from exposed females had concentration-dependent increases in Cd concentrations, expression of metallothioneins (mt2 and smtB), and metallothionein (MT) protein levels (Wu et al., 2012). Larvae from female fish in the higher Cd treatments also had increased survival when exposed to a toxic concentration of waterborne Cd for 48 h (Wu et al., 2012). The mechanism offering protection to larvae from Ni-exposed adults against waterborne Ni challenges is unknown. Ni is not thought to induce MTs to the same extent as metals such as Cd. For example, while whitefish fed Ni-supplemented diets accumulated large amounts of Ni in all tissues analyzed (Ptashynski and Klaverkamp, 2002), MT protein levels were not elevated in the kidney, liver or gill (Ptashynski et al., 2002). In addition, there were only two instances of elevated MT in the intestines and pyloric caeca across all times and dietary Ni concentration (and one decrease in MT with Ni exposure) (Ptashynski et al., 2002). Regardless, it would be interesting to examine the embryonic and larval expression of some of the many genes that are modulated with Ni exposure in fish (e.g., Bougas et al., 2013).

## 5. Conclusion

There are limited dietary data pertaining to the chronic effects and accumulation of Ni in fish. A previous study on dietary Ni with lake whitefish observed widespread Ni accumulation but few effects (primarily histopathology; Ptashynski et al., 2002). In contrast, we observed relatively little Ni accumulation coupled with a variety of effects in zebrafish. These include population-level effects such as reproductive capacity, growth of males and changes in offspring physiology. There are many possible reasons underlying this disparity between the two studies, which highlights the need for more data pertaining to the chronic accumulation and effects of dietary metals.

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