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Developmental exposure to heavy metals alters visually-guided behaviors in zebrafish

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Abstract

This laboratory-based study determined the consequences of heavy metal exposure using zebrafish *Danio rerio*. Embryos were transiently exposed to environmentally-relevant concentrations of cadmium or nickel until 72 h postfertilization (hpf), then they were returned to system water and allowed to grow until 7, 9, and 11 days postfertilization (dpf), when they were examined morphologically and behaviorally. Morphological measures of notochord length, eye diameter, and inter-eye distance were not different across treatments; however, significant differences in optomotor responses were observed in treated larvae at all ages tested. These results suggest that initial developmental exposure to cadmium and nickel significantly impacts visually-guided larval behavior. The absence of significant differences in gross morphology suggests that the effects of these metals are subtle and may occur at the cellular level. By using this ecologically relevant model and pollutant type, this study has broad application and implications with regard to safe levels of contaminant in drinking water and freshwater ecosystems.

Key words: cadmium, nickel, optomotor response, zebrafish.

Industrialization has increased the concentrations of toxins and toxicants found in aquatic and terrestrial ecosystems. These increases include both man-made compounds, such as endocrine disrupting chemicals and pharmaceuticals, and toxicants, naturally occurring molecules, such as metals and metalloids. Toxicants are typically present in low amounts and the additional industrial influx causes their levels to increase to potentially lethal levels.

Heavy metals, naturally-occurring environmental substances defined as having a density that is at least 5 times greater than water (Tchounwou et al. 2012), can be either nutritionally or non-nutritionally essential, depending on their involvement in normal cell functions (Overhoff and Forth 1978; Chang et al. 1996). Given that heaviness and toxicity are interrelated, it is not surprising that small concentrations of heavy metals have been shown to induce environmental and physiological toxicity (Duffus 2002). Indeed, nonessential heavy metals are reported to have toxic effects at fairly low doses through a variety of cellular metabolic pathways (Chang et al. 1996; Goyer 1997; Tchounwou et al. 2001; Wang and Shi 2001; Tchounwou et al. 2004; Bertin and Averbeck 2006). In addition, heavy metals bioaccumulate, exerting increased damage when exposure occurs for long periods of time (Mountouris et al. 2002). Heavy metal exposure to both human and nonhuman organisms primarily results from anthropogenic sources of pollution such as mining, industrial production, and agricultural use (Nriagu 1989; Herawati et al. 2000; He et al. 2005); additional metal exposure can come from natural forces such as volcanic eruption, terrestrial weathering, and glacial effluent (Duffus 2002; Bradl 2005). Heavy metals have been measured in both the water column and the sediment, with recent reports indicating that the amount of metal(s) in the water is a much better predictor for environmental health in aquatic ecosystems than sedimentary metal levels (Tao et al. 2012; Abdel-Baki et al. 2013).

Here, the impact of developmental exposure to heavy metals on visually-guided behaviors was assessed using the divalent cations cadmium and nickel. Cadmium is used frequently in the production of alloys and batteries (Flick et al. 1971). Nickel is also used in industry as it has a slow oxidation rate at room temperature and is therefore considered an anticorrosive agent. Consequently, nickel is used in metal platings to prevent rusting or tarnishing (Dallinger

et al. 1987). Both are reported to have damaging effects to ecosystems and the organisms within them (Moore and Ramamoorthy 2012), including negatively impacting development and anatomy of fish (Cheng et al. 2000; Kienle et al. 2009; Jarić et al. 2011; Oliva et al. 2012; Squadrone et al. 2013). For example, swimming activity in zebrafish larvae is adversely affected by waterborne nickel exposure occurring either acutely (7.5–15 mg/L Ni) or over time (>10 mg/L Ni) (Kienle et al. 2009). Exposure to 100 μM cadmium from 5 h after fertilization until 24 h postfertilization affects retinal, brain, and muscle development in zebrafish (Chow and Cheng 2003; Chow et al. 2008, 2009). Zebrafish developmentally exposed to another heavy metal, methylmercury, have altered visually-guided behaviors and retinal physiology as adults (Weber et al. 2008). Thus, developmental processes are highly sensitive to heavy metal exposure. Building on this information, this study will examine how exposure to environmentally relevant concentrations (ppb) of waterborne cadmium and nickel affect visually-guided behaviors in larval zebrafish. Our expectation is that transient exposure to sublethal doses of cadmium and nickel will have a significant effect on growth and/or behavioral development as evidenced through a visually-guided behavioral assay.

Materials and Methods

General

Embryos for this project were obtained by spawning in-house zebrafish adults at the Zebrafish Ecotoxicology, Neuropharmacology and Vision Laboratory (ZENV) at American University Fish Facility throughout 2015. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at American University.

Adult wildtype zebrafish, *Danio rerio*, were maintained in the laboratory at 28–29 °C on a 14-h light: 10 h dark photoperiod and fed daily (as in Westerfield (2000); Lawrence (2007)). To obtain embryos, adults were placed into a 16" breeding chamber with a mesh bottom the night before, with multiple males and females (up to 8–10 of each gender) to insure genetic variability. The following morning, within an hour of light onset, fertilized eggs were collected from the bottom of the breeding chamber and staged (at shield to 75% epiboly). Embryos were then placed in system water in 100 mm petri dishes and maintained at 28–29 °C and on the same photoperiod as adults until transfer into the designated experimental containers.

Treatment groups

Petri dishes (100 mm) in water baths, at the same environmental conditions as adults, were used to house the eggs and embryos, both treated and control, for the duration of the experimental time period. At 6 h postfertilization (hpf) (Shield Stage), zebrafish ($N = 20$ per dish $\times$ 2 dishes per treatment) were placed into one of five treatments until 72 hpf: (1) system water control, (2) low cadmium (0.05 PPB), (3) high cadmium (2.0 PPB), (4) low nickel (0.5 PPB), or (5) high nickel (15 PPB). The cadmium concentrations were selected as low, but nominal variations of concentrations measured in Spain (0.7–8.9 μg/L; Oliva et al. 2012). Waterborne nickel concentrations can range from 1 μg/L to 10 μg/L, but can increase to 100–1,000 μg/L in waterways near urban areas so the concentrations chosen were low spectrum, nominal concentrations (Ku et al. 2015). Additional sources from community and county water reports from Sudbury, Ontario, Canada and Washington, DC, United States of America, further validated our concentrations as ecologically relevant (Annual Water Quality Reports; 2016 Integrated Report, DC). In all locations sampled, the concentrations of metals were either equivalent or higher than the concentrations used in this study. System water consisted of Deerpark® Brand 100% Natural Spring Water with methylene blue added to reduce fungal growth. Methylene blue was not added to the behavioral testing dishes. Information collected from Deerpark® demonstrated no detectable levels of cadmium or nickel ions in the water and that levels of calcium (which may act as an internal competitive binding agent with both cadmium and nickel) could be between 4.2 mg/L and 66 mg/L (Bottled Water Quality Report 2015). Levels of other minerals and inorganic materials in the Deerpark® water were well within the EPA standards as reported in the Bottled Water Quality Report and were not perceived as likely to act as a competitive binding agent or alter the ability of the cadmium or nickel to be absorbed into the fish.

Once treatments began, experimental dishes were checked and replenished daily to ensure constant water metal concentration and to remove any debris. Concentrations were not verified through any additional measures other than the original dilution of stock solution (Sigma-Aldrich chemical; product 265330. CAS 7440-43-9; product 266965. CAS 7440-02-0.) All stock solutions were fully dissolved in water and made bioactive as a chloride solution. The stock solution was diluted into working solutions in the PPB concentrations, which supplied water for the duration of the experiment. It has been demonstrated that neither cadmium nor nickel degrades in neutral pH water (Boyle et al. 1976; Kienle et al. 2009).

At 72 hpf, all larvae were removed from treatment, transferred to new petri dishes containing system water until 11 d (days) pf. During this time, larvae were fed AP100 (Aquatic Habits/Pentair) once per day with excess food removed 45 min after feeding.

Optomotor response

At three ages (7 dpf, 9 dpf, and 11 dpf), larvae were tested behaviorally by assessing their optomotor response (OMR). These ages were selected based on (1) the development of the OMR (which begins at around 7 dpf; Neuhauss 2003), and (2) prior zebrafish vision-based behavioral studies (Kienle et al. 2009; Orger et al. 2004). The OMR was assessed at rearing temperature (28–29 °C). In particular, at each age, 10–20 randomly-chosen larvae were transferred to a separate 100 mm petri dish and placed into the behavioral chamber. Larvae were allowed to acclimate for 2 min, after which time a startle response, a well-known behavior in fish (Kimmel et al. 1974), was elicited to ensure that all the larvae being tested were able to swim (Parker and Connaughton 2007; Reider and Connaughton 2014). This was done twice before each behavioral analysis and qualitatively scored. Any larvae that did not respond to the startle stimulus were noted and removed from the behavioral chamber.

Larval OMRs were elicited using a Fourier motion stimulus in the form of a rotating radial grating presented beneath the fish. Our rotating, radial, black and white grating is modified from a classic OMR behavioral task (Rock and Smith 1986). In brief, the recording petri dish was placed on top of a 36-inch Dell computer monitor. The monitor was color calibrated using a Spectroscan 670 (Photoresearch Corporation). A 12-inch diameter-rotating black and white radial grating stimulus was shown on the monitor and rotated (clockwise and counterclockwise) with a control period of white light shown in between. Each direction and the white light were shown to the fish for 30 s. The white screen acted as a negative control and allowed the fish to rest between stimuli. The sequence was run twice per group of larvae to ensure behavioral consistency. The response of the larvae during the entire protocol was videotaped using a Canon FS40 handheld video camera mounted directly above the OMR apparatus.

A positive OMR response occurs when the fish swim in the direction of the stimulus, whereas a negative OMR response occurs when fish swim in a direction opposite to the stimulus (Neuhauss 2003). In our assay, we assessed positive OMR responses by documenting, using scan sampling (for review see Altmann (1974)) the orientation of each individual larva in the dish at 5-s intervals during the 30-s stimulus presentation. Larval orientation was scored from playback of video recordings that were watched in real time using VideoLAN VLC player (Version 2.2.1.). The 5-s interval was measured using the time indicated on the recordings and confirmed using a repeated stopwatch. To avoid observer bias, recordings were analyzed "blind" in that the observer was unaware which trial was being assessed until after all the data were collected.

Larvae were counted as swimming with the stimulus (positive OMR) if the head of the larva was pointed in the same direction as stimulus movement and/or oriented at a 55°–105° angle to the midline of the closest white bar, also in the direction of stimulus movement. Larvae that were either not swimming or were swimming with the stimulus, but their head was not at the indicated angle, were scored as "no response." Larvae that were actively swimming against the stimulus were recorded as showing a negative OMR, but were pooled with the "no response" category prior to analysis.

Once the responses of individual larvae were determined, the percentage of larvae with a positive OMR for both stimulus directions (clockwise and counterclockwise) in each testing dish was calculated. In an effort to reduce our inherent pseudoreplication presented in the data, statistical differences were analyzed using a generalized linear mixed effects model fit by maximum likelihood assuming a binomial (logit) distribution was used to assess optomotor behavior. The link function is represented by $g(x) = \log(x/(1-x))$. This model described the effect of the stimulus and the interaction between treatment and age on OMR. A generalized linear mixed effects model uses both random and fixed effects to better deal with missing values than other analyses such as ANOVA. The model estimated random effects for the interaction between treatment and age among fish from the same petri dish. The repeated measures of orientation were also taken into account by the random effects in the model. The model estimated fixed effects for both treatment and the interactions between treatment and age. The model assumes errors have constant variance, are independent, and are normally distributed as per standard generalized linear mixed effects model. The test statistic for which the P values are reported is the Wald test for regression coefficients. All analyses were conducted in R (lme4 package) at an α-level of 0.05.

Anatomical analysis

At the completion of the behavioral tests, 5 randomly selected specimens from each age and treatment group were euthanized in a 0.02% tricaine solution and fixed in 4% paraformaldehyde for anatomical analysis. The total sample size for morphological measures was 75 specimens. Fixed specimens were examined using a stereomicroscope (Olympus SZX16 Stereo Microscope) and photographed using an Olympus DP72 color camera and MetaMorph software. Growth measurements, including notochord length, eye diameter, and intraocular space were measured with Image J. Notochord length (overall body length) was measured as the most anterior part of the head to the posterior portion of the tail. Eye diameter was measured as the length from the most anterior to the most posterior part of the eye. Intraocular space was measured as the left–right length between the midpoint of each eye. Anatomical measurements were collected for all treatment groups except the high nickel 9 dpf exposure group because these collected specimens degraded in postmortem fixative. Each measurement of external anatomy was conducted three times and averaged to reduce error. Statistical differences in morphological measurements were assessed across age and treatment using a 2-way ANOVA (SPSS, version 20), with $\alpha = 0.05$.

Results

Overall, survival in the experimental containers was comparable across treatment groups. In all, the concentrations of cadmium (0.05 PPB and 2.0 PPB) and nickel (0.5 PPB and 15 PPB) used were lethal to only ~5% of experimental larvae. Control larvae experienced a similar mortality rate of ~4%. Interestingly, overall growth was not severely affected by early developmental exposure to heavy metals (Figure 1); however, visually-guided behaviors were significantly altered (Figure 2), suggesting heavy metal exposure causes subtle effects that impact neuronal circuitry.

Notochord length

No significant differences in notochord length were observed across age or treatment (Figure 1A–C) ($F = 2.24$, $df_1 = 74$, $df_2 = 72$, $P = 0.170$).

Inter-eye distance

Prior to statistical analysis, raw inter-eye distance measurements were normalized to notochord length to adjust for individual variability. Mean inter-eye distance measurements were highly variable, particularly when measured in the 7 dpf and 11 dpf groups (Figure 1D–F). For both these ages, measurements from the low cadmium and high nickel treatment groups were lower than controls. However, there were no significant differences noted across age or treatment ($F = 0.785$, $df_1 = 74$, $df_2 = 72$ $P = 0.488$).

Eye diameter

To account for individual variability in growth, raw eye diameter measurements were normalized to notochord length prior to statistical analysis. There were no statistically significant differences in eye diameter (Figure 1G–I) ($F = 1.23$, $df_1 = 74$, $df_2 = 72$ $P = 0.344$).

OMRs

Age significantly impacted the OMR. Control fish responded with a more robust OMR as age increased ($P < 0.001$) (Figure 2A–C) and a greater percentage of fish were responding as age increased. For example, at 7 dpf, ~50% of control fish displayed a positive OMR (Figure 2A). By 9 dpf, the percentage increased to 75% and by 11 dpf ~92% of control fish displayed a positive OMR.

Treatment also significantly affected the OMR. At 7 dpf, fish exposed to either low cadmium, high cadmium, or low nickel had a reduced OMR, with < 25% of larvae displaying a positive response to the test stimulus. However, 7 dpf larvae in the high nickel group demonstrated a robust OMR with 82% of larvae showing a positive response. At 9 dpf, the number of larvae displaying a positive OMR decreased in all treatment groups, with the larvae from the high nickel group showing the greatest reduction in positive responders with only ~13% displaying a positive OMR (Figure 2B). By 11 dpf, all metal exposed larvae displayed a reduced OMR, with the high nickel group demonstrating the lowest percentage of positive OMR (~5%; Figure 2C). Statistical analysis revealed that the percentage of larvae within the low ($P < 0.01$) and high ($P < 0.001$) nickel

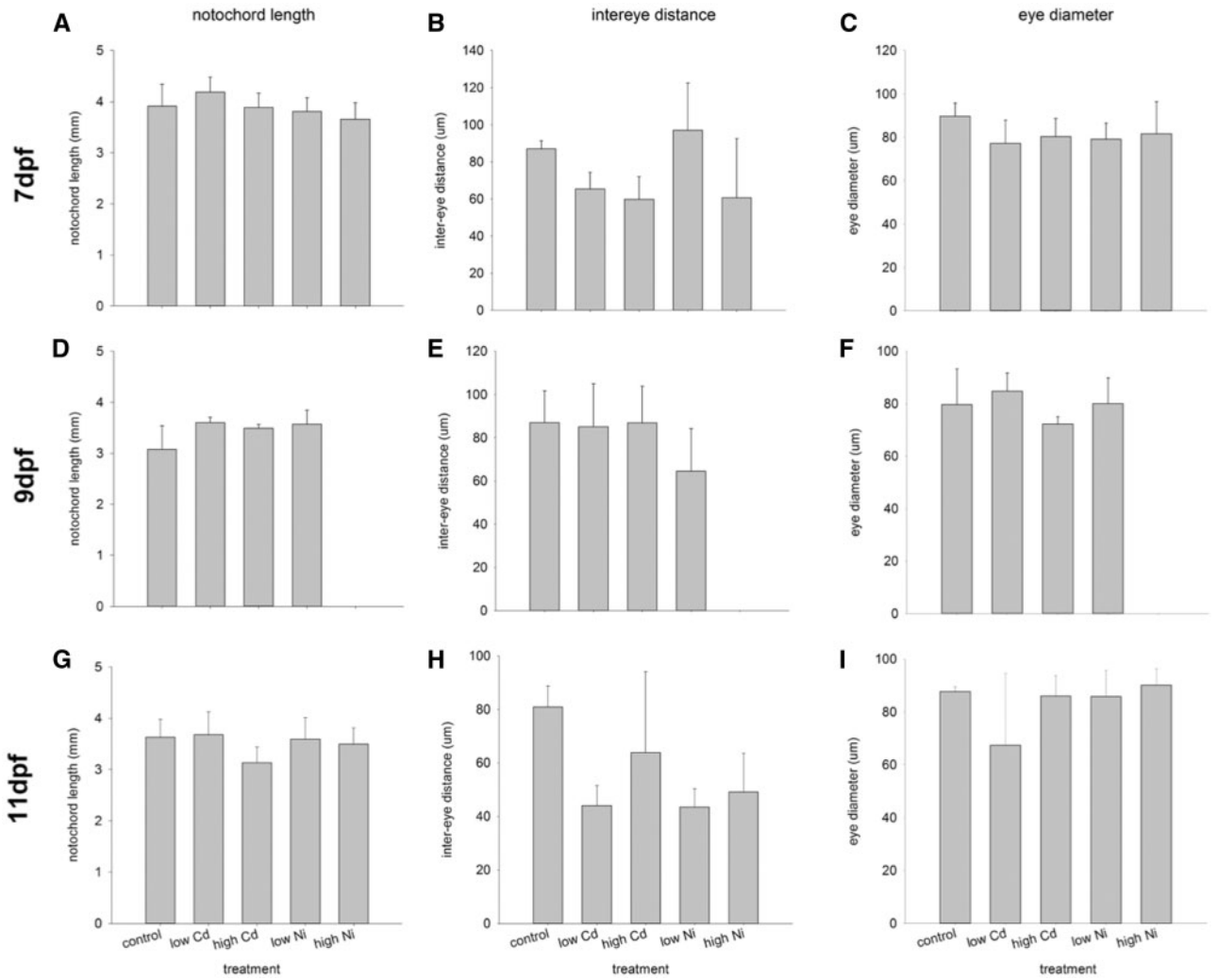

Figure 1. There were no statistical differences in morphological parameters after developmental exposure to either cadmium or nickel. Morphological measurements collected from zebrafish larvae at 7 dpf, 9 dpf, and 11 dpf after early developmental exposure (24–72 hpf) to low cadmium (Cd), high cadmium, low nickel (Ni), and high nickel treatments. Values presented are sample group means (in either mm or μm) ± SD. Inter-eye and eye diameter measurements are normalized based on individual Notochord length.

treatment groups giving a positive response was significantly reduced compared with the control fish. However, no statistically significant differences were observed in the responses of the cadmium treatment groups ($P = 0.402$).

An interaction term consisting of both age and treatment effects was also significant: older larvae within the low ($P < 0.001$) and high ($P < 0.001$) nickel treatment groups displayed greater reductions in OMR than younger larvae. A similar difference was not found in the cadmium exposure groups ($P = 0.113$).

Discussion

This study demonstrated that early developmental exposure to sublethal concentrations of cadmium and nickel did not significantly alter gross morphological parameters, but did significantly affect the OMR in larval zebrafish. This suggests that there has been a functional change at one or more levels of visual processing.

The morphological analyses used in this study are standard morphological measures used to identify differences in growth or overall morphology as a result of exposure to pollutants or toxicants in the aquatic environment (Brown 1997; Bar-Ilan et al. 2009; Jezierska et al. 2009). We observed no significant differences in any of the anatomical measurements, a surprising finding given that exposure to both cadmium and nickel are known to cause developmental abnormalities. Exposure to waterborne cadmium during the first 24 h of development reduces eye size (Chow et al. 2009), causes brain hypoplasia (Chow et al. 2008), and affects somite development (Chow and Cheng 2003) and nickel exposure decreases locomotor activity (Kienle et al. 2009). Differences between these studies and our results likely results from the chemical concentrations used. Our concentrations of nickel and cadmium are in PPB (μg/L), which is significantly lower than both the nickel (mg/L) and cadmium (100 μM = 18 mg/L) concentrations reported by these other investigators. The fact that we are able to see behavioral differences without significant differences in morphological measurements further suggests that environmentally relevant (i.e. PPB) concentrations of these heavy metals have subtle deleterious effects. There was significant individual variation; however, suggesting differential individual growth of larvae across treatment groups, including control larvae. Although no consistent statistical differences in

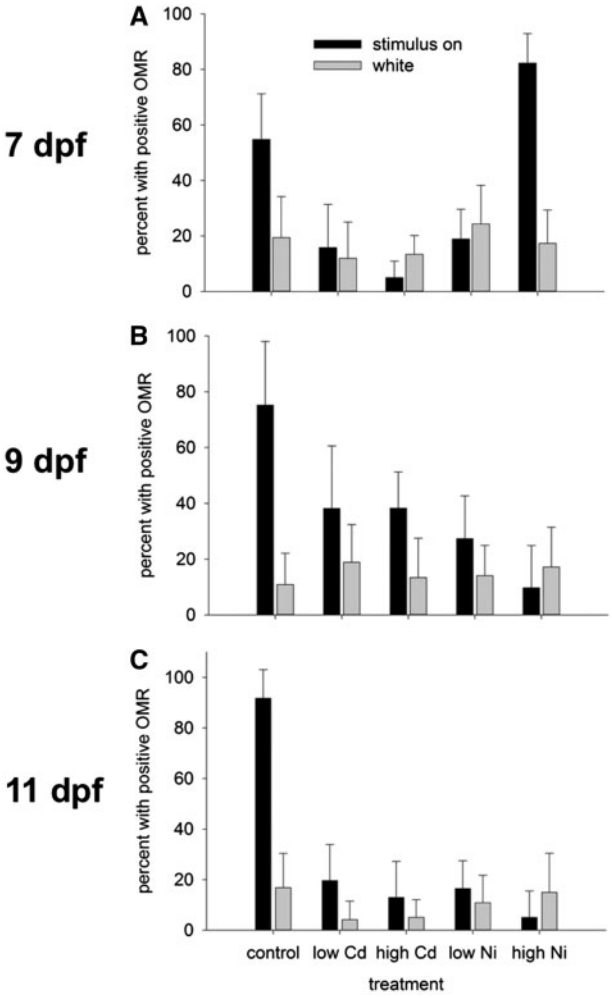

Figure 2. Differences in OMRs were observed in larvae after embryonic exposure to cadmium and nickel. Percentage of larvae responding to either the moving OMR stimulus (dark bars) or a white, stationary background (light bars). Values are mean ± SD. (A) Responses at 7 dpf. The percentage of larvae from the low nickel (0.5 PPB) exposure group was significantly decreased compared with controls ($P < 0.001$). (B) At 9 dpf, the percentage of larvae with a positive OMR was significantly reduced in all nickel treatment groups ($P < 0.01$). (C) By 11 dpf, most (>85%) control larvae were positively responding to the moving stimulus and all nickel treatment groups demonstrated a significantly reduced positive OMR ($P < 0.01$). Cd = cadmium; Ni = nickel.

morphological measurements were noted, the trends observed correspond to other reported results (Jezierska et al. 2009; Kienle et al. 2009) suggesting that cadmium and nickel act as calcium mimics in several locations in the vertebrate body.

However, there were qualitative differences in the timing of developmental events. For example, larvae in the low cadmium group hatched ~1 day earlier than larvae in any other treatment group and these larvae consistently had some of the largest notochord lengths. This is consistent with other cadmium exposure studies (Chow and Cheng 2003). The presence of cadmium ions, in low concentrations such as in poorly treated drinking water, can increase calcium uptake in red blood cells and bone (Nriagu and Andreae 1984; Plishker 1984), increasing growth. In contrast, larvae from the high cadmium group did not hatch early, which is consistent with the altered calcium metabolism and a reduction of cellular respiration reported to occur after exposure to high levels of cadmium, such as in industrial effluents (Bertin and Averbeck 2006). Though we did not find any differences in hatching rate in the nickel-exposed groups (in contrast to Dave and Xiu (1991)), nickel exposure seemed to be more deleterious overall than cadmium with regard to growth. Reported findings with rodents show that acute exposure to high concentrations of nickel inhibits calcium reabsorption in bone tissue (Zaidi et al. 1991), which would reduce growth overall, similar to our findings.

The OMR is an innate visually-guided behavioral response present in most teleosts (Pitcher 1986; Saidel and Fabiane 1998; Carvalho et al. 2002). OMRs have been used extensively with zebrafish (both larval and adult animals) to identify visual system deficits in various mutant animals. Our data indicate that transient exposure to nickel during the critical developmental periods for retinal and retinotectal circuitry (i.e. 24–72 hpf) significantly impacts the larval OMR. Startle responses were not affected, however, suggesting the observed decreases in positive OMR was not a generalized effect on muscle function. Expanding our results to a broader spectrum, this reduction in OMR could lead to a reduction in fitness in a natural setting as tracking ability and therefore prey capture ability would be reduced. In contrast, there were no significant differences in positive OMRs resulting from cadmium exposure, though the data suggest possible biologically relevant trends. It is probable that the comparatively small sample size of the cadmium exposure group reduced the overall power of the statistical analyses. Further experimentation with ecologically relevant cadmium doses would reveal if the OMR would be impacted by this exposure.

The OMR stimulus developed for this study was a rotating black and white pinwheel stimulus projected onto a computer screen below the larvae. Zebrafish larvae do respond to an OMR stimulus presented directly below them (Neuhauss 2003; Yokogawa et al. 2012). In pilot experiments, the stimulus was optimized for larval zebrafish so that positive responses were consistently recorded from ≥75% of control larvae being tested. This threshold is consistent with the use of OMR in other fish species and in mouse (Kane et al. 2004; Prusky et al. 2004). Significant response differences were observed in both control and test fish during the stimulus on versus the white resting screen period, indicating that fish were responding only to the stimulus and further validating the experimental design.

Examination of control larvae also clearly showed that the percentage of fish displaying a positive OMR increased with age. In contrast, in the same 4 days, the OMRs from treated larvae were often either reduced or absent. This loss of a positive OMR suggests cellular/structural changes or a functional alteration caused by exposure to these metals, and that these changes worsen with age. These differences were significant in nickel-exposed larvae, though an age-related reduction in the number of larvae with positive OMRs was also observed in the cadmium exposure groups.

Though we did not measure tissue levels of either cadmium or nickel in our treated larvae, we believe that there was tissue incorporation over the 3-day exposure period for 3 reasons. First, we observed differences in hatching rate and overall development at 72 hpf (the end of the exposure period) in our treatment groups, suggesting specific effects. Second, other studies exposed zebrafish embryos and/or larvae to these heavy metals over a much shorter exposure period—2 h (Kienle et al. 2009) or 24 h (Chow and Cheng 2003; Ku et al. 2015; Chow et al. 2008, 2009)—with treatment-induced effects. Third, both metals are known to bioaccumulate, particularly in the liver and/or kidney (Jarić et al. 2011; Oliva et al. 2012; Squadrone et al. 2013; Ambreen et al. 2015).

This study shows considerable behavioral evidence in zebrafish for the harmful effects of sublethal cadmium and nickel concentrations found in drinking water. A no observed adverse effect level (NOAEL) indicates concentrations at which the organism can be exposed to the substance where no negative effects are found (Cockerham and Shane 1993). The observed behavioral changes suggest that the effective dose of aqueous substances are potentially at lower concentrations than previously thought and that using behavioral assessment as a method to determine toxicity can be an efficient determinant for finding the NOAEL.

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Author contributions

Study concept and design: M.K.L., V.P.C. Acquisition of data: M.K.L. Analysis and interpretation of data: M.K.L., V.P.C. Drafting of the article: M.K.L., V.P.C.

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