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Comparative effects of cadmium, zinc, arsenic and chromium on olfactory-mediated neurobehavior and gene expression in larval zebrafish (*Danio rerio*)

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**Abstract**

Comparative effects of cadmium, zinc, arsenic and chromium on olfactory-mediated neurobehavior and gene expression in larval zebrafish (*Danio rerio*)

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Studies have shown that olfactory-mediated behaviors that are critical to survival can be disrupted by exposure to certain metals. Aquatic Superfund sites often contain elevated levels of various metals, yet few have been characterized for their potential to cause olfactory toxicity. A larval zebrafish behavioral assay was developed to characterize concentration-response curves for zinc (Zn), hexavalent chromium (Cr), and arsenate (As) olfaction inhibition. Cadmium (Cd), an established olfactory toxicant, was used as a positive control. As expected, following a 24-hour exposure to Cd, we observed a reduced response to taurocholic acid (TCA), a behaviourally-active odorant, thus validating the behavioral assay. Zn exposure also decreased TCA response ( $IC_{50}$ : 34.9  $\mu$ g/L and 69.0  $\mu$ g/L respectively). Behavioral responses to another odorant, L-cysteine, revealed that Zn was potentially of specific toxicity to ciliated olfactory sensory neurons. No significant changes in olfactory responses were observed from Cr and As exposures, even at exposures far greater than observed environmental concentrations. Exposures to binary mixtures of Cd and Zn indicated that Zn had a protective effect against Cd toxicity at low concentrations, but this effect disappeared at higher Zn concentrations. Quantitative analysis of whole larvae gene expression of 8 genes

important in mitigating metal induced oxidative stress, confirmed an antioxidant response was evoked by Cd, but not for the other metals, suggesting that oxidative stress was not a primary mechanism of Zn induced olfactory dysfunction. In summary, our study identified that Zn inhibits olfaction at environmentally-relevant concentrations, and that it may reduce Cd when present in mixtures. Our study extends the utility of zebrafish as a model species to assess olfactory toxicity in fish.

**Abbreviations:** Nrf2: nuclear factor, erythroid 2-like 2, omp: olfactory marker protein b, dpf: days post fertilization, EM: E3 embryo medium, SRP: superfund research project, Zn: zinc, As: arsenic, Cr: chromium, Cd: cadmium, TCA: taurocholic acid, OE: olfactory epithelium, OSN: olfactory sensory neuron, OR: olfactory receptor, MOR: major olfactory receptor, TRPC2: Transient Receptor Potential Cation Channel Subfamily C Member 2, OB: olfactory bulb, hmox1a: heme oxygenase 1a, gstp: glutathione s-transferase pi, gclc: glutamate-cysteine ligase, catalytic subunit, nqo1: NAD(P)H dehydrogenase, quinone 1, prdx1: peroxiredoxin 1, gpx1a: glutathione peroxidase 1a, sod1: superoxide dismutase 1, soluble, sod2: superoxide dismutase 2, mitochondrial, hsp70: heat shock cognate 70-kd protein, tandem duplicate 3, actb1: actin, beta 1, gapdh: glyceraldehyde-3-phosphate dehydrogenase, hprt1: hypoxanthine phosphoribosyltransferase 1, gadd45bb: growth arrest and DNA-damage-inducible, beta b

**Keywords:** metals, olfaction, zebrafish, olfactory sensory neurons, antioxidant gene expression, metallothionein

## 1. Background

Waterborne contaminants have been implicated in the decline of wild salmon populations in the Pacific Northwest (Lackey, 2003). These contaminants, including metals and pesticides, have been found at elevated levels in critical fish habitat and are known to affect normal fish behavior. Many of these behaviors are mediated by the olfactory system, which detects dissolved odorants present in the water column. Depending on the odorant, the olfactory system can confer directional, conditional, tactical or genetic information to the brain (Tierney, 2010).

Different mechanisms are thought to underlie altered olfactory mediated behavior following exposure to various contaminants (Scott and Sloman, 2004; Tierney, 2010). Some may act as an odorant themselves or modify odorant perception. This is thought to be the case with carbamates, where exposure results in decreased sensitivity to natural odorants, in part due to a hypothesized thickening of the mucosal layer of the olfactory epithelium (Jarrard et al, 2004; Tierney, 2010). Other contaminants, like some metals, may act on the nervous system, degrading and ultimately destroying olfactory sensory neurons (OSN) (Wang, 2013; Williams, 2016). The investigation of these underlying mechanisms of toxicity has become a focus of research in recent years as model species like zebrafish have become more popular.

Multiple approaches exist through which one can assess olfactory toxicity and include molecular and biochemical indicators such as gene expression, enzymatic reactions, DNA or RNA adducts, or effects on cellular receptors (Tierney, 2010). These

and others allow for the study of potential mechanisms underlying changes in olfaction following chemical insult. Alternatively, anatomical indicators of olfactory toxicity can be utilized including measuring internal or external cellular appearance through the use of scanning electron microscopy, histology, and immunohistochemistry (Tierney, 2010). While the above methods have proved useful exploring mechanisms, physiological changes often provide a more sensitive endpoint and have historically been used when assessing novel contaminants for olfactory toxicity.

To measure physiological changes directly, neurophysiological and behavioral assays are used. Neurophysiological indicators like the electro-olfactogram (EOG) can directly measure the in vivo OSN electrical potential within the rosette while the electroencephalogram (EEG) detects integration of OSN activity into the olfactory bulb (Scott & Scott-Johnson, 2002; Tierney, 2010). Behavioral assays instead measure alterations in fish movement, swim speed, or location in response to known odorants. By comparing response of untreated and treated fish, olfactory dysfunction can be measured following contaminant exposure. Various behavioral assays exist for all stages of fish development including early larval stages (Vitebsky, 2005), something not possible with EEGs and EOGs.

## **2. Introduction**

A functional olfactory system is paramount for many aquatic organisms in mediating behaviors including prey selection, predator avoidance, homing, kin identification, and mate selection (Tierney, 2010; Cooper et al, 1976; Dittman and Quinn, 1996; Hara, 1992; Sutterlin & Gray, 1973). Teleost fishes comprise over 90% of all fish

species, and include most commercially relevant species such as salmon, trout, sablefish, pollock, and rockfish, as well as model organisms such as zebrafish. The olfactory system of teleosts is remarkably sensitive, with some species of salmonids able to detect amino acids in the nanomolar range (Yamamoto et al, 2013; Bando et al, 2011). Olfactory sensory neurons (OSNs) present in the olfactory rosettes are exposed to the environment and protected only by a mucosal lining, which is secreted by goblet cells within the olfactory epithelium (OE). Odorants are detected by OSNs within the OE which transmit information via the olfactory nerve to the glomeruli within the olfactory bulb, the first central nervous station for odor processing (Biechl, 2016).

To date, five OSNs have been identified in fish, including: ciliated, microvillous, crypt, kappe, and pear OSNs, which collectively detect five main odorant types; including amino acids, bile acids, steroids, prostaglandins, and nucleotides. Each of these five OSN types can express different classes of olfactory receptors (ORs), which are grouped into four major gene families: Trace Amine Associated Receptor (TAAR), Major Olfactory Receptor (MOR), Vomeronasal type 1 receptor-like (V1R), and Vomeronasal type 2 receptor-like (V2R). Each individual OSN expresses a single OR, and OSNs expressing the same OR converge to the same glomeruli within the OB (Morita & Finger, 1998; Hansen et al 2003; Hansen et al 2004; Sato et al. 2007). Microvillous and ciliated OSNs are the most numerous OSNs in teleosts, and are responsive to amino acids, and bile acids respectively. Amino acids are potential feeding cues, while bile acids, putative social pheromones (Sato, 2005).

The fish olfactory system is highly conserved among teleosts, including the model species zebrafish (*Danio rerio*). Rapid development, access to powerful genetic tools, optically clear embryos, ease of husbandry, and phenotype of chemical-induced olfactory injury make zebrafish an attractive model species to assess olfactory function and mechanisms relevant to wild fish (Sato et al 2005; Wang & Gallagher, 2013; Ahuja et al 2014). Even at the early stages of development, larval zebrafish respond to amino acids and bile acids that may activate, with little overlap, distinct areas of the olfactory bulb predominantly in the lateral and medial OB, respectively (Li et al 2005). Microvillous OSNs

express V1R and V2R-type olfactory receptors, transient receptor potential channel C2 (TRPC2) and innervate the lateral chain glomeruli (Biechl, 2016; Sato, 2007). Ciliated OSNs express MOR and TAAR-type olfactory receptors as well as olfactory marker protein (OMP), and predominantly target the anteromedial glomeruli (Sato, 2005; Li et al, 2005).

As the peripheral olfactory system is in contact with the surrounding environment it is vulnerable to the adverse effects of contaminants. Of particular concern are the myriad environmental chemicals present in aquatic systems, including a number of metals and pesticides that inhibit olfaction (Lürding & Scheffer, 2007; Sandahl et al, 2007; Tierney et al, 2010). Many of these chemicals are detected at elevated levels in the surface waters of important fish habitat and may impair or completely oblate olfactory mediated behavior at ecologically-relevant exposures (Tierney, 2010). In the Pacific Northwest, short-term exposure to copper (Cu) at concentrations found in surface waters, and urban runoff can decrease predator detection in coho salmon (McIntyre et al, 2012). Cu and cadmium (Cd) are among the most well-studied olfaction impairing metals in teleosts (Baldwin et al, 2011; Green et al, 2010; Sandahl et al, 2007; Sloman, 2007; Tierney et al, 2010; Williams, 2016), however, few other metals present in surface waters have been investigated for their ability to interfere with fish olfactory function. Furthermore, the effects of metals on olfaction have predominantly been assessed individually (Tierney et al, 2010), whereas environmental exposures often occur as mixtures (Tierney, 2016). Historically, metal mixtures were chosen by identifying concentrations present in contaminated lakes, and these studies demonstrated that mixtures could impair the olfactory response of fish, but the effects of specific metals were in the mixture were not identified (Thompson and Hara, 1977; Azizishirazi et al, 2013). Recent work by Dew et al. (2016) demonstrated that additive toxicity cannot be assumed, and this further highlights the need to assess metal mixtures.

In the present study, a larval zebrafish behavioral assay was validated and used to assess the olfactory toxicity of a select group of metals, including a known olfactory toxicant, Cd. Following assay validation, the olfactory toxicity of three relatively

understudied metals (Zn, As and Cr) were assessed using responses to two odorants (taurocholic acid (TCA) and L-cysteine) that relate to two distinct OSN types. The metals were chosen primarily based on their presence in a Superfund site, the lower Duwamish waterway (where). The lower Duwamish waterway comprises the lower section of the Green river, a productive river system for Chinook salmon (WDFW, 2017) and was designated a Superfund site in 2001 due to over a century of heavy industrial use. Metals associated with these industries include zinc (Zn), hexavalent chromium (Cr), arsenate (As), and Cd; they were noted at elevated concentrations in the sediment and water (Conn, 2015; Paulson, 1989; Windward, 2010). The metals and exposure scenarios are not unique to our local site, and are among the top 6 most common metal contaminants found at EPA National Priority List sites. Our study also included a titration-based approach to assess non-additive effects of binary mixtures of Zn and Cd, which commonly occurs in Superfund sites. We hypothesized that the degree of metal induced oxidative stress would be an underlying mechanism associated with the potency of olfactory inhibition, and used antioxidant, stress response, and metallothionein gene expression to characterize metal effects.

### **3. Materials and methods**

#### *Zebrafish maintenance*

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Washington. Adult wildtype zebrafish were housed in recirculating aquaria maintained at  $28 \pm 0.5$  °C in a 14 h light/10-hr dark cycle. Fish received 2% of their body weight in flake food per day and were provided with supplemental artemia twice daily. Source water from city municipal water was passed through a reverse osmosis filtration system and adjusted to  $1000 \pm 100$   $\mu$ S (pH 7.2) using Instant Ocean® salt and Na<sub>2</sub>HCO<sub>3</sub>. Critical water quality parameters (ammonia, nitrite, pH, and temperature) were checked once daily. Paired male and female adult zebrafish were placed in divided spawning tanks the evening prior to spawning. Embryos were collected in the morning and placed into petri dishes containing fresh E3 embryo medium (EM; 5

mM NaCl, 0.17 mM KCl, 0.33 mM CaCl<sub>2</sub>, and 0.33 mM MgSO<sub>4</sub>, pH 7.2–7.4). Static renewal of EM occurred once daily until exposure at 5 days postfertilization (dpf).

### *Metal exposures*

Metal salts (>95% pure sodium arsenate dibasic hepta hydrate, zinc sulphate, cadmium chloride, and potassium dichromate) obtained from Sigma-Aldrich (St. Louis, MO) were used to make 1.0 mM stock solutions. On the day of exposure, stock solutions were diluted to working concentrations with EM (Table 1) and added to 90 mm petri dishes prior to transferring 4 dpf larval zebrafish (50 fish per 25 mL solution). Zebrafish larvae were exposed to metal solutions for 24 hours prior to removal into fresh EM. To assess effects of binary mixtures of Zn and Cd, an approach analogous to a chemical titration of the two metals was used (Meyer et al, 2015). In this approach, one metal was held constant at its IC<sub>50</sub> concentration, while the concentration of the other metal is modulated over successive metal mixtures (**Table 1**).

### *Behavior assay*

An assay similar to that described by Shamchuk et al. (2017) was used to observe behavioral changes in larval zebrafish in response to the aversive odorants, TCA and L-cysteine, following metal exposure. Briefly, a clear acrylic trough (10.5 cm x 3.5 cm x 1.7 cm) was placed on top of a leveled light box for stable illumination. Two clear removable dividers divided the trough into three equally-sized zones. For each experiment, 15 mL of fresh EM was added to the trough, dividers inserted, and ten 5 dpf larval zebrafish transferred to the middle zone. Following a 15 min acclimation period, 30 µL of odorant solution was added to either the left or right zone, and mixed thoroughly. After a 5 min odorant equilibration period, dividers were carefully removed, and video was recorded for 10 min using an overhead camera (details). Addition of an odorant to the left or right zone of the trough was randomly selected. Behavioral trials were conducted between 9:00 and 18:00 to ensure light exposure occurred within the normal rearing light cycle, and trials were randomized throughout this time period as zebrafish embryo activity varies throughout the day (MacPhail et al, 2009). Videos of the trials were analyzed using

ImageJ (National Institutes of Health, USA) using a protocol similar to those of Samchuk et al. (2017) and Wyeth (2011). In each trial, the movement of all 10 zebrafish larvae over time were treated together as a single mass. To reduce background and eliminate stationary fish, every 100<sup>th</sup> frame was averaged together to create a background image that was subtracted from each frame. Individual frames were summed together resulting in a videogram representing 5 min of fish movement (**Fig. 1**). The mean center of mass was calculated for each videogram and the shift distance, the difference between the center of mass and the center of the trough, was determined. Results were normalized by dividing the average aversive response of control larvae to a given odorant, and the shift distance converted to percent response, with 100% indicating full sensory function was retained and 0% indicating complete loss of olfactory mediated behavior. Data is presented as either mean mass shift  $\pm$  standard error (SEM) or % response  $\pm$  SEM.

#### *Antioxidant and metallothionein gene expression*

To investigate if Cr, As, and Zn share a similar mechanism of toxicity through whole larval oxidative stress, targeted gene expression was performed. Analysis of eight putative Nrf2 (Nfe2l2a)-dependent antioxidant genes (hmox1a, gstp1, gclc, nqo1, prdx1, gpx1a, sod1, sod2), a stress response gene (hsp70), an inducible DNA damage repair gene (gadd45bb), and three reference genes (actb1, gapdh, hprt1) was completed after 24 hr metal exposures using the QuantiGene Plex (QGP) assay following procedures described in Mills & Gallagher (2017). To determine if the protective effect of Zn on Cd-induced olfactory toxicity was the result of alterations in the induction of metallothionein, an additional QGP gene array, comprising two homologs of metallothionein (mt2 and mtbl) and two reference genes (actb1, and hprt1) was also completed following metal exposures using the same protocol. Briefly, larvae (n=10) were euthanized following chemical exposure and behavioral analysis with 0.016% MS-222 in EM. Whole larval fish were homogenized in 400  $\mu$ L homogenizing solution with 4  $\mu$ L Proteinase K (Affymetrix, Santa Clara, CA) for 2 min at 40 Hz on a TissueLyzer shaker (Qiagen). Homogenized samples were incubated at 65° C for 5 minutes, centrifuged at 14k rpm for 5 min, then incubated at 65° C for another 30 min with occasional vortexing. A final centrifugation

step at 14k rpm for 10 min was used to pellet cellular debris. Supernatant samples were diluted in additional homogenizing solution to reach the linear range of the QGP assay and stored at -80° C.

The QGP assay was run according to manufacturer directions, using 40 µL of diluted homogenate per well and two technical replicates per biological replicate. Probes against genes listed above were designed by Affymetrix (info). Completed assay plates were immediately run on a MagPix luminometer (Luminex, Austin, TX). Median fluorescent intensity (MFI) readings were recorded for all samples. A baseline 2× the limit of detection (as recommended by the manufacturer) was used as the minimum signal allowed for the gene analyzed. Expression of the target antioxidant genes of interest were normalized by dividing by the geometric mean of three reference genes (actb1, gapdh, and hpert1).

### *Statistical analyses*

GraphPad Prism Ver 7.0 (Graph Pad Software Inc, San Diego, CA, USA) was used for all statistical analysis. Normality of behavioral data was confirmed using Shapiro-Wilk's normality test, and the effects of metals on olfaction was determined using one-way ANOVA followed by Fisher's LSD post hoc test. No heterogeneity was present in the datasets. The effects of chemical exposure on gene expression were assessed using one-way ANOVA followed by a Tukey's hsd post hoc test for multiple comparisons. All statistical data were considered significant at  $p \leq 0.05$ .

## 4. Results

### *Zn and Cd exposure decreases olfactory-mediated behavior*

Videograms generated from video recordings of behavioral assays analyzed for center of mass shift distance are shown in **Fig. 1A-D**. Concentration-response curves were generated for both TCA and L-cysteine (**Fig. 1E-F**), and the results were used to determine the lowest concentration at which the maximum aversive response was reached for both odorants. Peak aversive behavioral response to TCA was  $4.0 \times 10^{-4}$  mol/L, which resulted in a mean shift of 1.58 cm, while the maximum aversive response to L-cysteine was  $8.0 \times 10^{-4}$  mol/L, with a mean shift of 0.88 cm (**Fig. 1E-F**). Both of these odorants showed a significant response over the embryo media control odorant (EM; mean shift = 0.3 cm). As shown in **Fig. 2B**, 24 hr exposure to various Cd concentrations resulted in a concentration-response curve to TCA ( $IC_{50}$  34.9  $\mu$ g/L, CI:20.9-58.5), similar to those described in previous studies (Wang, 2012). Following validation of the behavioral assay with Cd, we assessed three additional metals, Zn, As and Cr, for changes in response to TCA under the same experimental conditions (**Fig. 2A**). Zebrafish larvae exposed to Zn exhibited a dose-response relationship similar to Cd, although at higher concentrations ( $IC_{50}$  69.0  $\mu$ g/L, CI: 46.3-99.6; **Fig. 2C**). Exposure for 24 hr to As and Cr resulted in no statistically significant changes in behavior, even at concentrations exceeding those of environmental relevance (**Table 1; Fig. 2A**).

To observe potential differences in response to different odorants classes, we compared larval behavior between TCA and L-cysteine after 24 hr exposure to a single concentration of each metal. We tested Zn and Cd at their  $IC_{90}$  concentrations (240 and 312  $\mu$ g/L respectively) due to their altered response to TCA at  $IC_{90}$ , while for As and Cr, we used the highest concentrations assessed with TCA (400 and 2600  $\mu$ g/L respectively). A 24 hr exposure to Cd (312  $\mu$ g/L) resulted in an 84% decrease in larval response to L-cysteine, similar to the 90% decrease of response to TCA. Neither of these responses statistically differed from the control odorant (EM) response of Cd-exposed larvae, thus demonstrating a complete loss of olfaction-driven behavior (**Fig. 3A**). A 24-hr exposure

to Zn (240  $\mu\text{g/L}$ ) resulted in a 60% decrease in response to L-cysteine, which is significantly less than observed for TCA (90% decrease, **Fig. 3B**). Exposure to high concentrations of As and Cr resulted in no change in the aversive response to L-cysteine or TCA when compared to unexposed larvae (**S1**).

#### *Zn protects against Cd-induced loss of olfactory-mediated behavioral response*

Following exposures to single compounds, we investigated the effects of Zn against Cd-induced olfactory injury in larval zebrafish. We performed two sets of exposure experiments investigating binary Zn:Cd mixtures. In the first scenario, all mixtures contained Zn at its  $\text{IC}_{50}$  ( $\text{IC}_{50} = 69 \mu\text{g/L}$ ), while the Cd concentration varied (**Fig. 4A**). In the reciprocal experiment, all mixtures contained Cd at its  $\text{IC}_{50}$  ( $\text{IC}_{50} = 35 \mu\text{g/L}$ ), while the Zn concentration varied (**Fig 4B**). As with single metal exposures, all exposures were conducted for 24-hr prior to behavioral analysis. We observed a statistically significant decrease in olfactory response to TCA as Cd concentrations increased beyond its  $\text{IC}_{50}$  when Zn held at its  $\text{IC}_{50}$  concentration (**Fig 4A**). When Cd was held constant at its  $\text{IC}_{50}$  and Zn concentration varied, we observed a significant increase in response between an  $\text{IC}_{50}$  concentration of Cd alone and that of a  $\text{IC}_{50}$  concentration of Cd plus a  $\text{IC}_{10}$  (20  $\mu\text{g/L}$ ) concentration of Zn. At higher concentrations of Zn this protective effect was removed, and complete loss of response to TCA observed at the = 112  $\mu\text{g/L}$  Zn ( $\text{IC}_{70}$ ), (**Fig 4B**).

#### *Antioxidant gene response in the presence of metals*

As shown in **Fig. 5A**, antioxidant genes were induced, including *hmox1a* (-2.1 fold), *prdx1* (-3.2 fold), and *hsp70* (-1.9 fold) following 24-hr exposure to 1  $\mu\text{M}$  Cd (**Fig. 5A**). In contrast, exposure to As, Cr, and Zn did not modulate any of the other genes (**Fig. 5B**). Furthermore, in the binary mixtures (including the lowest and highest Zn concentration 35  $\mu\text{g/L}^{-1}$  Cd, 20  $\mu\text{g/L}^{-1}$  Zn, and 25  $\mu\text{g/L}^{-1}$  Cd, 69  $\mu\text{g/L}^{-1}$  Zn), increasing the Zn concentrations did not alter the expression levels of any of the antioxidant genes on the panel (**S2**). Expression of the inducible DNA repair gene, *gadd45bb*, was well below the linear response range for the assay (data not shown). As observed in **Fig. 6**, exposure to Cd

and Zn increased the expression of *mt2* (5.9-fold and 1.9-fold respectively), but had no effect on *mtbl* expression.

## 5. Discussion

The primary odorant used in our assay, TCA, is known to preferentially activate ciliated OSNs, and as such, our assay was insensitive to damage of other OSN cell types. By contrast, L-cysteine is an amino acid odorant known to preferentially activate microvillous OSNs (Sato, 2005). Thus, our trough assay system was able to assess olfactory dysfunction towards the two predominant OSN cell types present in larval zebrafish. Although Cd and Zn caused a loss of olfactory function, the fact that neither Cr nor As affected zebrafish olfaction, even at high concentrations using two different odorant classes was somewhat surprising. Also of interest, was the fact that behavioral analysis following Zn exposures revealed a greater disruption of response to TCA than to L-cysteine. Previous studies in zebrafish, goldfish, and coho salmon have shown metal exposures may target distinct classes of OSNs, resulting in differential toxicity (Dew et al, 2014; Hentig & Byrd-Jacobs, 2016; Williams et al, 2016). Similarly, it is likely that our observed difference in disruption between TCA and L-cysteine behavioral responses following Zn and Cd exposure were the result of toxicity to ciliated OSNs. Previously, Hentig & Byrd-Jacobs (2016) found differential toxicity of Zn towards ciliated and microvillous OSNs following brief exposure to 1M Zn in adult zebrafish. A recent study in coho salmon (Williams, 2016) found a specific inhibition of ciliated OSNs exposed to Cd, and the damage persisted following the cessation of exposures. However, the mechanism by which preferential dysfunction of ciliated, but not microvillus OSN occurs, is unknown.

While previous studies have identified Zn as an olfactory toxicant, the concentrations used in these studies were in excess of 1 mM (65,000 µg/L) and thus, orders of magnitude greater than those typically encountered in polluted sites (Hem, 1972). Accordingly, our study represents the first time that olfactory mediated behavioral alterations have been observed in a fish species at environmentally relevant Zn concentrations (Hentig & Byrd-Jacobs, 2016; Cancalon, 1982). Previous studies have used intranasal infusion to investigate Zn induced olfactory inhibition in adult zebrafish

and channel catfish and found exposure resulted in a reduction of sensory neurons, loss of cilia on the apical surface, and disruption of olfactory-mediated behavioral responses (Hentig & Byrd-Jacobs, 2016; Cancalon, 1982). While these studies have shown that Zn can be an olfactory toxicant in fish, the exposure method, duration, and concentrations were significantly different than that which may occur in the environment. We calculated an IC<sub>50</sub> for olfactory inhibition of 69.0 µg/L for Zn, this low ppb concentration of Zn is well within range of Zn concentrations reported in polluted waterways, and suggests that altered olfactory mediated behaviors is possible. Salinity and water hardness, are known to be important modulators of olfactory toxicity; differences between our water conditions and those found in river systems need to be taken into account. Additionally, interspecies variation, as well as intraspecies variation, can result in significant differences in metal susceptibility. Zebrafish are somewhat more resistant than wild fish to metal induced olfactory injury, at least in the case of Cd and Cu (Wang et al, 2013; Williams et al, 2016; Tilton et al, 2008).

The modulating effect of Zn on Cd-olfactory injury, at least under one of the exposure conditions analyzed in our study, was also interesting. A potential cellular mechanism underlying our observation is that some OSN populations contain metalloproteins that have either Cu or Zn binding sites. Cd, Zn, and Cu, divalent cations, can displace each other from metalloproteins, which would lead to altered function of the site and olfaction dysfunction (Hartwig, 2001). Binding constants for these metals on ORs are unknown, however Cd is known to be able to displace Cu and Zn from other metalloproteins, as well as metallothionein, and is a known mechanism of toxicity for Cd in other tissues. Dysregulation of this important step in odorant signaling could lead to olfactory dysfunction (Wang et al, 2003) and would help to explain our findings that Zn can alter function independent of gene expression. Another potential mechanism is that calcium channels, critical for cell signaling in OSNs, may be blocked by large divalent cations such as Zn, Cd, and Cu, and was used to explain findings in rodents where Zn and Cu, but not Mg resulted in reduced olfaction mediated behaviors (Block et al, 2017). Both of these suggested mechanisms could help to explain why the two cations in this

study (Cd and Zn) were more potent olfactory deregulators than the two anions (Cr and As).

An objective of our study was to better assess the environmental risk of As and Cr on fish olfaction at environmentally-relevant concentrations. Arsenic is a known neurotoxin in mammalian species, and in fish, golden shiners avoid As-containing waters (Hartwell et al, 1989). In aquatic environments, As is typically found as arsenate ( $\text{As}^{5+}$ ) in surface waters and rivers and is less toxic than the more reduced arsenite ( $\text{As}^{3+}$ ). Arsenite is generally accepted to be the more toxic form, however, in the limnetic and pelagic zones of lakes and the surface waters of rivers, the more oxygenated water favors arsenic in the pentavalent state, and was therefore the form of arsenic chosen for this study. Absorption rates of arsenate are 4-fold lower than arsenite in BALB/3T3 Cl A31-1 cells (Bertolero et al, 1987). The lack of behavioral alterations following arsenate exposure was therefore not altogether unexpected. Similarly, the valence state of chromium strongly influences its toxicity with hexavalent Cr being the more toxic form. This is thought to be mainly the result of the higher absorption rate of hexavalent Cr (Casadevall & Kortenkamp, 2002). Hexavalent Cr is the predominant species of dissolved Cr in surface waters and is favored as  $\text{CrO}_4^{2-}$  due to the concentrations present at polluted sites (Markiewicz et al, 2015). Previous studies have found hexavalent Cr to alter the swim speed of adult zebrafish (Lu et al, 2017), accumulate within Chinook salmon (Frago et al, 2006; Patton et al, 2007), and induce hyposmia in foundry workers (Sunderman, 2001; Doty & Hastings, 2001). However, the exposure scenarios cannot be equated to wild fish due to the extremely high concentrations of metalloid vapors that can occur in industrial settings (Wilbur et al, 2012).

To test the hypothesis that the degree of metal induced olfactory dysfunction was associated with whole embryo oxidative stress, we analyzed the expression of an antioxidant gene panel comprising genes shown previously to be important in Cd induced oxidative stress of larval zebrafish. Previous studies in our lab have demonstrated that oxidative stress is a key mechanism of Cd induced olfactory toxicity in teleosts (Wang & Gallagher, 2013; Espinoza et al, 2012; Williams et al, 2016). Additionally, morpholino

knockdown of nuclear factor (erythroid-derived 2)-like 2 (Nrf2a), a transcription factor important in maintaining the redox status of cells, resulted in increased sensitivity to Cd, altered olfactory-mediated behavior, and reduced expression of Nrf2a responsive and putative Nrf2a responsive antioxidant genes including *gst pi*, *gclc*, *hmx1*, and *prdx1* in whole larval zebrafish (Wang & Gallagher, 2013). As expected the positive control Cd, induced an antioxidant response. In contrast, As, Cr, and Zn did not. Zn was shown in our behavioral assays to be a potent olfactory inhibitor, which suggests that whole larvae expression of antioxidant genes is not predictive of Zn induced olfactory dysfunction, and that oxidative stress is not a mechanism of olfactory toxicity at the concentrations used. Organ-specific gene induction was not possible at this stage of development due to the size of larval zebrafish. A future study assessing oxidative stress in olfactory specific tissue is recommended.

Chronic low dose exposure to arsenite has been shown to trigger oxidative stress in the brain of adult zebrafish, as well as induce expression of Nrf2a and antioxidant genes including *sod1*, *sod2*, and *gpx1* (Sarkar et al 2014). Our gene induction panel, using whole larvae, provided further evidence that arsenate is the less toxic form. As with arsenic, the valence state of chromium strongly influences its toxicity. Hex-chromium induces oxidative stress in larval zebrafish and decreases hatchability at high concentrations (Dave et al, 1987; Jin et al, 2015). It was therefore surprising to see no change in response to odorants following 24 hr exposures to hex-chromium. The exposure duration, and concentrations used in this study are lower than previous studies, and it was expected that this was why we observed a... the difference in induction of oxidative stress genes observed. Ongoing work using laser ablation inductively coupled mass spectrometry (LA-ICP-MS) will investigate if the extent of absorption, distribution, and accumulation is associated with toxicity, and should help to explain differences observed between Cd, Zn, As, and Cr.

Although the protective effect of low Zn concentrations against Cd induced olfactory toxicity does not appear to be driven by oxidative stress, an increased expression in metallothionein mRNA, and the expected increase of metallothionein was

hypothesized to have contributed to the protective effect. Metallothionein, an intracellular metal-binding protein rich in cysteine, is important in homeostasis of essential metals (Zn and Cu), and detoxification of non-essential metals (Cd and Hg) in teleost species. Two metallothionein homologs, metallothionein-2 (mt2) and metallothionein-B-like (mtbl), represent the predominant forms of metallothionein present in larval zebrafish (Wu et al, 2008; Teoh et al, 2015; Roesijadi, 1992; Andrews, 1990, 2000; Kägi, 1991; Palmiter, 1998). Similar to antioxidant expression results, variation of Zn concentration within binary mixtures of Cd and Zn, did not alter mt2 expression. This suggest that protection occurred via a different mechanism. Due to the lack of antioxidant response and mt2 induction, it is likely that competition at binding sites in olfactory tissue between Cd and Zn is driving this protective effect.

## **6. Conclusion**

The zebrafish behavioral trough assay, incorporating the odorants L-cysteine and TCA, proved to be an effective system to assess under studied, but relevant metals, on olfactory function in larval zebrafish. To our knowledge, we provide the first observation of Zn induced olfactory dysfunction in a fish species at environmentally relevant concentrations. The fact that Zn exposures resulted in differences in potency of disruption of behavioral responses towards the two odorants TCA and Cys is consistent with increasing evidence from our laboratory of a preferential toxicity of some metals towards ciliated OSNs. We also show that binary mixtures of Zn and Cd can result in non-additive mixture effects, and a protective effect of low level Zn concentrations towards Cd induced olfactory dysfunction. This protective effect did not involve an antioxidant response as measured by gene expression. In general, the lack of a potent antioxidant response by Zn, a known olfactory inhibitor using a highly sensitive high throughput platform, suggests the degree of whole embryo oxidative stress is not associated with behavioral dysfunction. Ongoing studies in our laboratory are directed towards understanding the uptake and distribution of metals into the various components of the fish olfactory system, and identifying olfactory protein targets of metals.

## **Acknowledgements**

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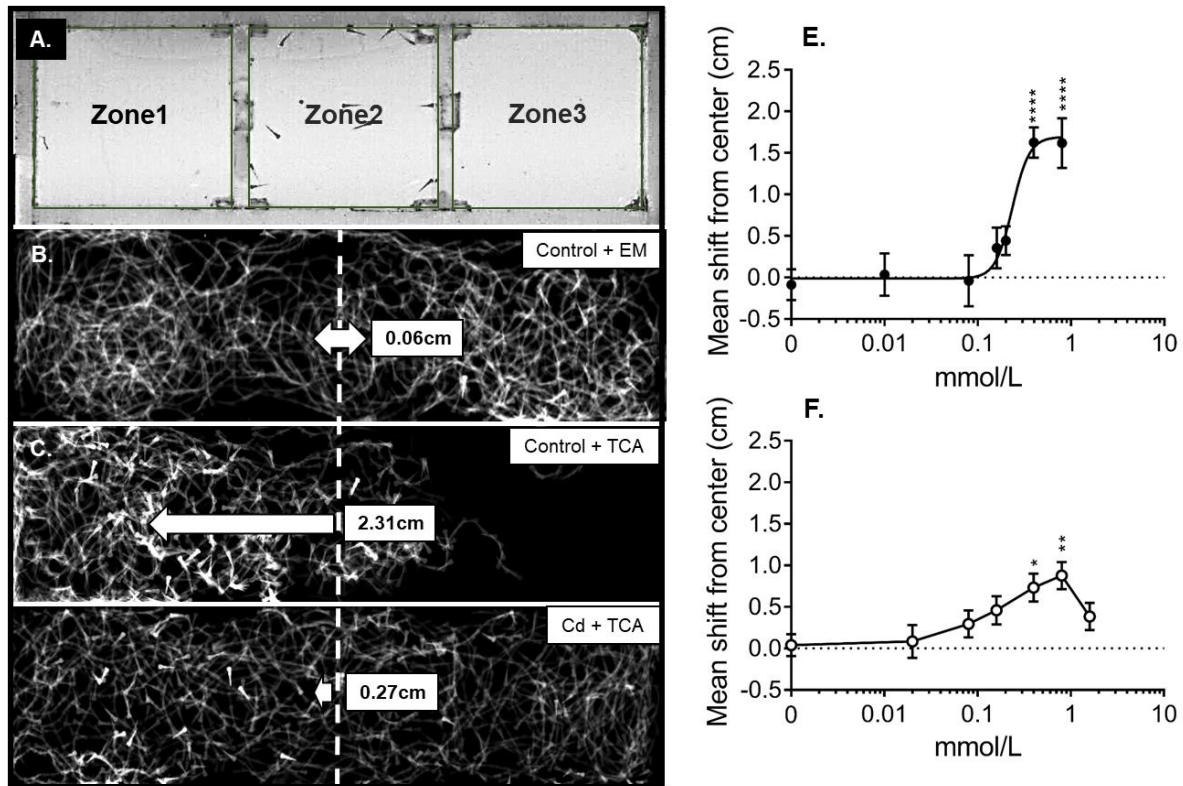
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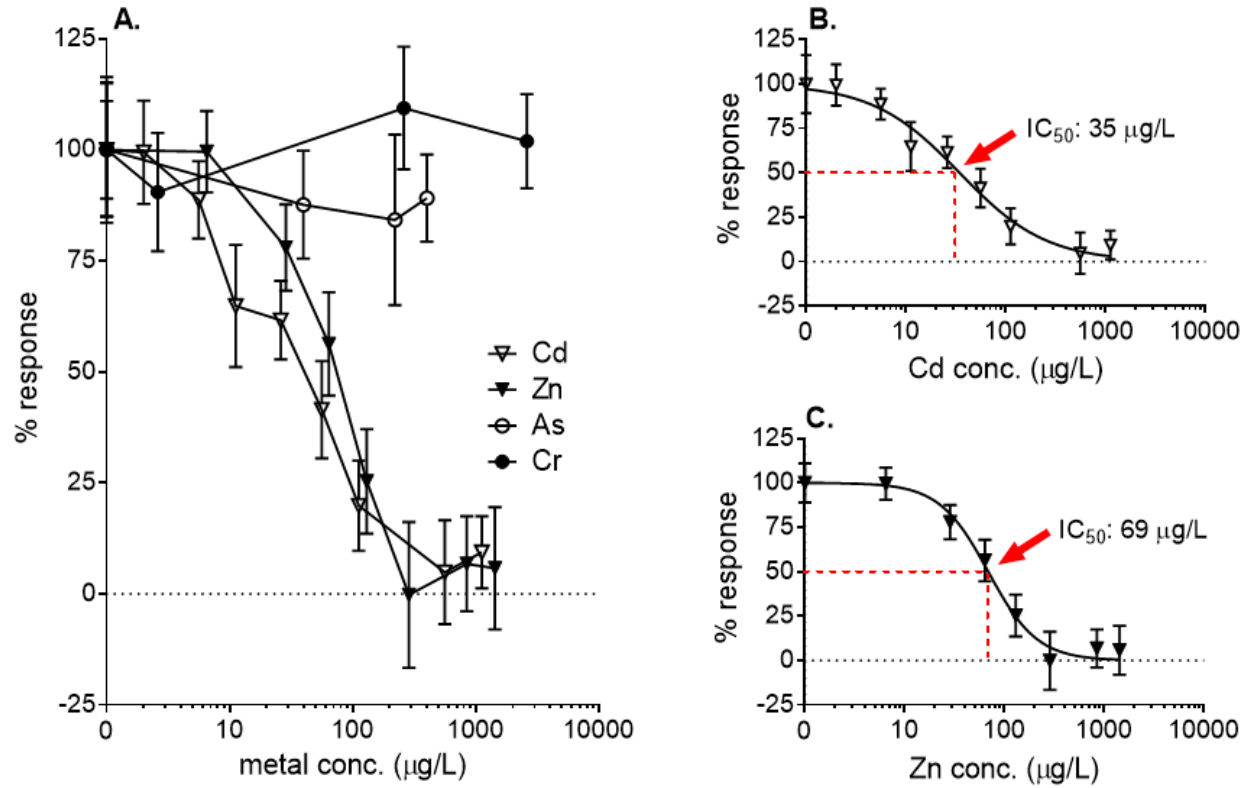
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Metal	Concentration ( $\mu\text{g/L}$ )
Zinc ( $\text{Zn}^{2+}$ )	7, 29, 64, 130, 286, 845, 1430
Cadmium ( $\text{Cd}^{2+}$ )	2, 6, 11, 26, 56, 112, 560, 1120
Chromium ( $\text{Cr}^{6+}$ )	3, 260, 2600
Arsenic ( $\text{As}^{5+}$ )	40, 220, 400
Binary Mixtures (Cd:Zn)	69 Zn + [0, 4, 15, 35, 81, 312 Cd] 35 Cd + [0, 20, 43, 69, 112, 240 Zn]

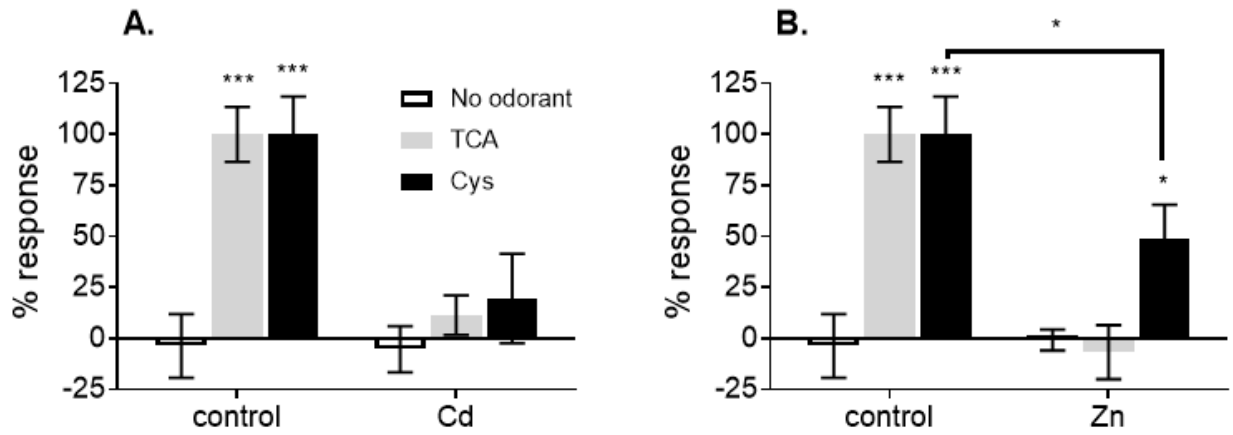
**Table 1.** Concentrations ( $\mu\text{g/L}$ ) of metals used in both behavioral and gene induction experiments. The low exposure concentration for each metal was chosen to represent an environmentally relevant concentration based off of the dissolved metal concentrations present at the Lower Duwamish Waterway. Binary Cd:Zn mixture concentrations were selected using  $\text{IC}_{50}$  values calculated from individual dose response curves (**Fig. 2**).



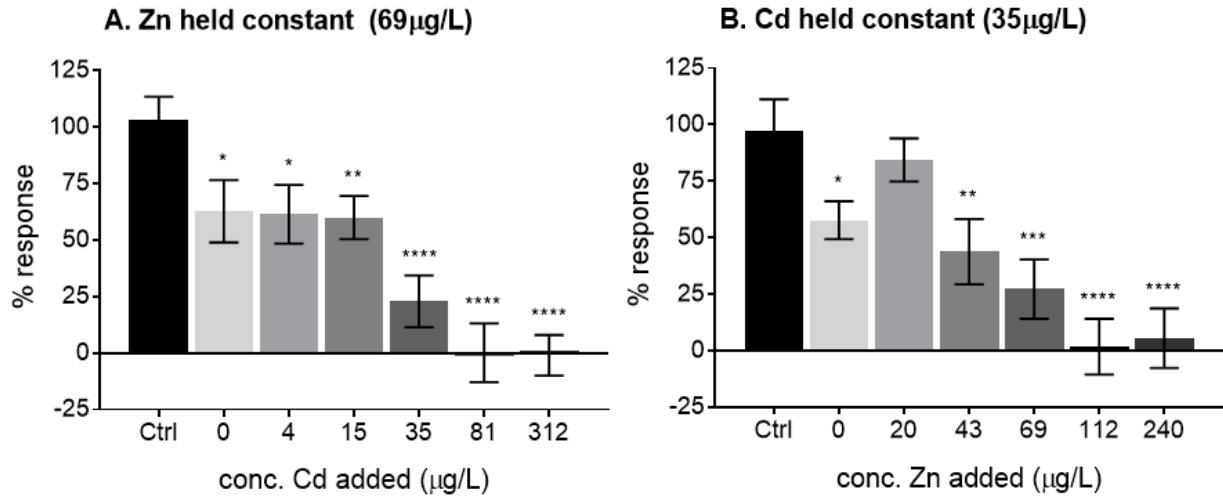
**Fig. 1.** Behavioral assay for odorant-driven response in zebrafish larvae to TCA and L-cysteine. **A.** Apparatus setup used in assessing behavioral response to the aversive odorants TCA and L-cysteine. Fish are placed in center zone (Zone 2) and odorants are added to either Zones 1 or 3 (randomized). **B-D.** Representative videograms generated from 5 min recordings following divider removal, arrows denote mean shift from center. Videograms show behavioral response of **(B)** unexposed 5 dpf larval zebrafish to EM control odorant, **(C)** TCA, and **(D)** 5 dpf larvae to TCA following a 24-hr exposure to 112  $\mu\text{g/L}$  Cd. **E-F.** Dose response of unexposed 5 dpf larval zebrafish to TCA **(D)** and L-Cysteine **(E)**. Points represent the mean value of 11 individual trials  $\pm$  SEM. An asterisk denotes a significant difference from fish given control odorant of EM ( $p \leq 0.05$ ).



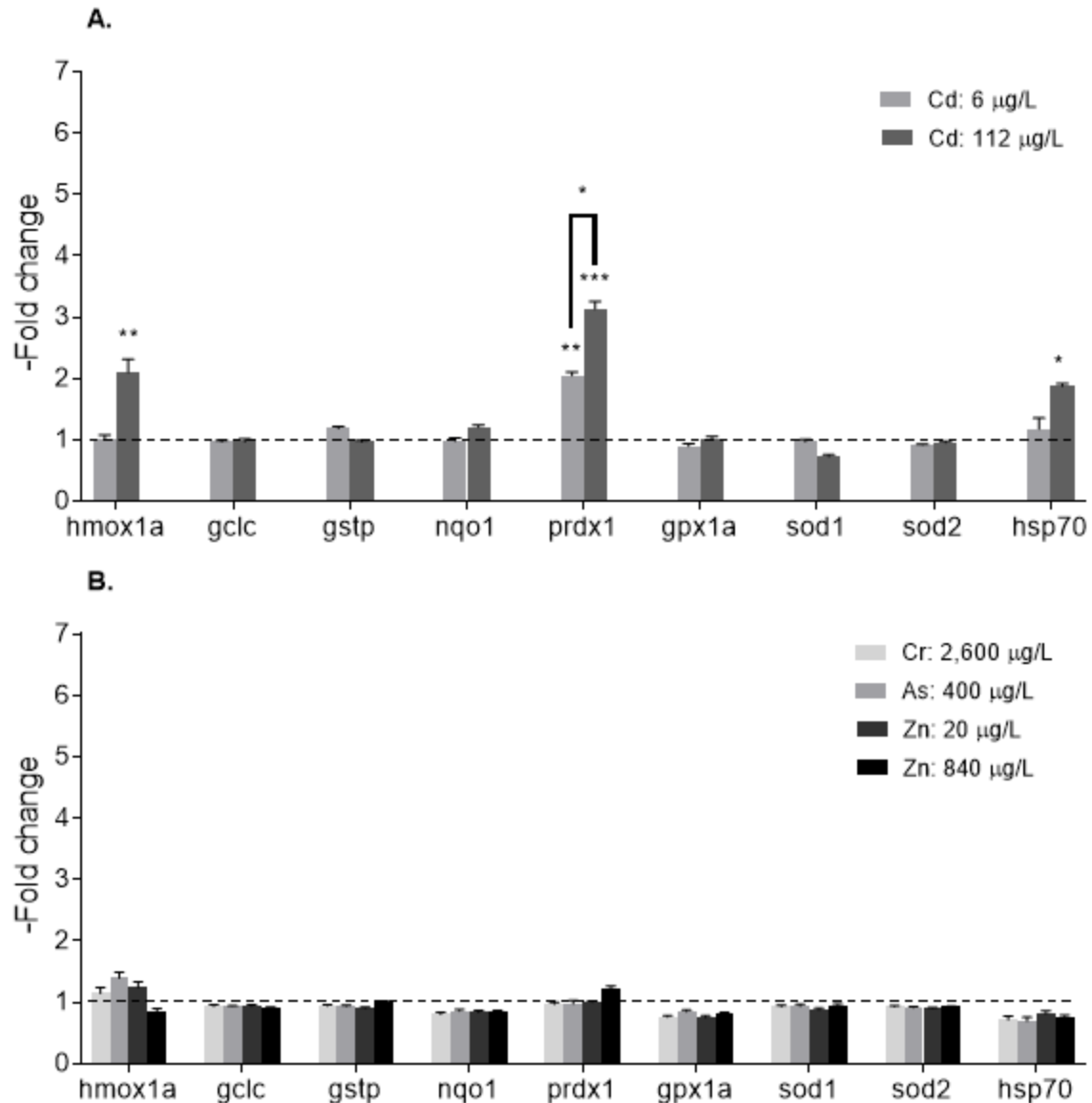
**Fig. 2.** Behavioral response of 5 dpf larval zebrafish to the aversive odorant TCA following 24-hr exposure to various metals, each point is the mean of N=11 individual trials  $\pm$  SEM. **A:** Behavioral response to understudied metals Zn, As, and Cr as well as positive control Cd. **B:** Dose response curve of 5 dpf zebrafish to Cd IC<sub>50</sub>= 35  $\mu$ g/L, CI: 20.9-58.5. **C:** Dose response curve of 5 dpf zebrafish to Zn IC<sub>50</sub>= 69  $\mu$ g/L, CI: 46.3-99.6.



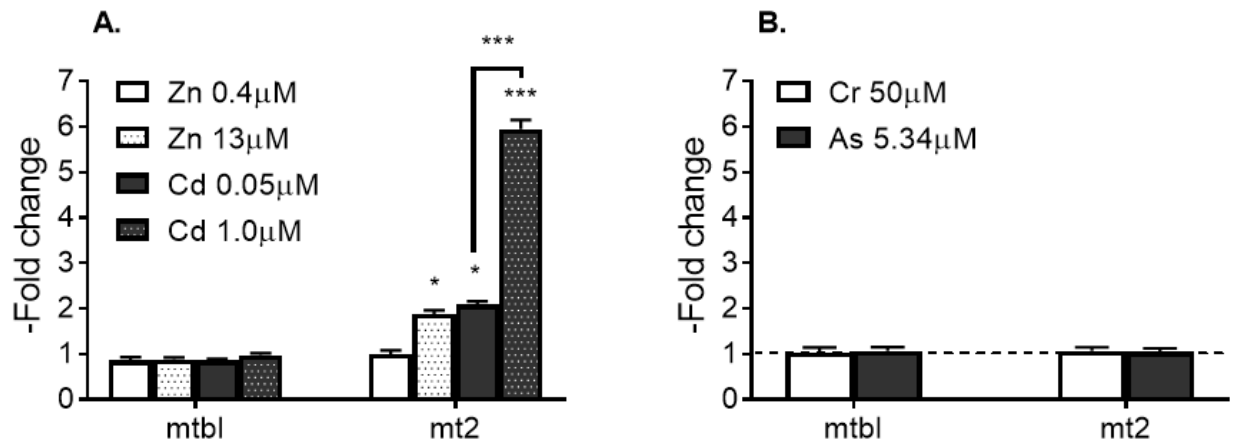
**Fig. 3.** Behavioral response of 24-hr metal-exposed larvae to two odorant classes: bile acids (TCA) and amino acids (L-cysteine). **A:** Behavioral response of control (EM) and Cd treated 5 dpf larval zebrafish, **B:** control and 240  $\mu\text{g/L}$  Zn treated 5 dpf larval zebrafish. **C:** 400  $\mu\text{g/L}$  As and 2600  $\mu\text{g/L}$  Cr, see supplemental. Asterisks indicated bars that are statistically different from no-odorant controls  $p \leq 0.05$ . Error bars represent  $\pm$  SEM of  $n=11$  individual trials.



**Fig. 4.** Behavioral response of 5 dpf larval zebrafish to TCA after a 24-hr exposure to Zn:Cd binary mixtures. **A.** Zn concentration held at  $IC_{50} = 69 \mu\text{g/L}$  with increasing Cd concentrations graphed as % response normalized to unexposed controls' response to TCA. **B.** Cd concentration is held constant at  $IC_{50} = 35 \mu\text{g/L}$  with increasing Zn. Graphs are of mean  $\pm$  SEM of 11 individual trials. Asterisks above bars indicate results that are significantly different from controls at  $p < 0.05$ . See **Table 1** for list of Zn and Cd concentrations.

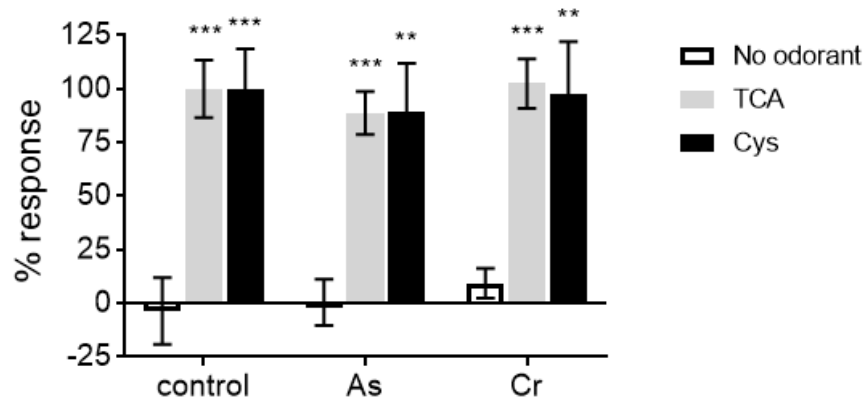


**Fig. 5.** Normalized fold change of expression of oxidative stress-related genes of 5 dpf larval zebrafish following 24-hr exposure to metals. **A.** Normalized graph for Cd at high and low concentrations show a small induction of hmox1a (2.1fold), prdx1 (3.2 fold), and hsp70 (1.9 fold) in zebrafish exposed to 1 µM Cd for 24-hr. **B.** Normalized graph for Cr, As, and Zn exposures shows no change in gene expression. Results were standardized to control fish and reported as mean  $\pm$  SEM of 5 individual samples. Asterisks above bars indicate results that are significantly different from controls at  $p \leq 0.05$ .

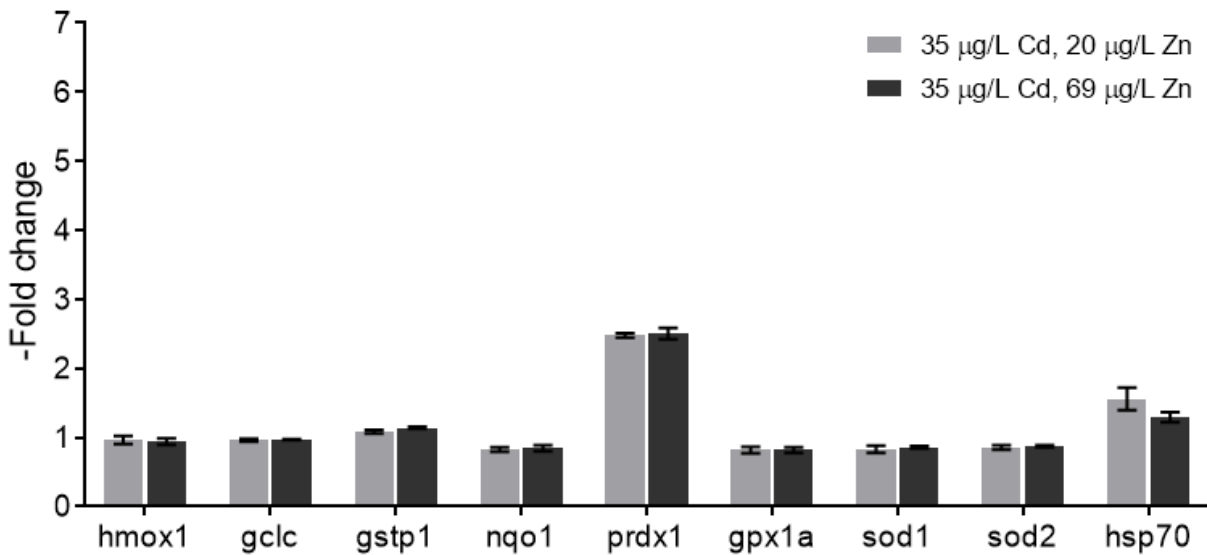


**Fig. 6.** Corrected fold change of expression of metallothionein homologs in 5 dpf larval zebrafish following 24-hr metal exposure. Results were standardized to control fish and reported as mean  $\pm$  SEM of 5 individual samples. Asterisks above the bars indicate results that are significantly different from controls  $p \leq 0.05$ . **A.** fold change following Cd and Zn exposure. **B.** As and Cr exposure did not induce significant changes in whole body expression of mt1b1 and mt2.

## Supporting information



**S1 Fig.** Behavioral response of 24-hr metal-exposed larvae to two odorant classes: bile acids (TCA) and amino acids (L-cysteine). **C:** 400 µg/L As and 2600 µg/L Cr. Asterisks above the bars indicate results that are significantly different from controls  $p \leq 0.05$ . Error bars represent  $\pm$  SEM of  $n=11$  individual trials.



**S2 Fig.** Normalized fold change of expression of oxidative stress-related genes of 5 dpf larval zebrafish following 24-hr exposure to two binary mixtures, Cd:Zn with 20 µg/L Zn, and Cd:Zn with 69 µg/L Zn. Results were standardized to control fish and reported as mean  $\pm$  SEM of 5 individual samples. Asterisks above the bars indicate results that are significantly different from controls  $p \leq 0.05$ .