Mechanisms and biomarkers of cadmium induced neurobehavioral impairment in the olfactory system of coho salmon (*Oncorhynchus kisutch*)

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Abstract

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Environmental and Occupational Health Sciences

Pacific salmon populations on the west coast of the U.S. are increasingly threatened by anthropogenic activities. One potential factor driving the population declines is disruption of olfactory function via waterborne pollutants. The salmon olfactory system is highly susceptible to disruption by waterborne pollutants such as metals. A majority of the current research has focused on the olfactory toxicity of waterborne copper, while other metals such as cadmium (Cd) have gone relatively understudied. This is a large concern as Cd is a common pollutant in many urban and agricultural waterways, some of which can be important salmon migratory routes. For my doctoral research, I focused on understanding the mechanisms underlying Cd mediated olfactory dysfunction using juvenile coho salmon parr (*Oncorhynchus kisutch*). The research goals for aim 1 of my project were to analyze olfactory-mediated alarm responses, epithelial injury and recovery, and a suite of olfactory molecular biomarkers encoding genes critical in

maintaining olfactory function in juvenile coho salmon receiving acute exposures to Cd. The mRNA molecular biomarkers analyzed included four G-protein coupled receptors (GPCRs) representing the two major classes of odorant receptors, as well as markers of neurite outgrowth and metal/antioxidant responses to metals. Coho received acute (8-168hr) exposures to 3.7 µg/L and 347 µg/L Cd, and a subset of fish was analyzed following a 16-day depuration. Coho exposed to 347 µg/L Cd over 48hrs exhibited a reduction in freeze responses, and an extensive loss of olfaction accompanied by histological injury to the olfactory epithelium. The olfactory injury in coho exposed to 347 µg/L Cd was accompanied at the gene level by significant decreases in expression of the olfactory GPCRs and increased expression of metal and antioxidant response markers. Persistent behavioral deficits, histological injury and altered expression of a subset of olfactory biomarkers were still evident in Cd-exposed coho following a 16-day depuration in clean water. Exposure to 3.7 µg/L Cd also resulted in reduced freeze responses and histological changes to the olfactory epithelium within 48hrs of Cd exposure, although the extent of olfactory injury was less severe than observed for fish in the high Cd exposure group. Furthermore, adverse behavioral effects were present in some coho receiving the low Cd exposure following a 16-day depuration. Based on these findings, the goals for Aim 2 of my research were to investigate the toxicity of Cd on salmon olfactory function that modeled a transient sub-chronic exposure scenario. Coho were exposed to environmentally relevant concentrations of waterborne Cd (0.3, 2, and 30 µg/L) for 48hrs and 16 days, followed by a 16-day depuration. Exposures to Cd significantly and persistently altered behavioral responses towards L-cysteine and conspecific odorants. Behavioral alterations following exposure to 30 µg/L Cd were associated with increased olfactory epithelial gene expression of stress markers metallothionein and heme oxygenase, altered protein expression of olfactory signal transduction

(OST) molecules critical in maintaining olfactory function, and altered gene expression of major coho odorant receptors (ORs). Exposure to Cd predominantly decreased expression of OST protein and OR molecular markers of ciliated olfactory sensory neurons (OSNs) compared to OST and OR markers of microvillus OSNs, suggesting a differential sensitivity towards Cd between the two major OSN populations. Behavioral alterations on exposure 0.3 and 2 μg/L Cd did not coincide with major histological injury or molecular changes in expression of major OST or OR markers, likely indicating disrupted signal transduction as the major mechanism underlying the olfactory behavioral dysfunction at the low-level Cd exposures. In summary, transient exposures to very low-levels of Cd can induce significant and persistent alterations of olfactory function in juvenile salmon, which could lead to increased mortality and ultimately increased stress on already threatened populations.

Table of contents:

1. Title page

3. Abstract

6. Table of contents

7. Acknowledgments

8. Introduction

18. Chapter 1: Effects of cadmium on olfactory mediated behaviors and molecular biomarkers in coho salmon (*Oncorhynchus kisutch*).

40. Chapter 2: Cadmium exposure differentially alters odorant driven behaviors and expression of olfactory receptors in juvenile coho salmon (*Oncorhynchus kisutch*).

74. Conclusions

76. References

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Introduction:

Sensory systems such as the visual, auditory, mechanosensory, thermosensory, gustatory and olfactory systems, connect living organisms to the environment around them. A difference in environmental conditions and an organism's life history often puts an increased emphasis on certain sensory systems over others. In aquatic organisms, the olfactory sensory system serves a critical role in survival. Odorants in the water convey important environmental and biological information to an organism regardless of water conditions that impact other sensory systems, such as impaired vision due to decreased water clarity. This is important as fish species rely on olfaction for many essential survival functions such as navigation, prey capture, predator avoidance, and social and reproductive cue detection (Buchinger *et al.,* 2014; Hara 2012; Hara 2006). Salmon, in particular, rely on their highly acute sense of smell, which they use for their long migration from marine waters back to their natal freshwater river for spawning (Cooper *et al.,* 1976; Dittman and Quinn 1996; Sutterlin and Gray 1973). However, before adult salmon can utilize their sense of smell for the return migration, they must first imprint the "scent signature" of their natal stream as they migrate downstream towards the ocean. When juvenile salmon emerge from the gravel beds and migrate downstream, it is believed they sequentially imprint the scent of the waterway along the migratory route (Dittman and Quinn 1996; Quinn 2011). This system allows juvenile salmon to remember the scent of "waypoints", which increases their chances of a successful return navigation as adults. These outward and return migrations represent highly sensitive periods in the salmon lifecycle as they rely heavily on a fully functional olfactory system for survival. Unfortunately, the downstream and upstream migrations are increasingly occurring through human impacted waterways, which often have impaired water quality.

Salmon migrating through rivers with impaired water quality, such as rivers receiving input from urban and agricultural areas, have the potential of experiencing disruption of olfactory function due to waterborne pollutants (Johannessen and Ross 2002; Johnson *et al.,* 2007). As salmon rely on their sense of smell for survival, disruption of olfactory function could lead to increased mortality (McIntyre *et al.,* 2012). Indeed, while U.S. Pacific salmon populations are under increased pressure from a variety of anthropogenic factors such as over-fishing and habitat loss, the disruption of olfactory function from transient exposures to environmental pollutants is believed to be a factor contributing to population declines. The fish olfactory system, a major route of chemical exposure, is extremely sensitive to disruption by waterborne pollutants (Hernadi 1993; Tierney *et al.,* 2010). The paired peripheral olfactory sensory rosettes consist of olfactory cavities that directly connects the surrounding water with the olfactory rosettes (Hamdani el and Doving 2007). The surfaces of the rosettes are covered by both a non-sensory and sensory epithelium. The non-sensory epithelium produces a thin mucus layer, which covers the sensory epithelium as a protective measure from waterborne toxicants (Part and Lock 1983). The sensory epithelium contains thousands of olfactory sensory neurons (OSNs). Olfactory sensory neurons are responsible for detecting odorant cues in the surrounding aquatic environment and relaying the signal to the brain. It is this neuron to water connection that facilitates interaction with waterborne pollutants and makes the salmon olfactory system highly sensitive to disruption.

The potential for chemical disruption of fish olfactory function has long been known (Brown 1982; Sutterlin and Sutterlin 1971; Tierney *et al.,* 2010). While natural factors such as changes in

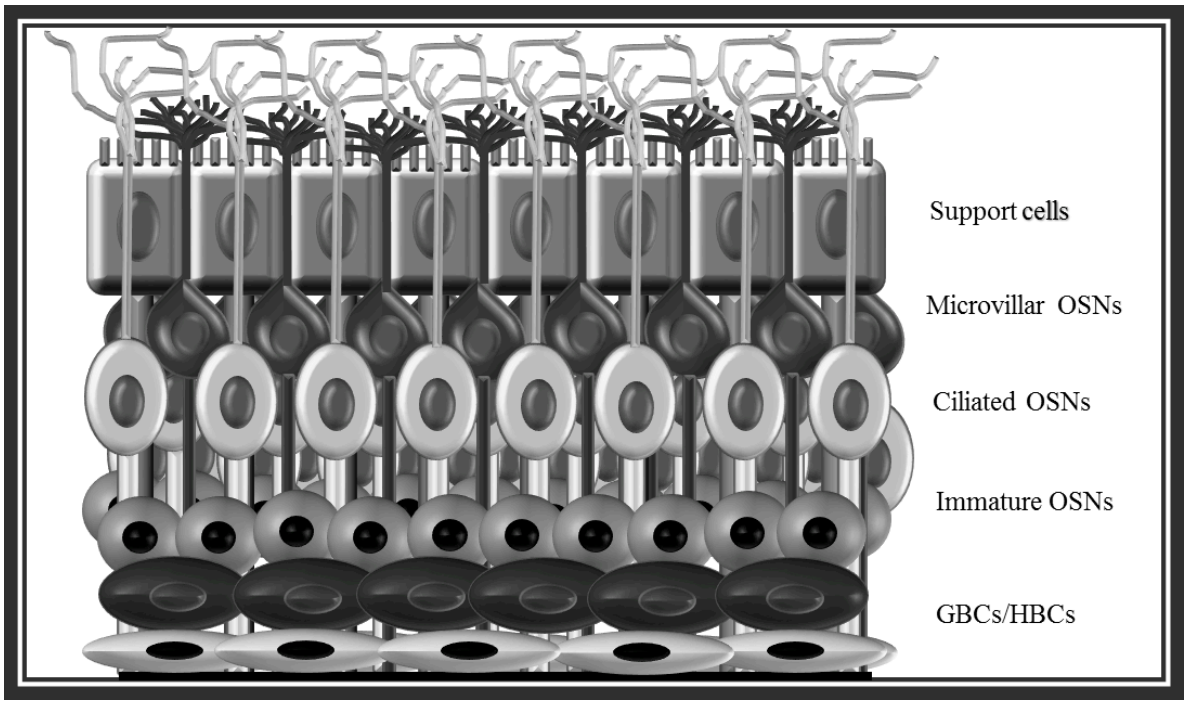
pH can have drastic effects on olfactory function, the effects of anthropogenic pollutants such as metals and pesticides have much more significant and long lasting effects on olfactory function (Leduc *et al.,* 2013; Maryoung *et al.,* 2015; Royce-Malmgren and Watson 1987; Tierney *et al.,* 2010; Tierney *et al.,* 2008). Dissolved waterborne metals, predominantly from anthropogenic sources, are common pollutants and known to be olfactory toxicants (Tierney *et al.,* 2010). Research on metals and fish olfactory toxicity has largely focused on copper, a very potent olfactory toxicant among metals (Baldwin *et al.,* 2011; Bettini *et al.,* 2006; Beyers and Farmer 2001; Hansen *et al.,* 1999; Hara *et al.,* 1976; Julliard *et al.,* 1996; Kolmakov *et al.,* 2009; McIntyre *et al.,* 2012; Sandahl *et al.,* 2007; Sandahl *et al.,* 2006; Saucier and Astic 1995; Saucier *et al.,* 1991; Saucier *et al.,* 1991; Wang *et al.,* 2013). Its ubiquitous use in products from brake pads to anti-fouling boat paint has introduced large amounts of copper into important salmon-bearing waters, with deleterious effects on fish survival (Schiff *et al.,* 2004). The olfactory toxicity of other metals, particularly cadmium (Cd), have been relatively understudied, and Cd has been shown to disrupt olfaction at levels comparable to that of copper (Chapman 1978; Scott *et al.,* 2003; Sloman *et al.,* 2003).

Waterborne Cd is predominantly derived from anthropogenic sources such as phosphate fertilizers, industrial runoff, discarded batteries, and deposition of particulate matter generated by pyrolysis, with a smaller fraction coming from natural sources. Waterborne Cd poses a significant risk for fish species such as salmon as waterborne Cd can readily accumulate within the peripheral salmon olfactory system and travel up to the olfactory bulb. Utilizing radiolabeled Cd, studies found that the Cd signal was about equal between the olfactory rosettes and the gills (Harrison and Klaverkamp 1989; Scott *et al.,* 2003; Tjälve *et al.,* 1986), a significant finding as

the gills of fish have long been thought to be the primary route of uptake of waterborne Cd. There are substantial numbers of ion channels in the gills for which Cd may use as a pathway of cellular entry. Cadmium can also rapidly enter the fish peripheral olfactory system, which has a high concentration of neuronal ion channels, and represents a serious risk for fish species exposed to waterborne Cd (Usai *et al.,* 1999). Specifically, the rapid uptake of Cd poses a significant danger for migrating juvenile salmon, due to the high toxicity of Cd and the period of exposure, which can last from several hours to up to two months (Ruggerone 2003). Once inside cells, Cd possesses the ability to induce oxidative and cellular stress, which can result in DNA damage, protein misfolding, and apoptosis (Cuypers *et al.,* 2010; Ercal *et al.,* 2001; Thévenod 2009). This disruption of cellular machinery and function can lead to significant losses of OSNs via cellular death. Indeed, studies investigating the effects of acute Cd exposures on fish olfactory systems found significant OSN injury and losses following waterborne exposures (Hernadi 1993; Matz and Krone 2007; Scott R. Blechinger and Douglas P. Chivers 2007; Wang *et al.,* 2013). Significant losses of OSNs can ultimately lead to impaired olfactory function. In addition to investigating the acute effects of Cd exposures, one study also investigated the potential long-term effects of Cd exposures on olfactory function. They found that juvenile zebrafish acutely exposed to Cd had olfactory deficits that persisted into adulthood (Scott R. Blechinger and Douglas P. Chivers 2007). However, while Cd exposures in the wild can last up to two months, our understanding of the effects of Cd on fish olfactory function is based only on acute exposures.

Despite what is known about the effects of Cd on fish olfactory function, the mechanisms underlying the Cd induced injury and functional disruption remain largely unknown. The

olfactory system is a highly complex and tightly coordinated signaling system with a remarkable ability for regeneration following injury. The salmon peripheral olfactory system is composed of a pair of rosette shaped structures, made up of 16 lamellae each. Each lamella is covered in non-sensory and sensory olfactory epithelia. The olfactory sensory epithelium is arranged in a pseudostratified layer and comprised of several major olfactory cell types (Figure 1). These include: support cells (SUS cells), which serve a glial like role in supporting the mature OSNs; goblet cells, which produce the mucosal layer that covers the sensory epithelial surface; mature



microvillar and ciliated OSNs, which detect odorant molecules; crypt cells, which are thought to detect odorant molecules; immature OSNs, which replenish the senescing/injured mature OSNs; and two multipotent olfactory stem cell (OSC) populations, the globose basal cells (GBCs) and horizontal basal cells (HBCs), which can generate all of the cells that make up the olfactory

sensory epithelium (Hamdani el and Doving 2007). While these cell populations work in concert to form a functional peripheral olfactory system, it is the role of both the ciliated and microvillar OSNs to detect odorants and send that signal to the brain. Initially, when an odorant binds to an odorant receptor (a G-protein coupled receptor, GPCR), located on the surface of cilia or microvilli of an OSN, it sets off a cascade of signaling molecules, ultimately opening calcium and chloride ion channels which sends a signal to the brain where the signal is decoded and a behavioral response is decided (DeMaria and Ngai 2010; Whitlock 2006).

The salmon olfactory system utilizes between 100-200 unique odorant receptors, but it is tasked with detecting several hundred unique odorants (Johnstone *et al.,* 2012). To bridge that gap, the olfactory system uses a combinatorial approach for odorant detection, wherein an odorant could activate several different odorant receptors at the same time (Malnic *et al.,* 1999). Having one odorant activate a set of unique odorant receptors provides each odorant a unique identifier, a "fingerprint". This combinatorial activation method can potentially produce billions of unique odorant "fingerprints", thus allowing the olfactory system to detect far more odorants than it has unique odorant receptors. Furthermore, the olfactory system generally employs the "one receptor-one neuron" rule, which means that each olfactory sensory neuron only expresses one

Figure 1. Olfactory sensory epithelial cell population and structure.

type of odorant receptor (Lewcock and Reed 2004; Sato *et al.,* 2007; Serizawa *et al.,* 2003). The salmon odorant receptors are divided into four main classes: the main olfactory receptors (mORs) and the trace-amine associated receptors (TAARs), which are located in ciliated OSNs and the vomeronasal type 2 receptor-like (OlfC) and vomeronasal type 1 receptor-like (ora),

located in the microvillar OSNs. Characterization of the odorant receptors within each of these classes has not been completed in salmon in salmonids, however research in zebrafish and rodents has provided insight into their possible functions (Behrens *et al.,* 2014; Jiang *et al.,* 2015; Liberles 2015). For example, it is thought that vomeronasal receptors detect pheromone odorants and a subset of amino acids, whereas the ciliated OSNs likely detect many more types of amino acids as well as other odorant cues not related to pheromones (Del Punta *et al.,* 2002; Hansen *et al.,* 2003; Leinders-Zufall *et al.,* 2000; Sato and Suzuki 2001; Zippel *et al.,* 1997). Another layer of complexity of olfactory function is that the ciliated and microvillar OSNs employ different molecules in their signal transduction pathways. The ciliated OSNs utilize a cAMP/adenylyl-cyclase III (ACIII) mediated pathway, whereas microvillar OSNs utilize an inositol triphosphate (IP3)/Phospholipase-C (PLC) mediated pathway. Each pathway utilizes calcium, chloride and sodium channels for signaling to propagate an odorant-induced signal to the olfactory bulb.

The combinatorial system utilized by the salmon olfactory system, the different signal transduction molecules used by each of the major OSNs classes, and the potential "specialization" of odorant receptors make odorant signaling and perception a potential target for disruption by metals. Theoretically, alteration of an odorant's "fingerprint" could completely change how a fish perceives what the odorant is. For example, if Cd predominantly inhibited one type of OSNs signaling, due to differences in signal transduction machinery, then an odorant that elicits a fear/escape response could have its "fingerprint" changed so that now when the fish detects the odorant, instead of escaping it may perceive the odorant as an attractive odorant. Indeed, there is some evidence that copper has a differential toxicity on the different classes of

OSNs (Dew *et al.,* 2014; Kolmakov *et al.,* 2009), however, those studies did not investigate the reasons for the possible differential toxicity.

The ability of Cd to disrupt OSN signaling is associated with its strong affinity for calcium and essential-metal binding sites within cells. Important cellular signaling events can be interrupted by Cd through the disruption/displacement of endogenous calcium, zinc and copper and their binding sites (Choong *et al.,* 2014; Hartwig 2001; Thévenod 2009). These binding sites can potentially be in important zinc-finger proteins, other metalloproteins, calcium activated proteins and channels involved in olfactory signal transduction. In particular, Cd has also been shown to disrupt adenylyl cyclase function, a molecule central in the neural signal transduction pathway used by ciliated OSNs, as well calcium ion channels used by OSNs (Lundberg *et al.,* 1987). As previously stated, both types of major OSNs classes utilize different signal transduction molecules with varying degrees of reliance on calcium signaling. Furthermore, the displacement of essential metals, such as zinc, from binding sites could inhibit neural function or induce cellular stress, which could affect neuronal signaling (Eckler *et al.,* 2011; Hartwig 2001; Huang *et al.,* 2006; Thévenod 2009). It was proposed that some olfactory GPCRs could have metal binding sites needed for proper function (Wang *et al.,* 2003). If Cd displaced the essential metal from these odorant receptors, it could theoretically disrupt the receptor's function. Despite all of these possible mechanisms in which Cd could disrupt and alter olfactory function, it has remained relatively understudied in fish species, especially in salmon.

In addition to inhibition of major OSN populations and signal transduction machinery, another potential target of Cd induced olfactory toxicity is the OSC population. Following natural

senescence of mature OSNs (roughly a two-week lifespan) and injury repair, the two populations of OSCs, HBCs and GBCs, mediate olfactory regeneration (Beites *et al.,* 2005; Carter *et al.,* 2004; DeMaria and Ngai 2010; Iwai *et al.,* 2008; Jang *et al.,* 2003; Leung *et al.,* 2007; Mackay-Sim 2010). Cadmium induced disruption of the natural OSN turnover, or regeneration following injury, could lead to significantly slower recovery of the olfactory epithelium, resulting in long-term olfactory dysfunction. Studies have shown that ablation of the olfactory epithelium, which killed many of the OSCs, resulted in significantly impaired recovery of the olfactory epithelium (Iqbal and Byrd-Jacobs 2010; Schwob *et al.,* 1995). One study found no regrowth of mature OSNs following exposure to methyl-bromide and instead observed an olfactory epithelium that most resembled respiratory tissue (Schwob *et al.,* 1995). Furthermore, zebrafish sub-chronically exposed to metals during adulthood and early juvenile stages have impaired olfactory epithelial regeneration and functional recovery. (Faucher *et al.,* 2012; Kusch *et al.,* 2008; Scott R. Blechinger and Douglas P. Chivers 2007). These studies indicate that it is possible for metals, such as Cd, can impair the regenerative capability of the olfactory sensory epithelium. It is known that Cd can rapidly accumulate throughout the salmonid olfactory sensory epithelium, indicating that Cd could potentially reach the OSC in the basal layer (Sloman *et al.,* 2003). Additionally, Cd exposures on juvenile salmon in the wild can persist for up to 2 months, which is a sufficient period of exposure leading to significant Cd accumulation and exceeding the OSN lifespan of two-weeks (Bettini *et al.,* 2006; Julliard *et al.,* 1996; Scott *et al.,* 2003). Accordingly, the presences of significant amounts of Cd within the salmon olfactory sensory epithelium could affect both OSN lifespan and the regeneration of lost OSNs by the OSCs. However, there are currently no studies that have investigated the effects of sub-chronic Cd exposures on juvenile salmon olfactory function and recovery, and if the OSC in their olfactory epithelia are impacted.

Based on the research gaps on the effects of waterborne Cd on juvenile salmon olfactory function, the focus of my research was to investigate the effects of Cd on juvenile coho salmon olfactory mediated behaviors in response to a suite of different odorants. In addition, I investigated regeneration of the olfactory epithelium following Cd induced injury and the potential differential toxicity of Cd on the two major classes of OSNs. The goal for aim 1 was to develop a suite of olfactory molecular biomarkers of Cd exposure and injury, with the hypothesis that exposures to Cd would significantly alter the expression of the olfactory biomarkers, and the altered expression would be associated with olfactory epithelial injury and decreased olfactory function. Aim 2 had two main research goals. Aim 2a was to elucidate the effects of Cd on salmon behavioral responses to a diverse array of olfactory odorants; my hypothesis being that Cd exposures, at environmental concentrations, would differentially impair responses to prototypical odorants. Aim 2b, was to investigate the effects of Cd on the different olfactory neuron populations; my hypothesis being that the differential odorant responses observed in aim 2a experiments would be associated with impacts to specific olfactory neuronal cell populations.

Chapter 1:

Effects of cadmium on olfactory mediated behaviors and molecular biomarkers in coho salmon (*Oncorhynchus kisutch*)

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Abstract

The olfactory system of salmonids is sensitive to the adverse effects of metals such as copper and cadmium. In the current study, we analyzed olfactory-mediated alarm responses, epithelial injury and recovery, and a suite of olfactory molecular biomarkers encoding genes critical in maintaining olfactory function in juvenile coho salmon receiving acute exposures to cadmium (Cd). The molecular biomarkers analyzed included four G-protein coupled receptors (GPCRs) representing the two major classes of odorant receptors (salmon olfactory receptor *sorb* and vomeronasal receptors *svra*, *svrb*, and *gpr27*), as well as markers of neurite outgrowth (*nrn1*) and antioxidant responses to metals, including *heme oxygenase 1* (*hmox1)*, and *peroxiredoxin 1* (*prdx1*). Coho received acute (8-168 hr) exposures to 3.7 ppb and 347 ppb Cd, and a subset of fish was analyzed following a 16-day depuration. Coho exposed to 347 ppb Cd over 48 hrs

exhibited a reduction in freeze responses, and an extensive loss of olfaction accompanied by histological injury to the olfactory epithelium. The olfactory injury in coho exposed to 347 ppb Cd was accompanied at the gene level by significant decreases in expression of the olfactory GPCRs and increased expression of *hmox1*. Persistent behavioral deficits, histological injury and altered expression of a subset of olfactory biomarkers were still evident in Cd-exposed coho following a 16-day depuration in clean water. Exposure to 3.7 ppb Cd also resulted in reduced freeze responses and histological changes to the olfactory epithelium within 48 hrs of Cd exposure, although the extent of olfactory injury was less severe than observed for fish in the high Cd exposure group. Furthermore, adverse behavioral effects were present in some coho receiving the low Cd exposure level following a 16-day depuration. In summary, acute exposures to environmental levels of Cd can cause olfactory injury in coho salmon that may persist following depuration. Mechanism-based biomarkers of oxidative stress and olfactory structures can augment the evaluation of olfactory injury manifested at the physiological level.

1.0 Introduction

Salmon populations have declined in the western United States, with several species being listed as extinct or endangered (Brown *et al.,* 1994; Nehlsen *et al.,* 1991; NOAA 2011). One of the factors implicated in these population declines is inhibition of olfactory processes associated with exposures to waterborne pollutants, including metals (Baldwin *et al.,* 2009; Brown *et al.,* 1994; Domagalski 1996; Feist *et al.,* 2011; Sandahl *et al.,* 2007; Scholz *et al.,* 2011). The olfactory sensory epithelium is in direct contact with the water column, making it highly sensitive to metal uptake and metal-induced olfactory injury (Baldwin *et al.,* 2003; Beyers and

Farmer 2001; Carreau and Pyle 2005; Julliard *et al.,* 1996; Kolmakov *et al.,* 2009; Moore and Waring 2001; Sloman *et al.,* 2003). Exposures to environmentally relevant concentrations of Cd, in particular, have been shown to alter important behavioral and physiological functions associated with the detection of olfactory odorants (Kusch *et al.,* 2008; Matz and Krone 2007; Scott *et al.,* 2003; Sloman *et al.,* 2003). Even a transient impairment of olfaction can lead to increased mortality through a loss of competence and increased susceptibility to predation (Beyers and Farmer 2001; Hamdani el and Doving 2007; Sandahl *et al.,* 2007). Collectively, these observations indicate that fish olfactory injury can serve as a relevant adverse outcome pathway (Ankley *et al.,* 2010; Carvan *et al.,* 2008) in assessing the effects of aquatic pollution.

At the cellular level, the salmon olfactory epithelium contains specialized olfactory receptor neurons (ORNs) of two sub-systems. ORNs in the olfactory system are responsive to amino acids, prostaglandins, steroids and bile acid odorants, whereas ORNs in the vomeronasal system are responsive to amino acids and nucleotide odorants (Sato and Suzuki 2001). These odorants bind to G-protein coupled receptors (GPCRs), located on the apical cilia and microvilli of the olfactory and vomeronasal ORNs (Hamdani el and Doving 2007). GPCRs play a central role in olfactory signal transduction and thus have the potential to serve as sensitive biomarkers of metal induced olfactory injury. However, olfactory GPCRs remain significantly under studied in the context of biomarker development and application.

The goals of the present study were to characterize the effects of acute Cd exposures on olfactory-mediated behaviors, histological injury, and molecular biomarkers in coho salmon, we employed a mechanism-based biomarker approach using gene markers associated with olfactory

injury and regeneration to link histological and behavioral impacts from exposures. Our biomarker genes included those encoding two major classes of odorant receptors (Dukes *et al.,* 2004; Dukes *et al.,* 2006), and biomarkers of antioxidant responses. These aforementioned pathways have been quantitatively altered in the olfactory tissues of fish following metal exposures (Berg *et al.,* 2010; Espinoza *et al.,* 2012; Tilton *et al.,* 2008; Wang *et al.,* 2013). As regeneration of lost ORNs is critical for olfactory function, we also utilized a biomarker of ORN neurite regeneration (Marron *et al.,* 2005; Naeve *et al.,* 1997). Our laboratory exposure paradigm modeled the scenario in which juvenile salmon out-migrating through contaminated waterways could be transiently exposed to metals followed by transition to unpolluted water (Ruggerone 2003).

2.0 Materials and Methods

2.1. *Chemicals.*

MS-222 (Tricaine methanesulfonate) was purchased from Argent Chemical Laboratories (Redmond, WA). Analytical grade cadmium chloride was purchased from Mallinckrodt Baker (Phillipsburg, NJ). RNeasy® mini kit was obtained from Qiagen (Valencia, CA). TRIzol® reagent and SuperScript® First-Strand Synthesis System were purchased from Invitrogen (Carlsbad, CA). Finnzymes® DyNAmo® SYBR Green 2-Step qPCR Kit was purchased from New England Biolabs, Inc. (Ipswich, MA). Quantitative real time PCR (qPCR) primers were obtained from Eurofins MWG Operon (Huntsville, AL).

2.2. *Fish husbandry and exposures.*

Juvenile coho salmon (1 yr of age, 15.0 g ± 5.7 mg) were raised in large cylindrical tanks with 8-10 °C recirculating freshwater from Lake Washington, Seattle under a natural photoperiod. The fish were fed BioVita Fry Feed (Bio-Oregon) and water quality conditions were typically 80-120 mg/L total hardness as calcium carbonate, pH 7.4 ± 0.2, 3mg/L DOC, and 9.1 mg/L dissolved oxygen content. Water chemistry was similar to those of other salmon-bearing rivers and streams in the Pacific Northwest (McIntyre *et al.,* 2008). Twenty-four hours prior to exposure the fish were transferred to 120 L glass aquaria, containing aerated lake water inside a large chilled water bath for acclimation. For the exposures, 8-12 juvenile coho per treatment were exposed for 8-168 hrs, with an equal number of control animals receiving carrier (DI water). The concentrations of waterborne Cd used were 3.7 ppb, a concentration below the 5 ppb EPA drinking water standard (EPA, water.epa.gov/drink/contaminants) and representative of urban storm water runoff concentrations (WSDOE 2008), and 347 ppb, representative of heavily polluted waterways (Maceda-Veiga *et al.,* 2012; Srinivasa Gowd and Govil 2008). Total waterborne Cd concentrations were analyzed prior and post exposures by ICP-MS (Espinoza *et al.,* 2012). Background levels of total Cd in lake water were below the limit of detection (0.1 ppb). Although the nominal Cd concentrations were 3.1 and 310 ppb, the measured total Cd concentrations (3.7 and 347 ppb) are used in all tables and figures. A 90% static renewal approach was implemented with water containing Cd replaced after a 24 hr period. Exposures were staggered for each of the four experimental periods (8, 24, 48, and 168 hrs) so that equal numbers of fish were continually exposed for the treatments, and to avoid ammonia buildup. Nitrate and nitrite levels were below detection for all treatment groups, and ammonia levels were $\leq$ 0.25 ppm during the exposures.

For the depuration study, Cd-exposed coho (and controls) were treated as described above with the following modification. Following 48 hrs of exposure, fish underwent behavioral testing before being returned to the large cylindrical tanks and allowed to recover for 16 days in clean water. At the termination of the 16-day depuration, the coho were transferred to 120 L glass aquaria and held in lake water for 48 hrs prior to behavioral testing and tissue collection.

2.3. *Neurobehavioral analysis of olfactory function.*

Olfactory-mediated behavioral analysis was conducted using a Y-maze with a black curtain enclosure to avoid frightening the fish during testing. The Y-maze was 100 x 40 x 25 cm and consisted of two arms, each of which measured 50 cm long and 20 cm wide. The arms terminated at a holding chamber, which was 40 x 40 cm. A transparent perforated gate separated the maze arms from the holding chamber. The maze received lake water at a constant flow rate of 8.3 L/min. A dye test was performed to confirm no mixing occurred between the arms. Coho from each Cd exposure group (n=12) were tested in groups of 4 and were acclimated for 15 minutes in the holding chamber prior to stimulus addition (skin extract or DI water). Stimuli were delivered via a peristaltic pump at 10 ml/min, and the gate was lifted 2 minutes post addition of the stimuli. Video recordings for behavioral responses were initiated 10 minutes prior to the gate lifting to establish baseline behavior, and for 5 minutes after gate opening to establish the behavioral response. The maze was flushed with clean water between trials and the arm receiving the skin extract was alternated.

For alarm testing, a coho skin extract was prepared by collecting 10 grams of skin tissue from 4 juvenile coho. The skin was rinsed in ice-cold PBS and homogenized in 40 ml distilled water on

ice and filtered. The skin extract was diluted in 1 L of DI water to provide a final stock concentration of 10 mg/ml. Behavioral endpoints analyzed were associated with alarm responses and included freeze responses and odor avoidance. The freeze response is a classic stress/alarm response which is characterized by the lack of movement for >2 sec (Egan *et al.,* 2009). Odor avoidance was characterized as time spent in the maze arm receiving DI water vs. time in the arm receiving the skin extract.

2.4. *Histological analysis of olfactory injury.*

Coho head tissues collected for histological analyses were fixed in 4% paraformaldehyde (PFA) at 4 ^{o}C. Olfactory rosettes were excised and embedded in a 2:1 mixture of 20% sucrose to Tissue-Tek® OCT™. Tissue sections (6 μm) were mounted on VWR® glass Superfrost Plus micro-slides. Sections were stained with Mayer's hematoxylin and eosin (H&E) and the slides were examined using a Nikon LABOPHOT-2 microscope. Images were captured using Nuance 3.0 imaging software. Histological sections were scored based on a 4-grade scale as follows; grade 0 (no visible difference relative to controls), grade 1 (clearly visible increase in the number of goblet cells), grade 2 (clearly visible increase in the number of goblet cells and decreased nuclei near the apical epithelial surface), grade 3 (clearly visible increase in the number of goblet cells, decreased nuclei near the apical epithelial surface and the presence of condensed nuclei). Three tissue sections were analyzed for each individual coho, and 3 coho were analyzed within each exposure group.

2.5. *Cellular apoptosis (TUNEL assay).*

Detection of apoptosis in the olfactory epithelium was performed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (In Situ Cell Death Detection Kit; Roche Diagnostics, Indianapolis, IN) using olfactory tissue sections. Images were collected using a Nikon LABOPHOT-2 microscope with a red filter set (Nikon G-1B, EX540/10, DM580, BA590) and captured at 610 nm with Nuance 3.0.1 software (Caliper Life Science, Hopkinton, MA). Image analysis was performed with Metamorph® software (Molecular Devices, Sunnyvale, CA). TUNEL positive nuclei detected above the threshold value (determined by negative controls) were counted and expressed as number of positive nuclei per 100 μm^2 of olfactory epithelium. Three tissue sections were analyzed for each fish and 3 individual coho were analyzed in each exposure group.

2.6. *Quantitative PCR analysis of olfactory genes.*

Eight individual coho were collected for analysis of molecular biomarkers. The fish were anesthetized with MS-222 prior to cervical dislocation, and the olfactory rosettes were quickly excised, transferred to TRIzol® reagent, frozen in liquid nitrogen and stored at -80 ˚C. Total RNA isolation and cDNA synthesis procedures have been described previously (Espinoza *et al.,* 2012). Candidate olfactory biomarker gene primers were generated by multiple sequence alignment of rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), and zebrafish (*Danio rerio*) target sequences. Primers for *hmox1*, *prdx1*, *nrn1* and *gpr27* were then designed using Oligo® Software, Version 6.71 (Cascade, CO). Dukes *et al,* (2006) previously published the *svra*, *svrb* and *sorb* primer sequences used in the present study (Primer sequences are presented in table 1). All PCR products were confirmed by sequencing. In addition to the above molecular markers, fish receiving the sub-chronic 168 hr exposure to 3.7 ppb Cd, as well

as depurated fish, were also analyzed for olfactory metallothionein-1a (*mt1a*) mRNA expression. We have previously shown *mt1a* to be responsive to acute Cd exposures (8-48 hr), but had not analyzed mRNA expression following longer-term low-level Cd exposures (Espinoza *et al.,* 2012). Quantitative PCR analysis for all genes was conducted in a 96-well format with SYBR Green using the relative standard curve method (Espinoza *et al.,* 2012). ß-actin expression did not differ between treatments and was used for normalization purposes.

2.7. *Statistical Analysis*.

Behavioral, histological and gene expression data were inspected for homogeneity of variances using D'Agostino and Pearson omnibus normality testing. Gene expression, histological and behavioural data data conforming to normal distributions were assessed for significance relative to control animals using a one-way ANOVA followed by a Dunnett's test. In some cases, data conformed to a non-parametric distribution, and these datasets were assessed using Kruskal-Wallis one-way ANOVA test followed by Dunn's non-parametric post-hoc test. All treatment-related differences relative to controls were considered statistically significant at $p \leq 0.05$. All statistical analyses were conducted using GraphPad Prism Ver. 5.0 (Graph Pad Software Inc., San Diego, CA, USA).

3.0 Results

3.1. *Effects of Cd on olfactory mediated alarm behaviors.*

As observed in Fig. 1, coho receiving 8 hr exposure to 347 ppb Cd exhibited significantly reduced freeze responses to skin extract ($p<0.05$). This trend continued, reaching a maximum

92% reduction in freezing behavior at 48 hrs of exposure ($p<0.05$, Fig. 1). Additionally, coho exposed to 347 ppb Cd for 48 hrs exhibited significantly impaired skin extract avoidance behaviors, which were not evident following a 16-day depuration ($p<0.05$, Fig.1). Although coho exposed to 3.7 ppb Cd exhibited significantly reduced freeze responses after 24 hrs of exposure, there were no observable effects on avoidance behavior ($p<0.05$, Fig. 1). Following the 16-day depuration, coho exposed to both the high and low levels of Cd continued exhibiting significantly reduced freeze responses to skin extract relative to control animals ($p<0.05$, Fig. 1). Behavioral responses were not recorded for the 168 hr Cd exposures due to mortality following 96 hrs of exposure.

3.2. *Histological effects of Cd on the olfactory epithelium*.

Histological examination of the olfactory epithelium revealed that exposure to 347 ppb Cd caused a decrease in ORN and sustentacular (SUS) cells. All tissue sections in the high exposure group were scored grade 3, indicating a reduced olfactory epithelial cell population and an increased number of condensed nuclei compared to controls ($p<0.05$, Fig. 2A, B). TUNEL analysis confirmed a significant number of apoptotic cells in fish receiving exposure to 347 ppb Cd ($p<0.05$, Fig. 2E, F). Similar to the behavioral observations, continued histological recovery in the high exposure group was still evident after the depuration, with several fish exhibiting grade 2 characteristics (Fig. 2C, D). By contrast, the olfactory epithelium of coho continually exposed to 3.7 ppb Cd for up to 168 hrs showed relatively minor histological injury as evidenced by an increase in the number of goblet cells in the olfactory epithelia (Fig. 2A, D and Fig. 3A, B).

3.3. *Effects of Cd on the olfactory molecular biomarkers*.

Similar to the observed behavioral deficits and histological injury, coho exposed to 347 ppb Cd exhibited a time-dependent decrease of mRNAs encoding olfactory and vomeronasal receptors (Fig. 4). Of the 4 olfactory GPCRs analyzed, *sorb* and *svra* were the most sensitive to Cd, with maximal 90% and 80% decreases in expression observed at 48 hrs of exposure (Fig. 4). Following the 16-day depuration, the expression of *sorb* and *svra* remained decreased, although some partial recovery of expression was observed ($p<0.05$, Fig. 4). *Svrb* expression was not as sensitive to Cd as the other olfactory receptor markers, although a loss of expression was observed following 48 hrs of exposure to 347 ppb Cd ($p<0.05$, Fig. 4). Expression of *gpr27* significantly decreased maximally to 25% of control levels in coho exposed to 347 ppb Cd for 48 hrs ($p<0.05$, Fig. 4). The mRNA expression levels of *svrb* and *gpr27* following the depuration were not different from controls (Fig. 4). By contrast, exposure to the low level of Cd caused only minor changes in mRNA expression of the 4 olfactory GPCRs (Fig. 4 and Table 2).

Expression of *nrn1* decreased in a time and exposure level dependent manner, with a maximum 68% reduction relative to control coho following 48 hrs of exposure to 347 ppb Cd ($p<0.05$, Fig. 4). After depuration, *nrn1* expression was still elevated by 1.9-fold over control levels ($p<0.05$, Fig. 4). Expression of *nrn1* in coho exposed to 3.7 ppb Cd rapidly decreased by 48% at 8 hr of exposure, but was similar to that of controls at the 24, 48, 168 hr exposures, and after depuration (Fig. 4 and Table 2). *hmox1* mRNA expression was induced on exposure to 347 ppb Cd at all time points analyzed, with a maximal 7-fold induction observed at 24 hrs ($p<0.05$, Fig. 5). By contrast, exposure to 347 ppb Cd markedly decreased mRNA expression of *prdx1* to 19% of control levels after 48 hrs ($p<0.05$, Fig. 5). Following depuration, the expression of both *hmox1*

and *prdx1* mRNA in the 347 ppb Cd exposure groups did not differ from controls (Fig. 5). Conversely, expression of *mt1a* mRNA in the depurated 347 ppb Cd group remained highly induced (21-fold over controls, Fig. 5). Similarly, the expression of *mt1a* was induced 7-fold and 2-fold above controls following 168 hrs of exposure to 3.7 ppb Cd and the depuration, respectively (Table 2 and Fig. 5). Although some minor modulation of *hmox1* and *prdx1* expression were observed in coho receiving exposure to 3.7 ppb Cd, these effects were not statistically significant (Fig. 5).

4.0 Discussion

To understand the cellular events underlying metal-induced olfactory injury, it is critically important to provide a substantial linkage among events at the molecular level to effects manifested at the behavioral and physiological levels. These linkages are especially important in the development of fish olfactory injury as an adverse outcome pathway for applications to the field of ecotoxicology (Ankley *et al.,* 2010). Our approach in the current study was directed towards filling these data gaps using a representative salmonid and a model olfactory toxicant relevant to field exposures. It also included an exposure scenario that modeled the environmental scenario, which salmon may receive transient metal exposures followed by a period in which exposures do not occur (Ruggerone 2003). Cadmium serves as a well-established olfactory toxicant and environmental pollutant in which exposures as low as 2 ppb in rainbow trout may impair behavior (Sloman *et al.,* 2003). The fact that we observed a rapid inhibition of olfactory behavior as manifested by freezing in the presence of an alarm signal in coho represents a novel finding that supports the sensitivity of salmonids to Cd. While the ecotoxicological consequence

of this behavioral impairment was not established in the present study, freezing behavior is an important anti-predator behavior that can affect predator avoidance and survival (Sandahl *et al.,* 2007; Smith 1992). In this regard, McIntyre *et al.,* recently reported similar neurobehavioral effects in juvenile coho exposed to environmental levels of copper that led to an increased susceptibility to predation (McIntyre *et al.,* 2012).

The impaired alarm responses observed in coho exposed to high, but environmental levels of Cd, was associated with histological injury to the olfactory epithelium, induction of apoptosis, and the extensive loss of GPCR gene expression. Among the biomarkers tested, the olfactory GPCR biomarkers *sorb* and *svra* proved to be the most strongly associated to neurobehavioral injury. These observations are consistent with those of our previous microarray analysis of olfactory gene expression in olfactory tissues of zebrafish (Tilton *et al.,* 2008) that indicated GPCRs as potential targets of copper, supporting the utility of these endpoints as effects-based biomarkers. The fact that exposure to the low 3.7 ppb Cd exposure did not cause extensive damage to the olfactory epithelium, but partially blocked alarm behavior suggests a relevant adverse outcome for salmon migrating through metal contaminated waterways (Mirza *et al.,* 2009). It is possible that the effects of Cd were partially mitigated by the strong inductive response by *mt1a* and also by the increase in goblet cells associated with increased mucus production, which are classic olfactory responses to metals (Bettini *et al.,* 2006; Espinoza *et al.,* 2012; Julliard *et al.,* 1996; Tallkvist *et al.,* 2002). In particular, increased mucus production can slow the diffusion of odorants, decreasing olfactory sensitivity, and protects against Cd inhibition of sodium and calcium ion channels essential for olfactory signal transduction (DeMaria and Ngai 2010; Elinder and Århem 2003; Pärt and Lock 1983; Tierney *et al.,* 2010).

Although the molecular mechanisms underlying metal-induced olfactory epithelial injury have not been fully established, it is apparent that oxidative stress is a major factor in this process (Cuypers *et al.,* 2010; Ercal *et al.,* 2001; Espinoza *et al.,* 2012; He *et al.,* 2008; Wang *et al.,* 2013). In this regard, *hmox1* is a potent antioxidant enzyme that is highly inducible during oxidative stress (Alam and Cook 2003; Ryter and Choi 2002). The rapid increase in *hmox1* expression in coho exposed to Cd in our study indicates an olfactory antioxidant response that implicates a role for oxidative stress in olfactory cell injury. Similarly, the decline of *prdx1* expression may represent an overwhelming of antioxidant defenses, associated with the loss of SUS cells on Cd exposure, as these cells preferentially express *prdx1* (Novoselov *et al.,* 1999; Yu *et al.,* 2005). Loss of *prdx1* could be especially deleterious to managing the harmful effects of increased ROS production on olfactory signal transduction processes (Maher 2006; Rhee *et al.,* 2005).

Recovery of olfactory function and olfactory epithelial injury occurs in fish following exposure to environmental pollutants and is a mechanism to ensure survival (Beyers and Farmer 2001; Hamdani el and Doving 2007; Sandahl *et al.,* 2007). The relatively slow, or only partial, recovery of olfactory function observed following Cd exposures has ramifications for predator avoidance in the wild. Sloman *et al.,* reported that brief exposures to Cd as low as 2 ppb can impair social behaviors for a period of up to 5 days (Sloman *et al.,* 2003). Others have reported olfactory epithelial recovery within two weeks of chemical exposures (Bettini *et al.,* 2006; Sloman *et al.,* 2003), however longer recovery periods may occur following sub-chronic exposure to metals (Saucier *et al.,* 1991). Our findings of acute Cd exposure causing a poor

recovery of the coho olfactory epithelium was somewhat surprising, albeit consistent with effects at the molecular level associated with decreased expression of the GPCRs, elevated *nrn1* and *mt1a* mRNA in several of our exposure groups. Rapid bioaccumulation of waterborne Cd into the olfactory system has been previously reported (Scott *et al.,* 2003) and may explain the lack of a full olfactory recovery following acute Cd exposures. Collectively, these aforementioned observations support using molecular biomarkers in conjunction with analysis of effects at the physiological level to evaluate olfactory function in wild fish exposed to metals.

5.0 Conclusion

In summary, our findings indicate that acute Cd exposures can have both rapid and persistent effects on olfactory neurobehavioral function. Similar neurobehavioral effects have been linked to impaired survival and increased susceptibility to predation in salmonids. Our approach involving molecular biomarkers, histological analysis, and behavior indicate that impairment of key olfactory sensory neurons and G-protein coupled receptors and the losses of ORN and non-neural supporting (SUS) cells may underlie impaired alarm responses. In the context of biomonitoring for olfactory injury in the field, the molecular biomarkers *mt1a* and *hmox1* may be of particular relevance for detecting metal exposures and the ability of fish olfactory tissues to mount a protective antioxidant response within the olfactory epithelium.

Figures

Table 1. Sequences of PCR primers used in this study

Gene	Primers (5' to 3')	(Source Species) accession # / reference
β-actin	Forward: GACCCACACAGTGCCCATCT	(*O. mykiss*) AF157514/ Matsuo et al. (2008)
	Reverse: GTG CCCATCTCCTGCTCAAA	
svra	Forward: ATGGCCTTCAGGGCTACGCT	(*S. salar*) DQ375532/ Dukes et al. (2006)
	Reverse: AGGCAGCTTCCGAGCCAGAA	
svrb	Forward: ATAGCTTTCCAGGCCACAAT	(*S. salar*) DQ375537.1/ Dukes et al. (2006)
	Reverse: AGGCAGCTTCCGAGCCAGAA	
sorb	Forward: TGGCCATAGTCTTAGTGGGG	(*S. salar*) DQ375529/ Dukes et al. (2006)
	Reverse: GTCAAATGTGTGCTGCAGGT	
gpr27	Forward: GGGATGCATTTGTATCACCA	(*D. rerio*) NM_001114434
	Reverse: GCCTAGGCCTATGTCAATTCT	
nrn1	Forward: GCTCTCACCGCCTGTAGCAG	(*S. salar*) BT058878
	Reverse: TGCCCAGACCTCAACATCGTT	
prdx1	Forward: TTCTTCTTCTACCCGCTGGA	(*S. salar*) NM_001140823.2
	Reverse: CTGGTCCTCCTTCAGCACTC	
hmox1	Forward: GATGCTGGCCTACCAGAGAG	(*S. salar*) BT046987
	Reverse: GACTCCAGCCGTGCTAGTTC	
mt1a	Forward: CAAGTGCTCCAACTGTGCAT	(*S. salar*) BT059876/ Espinoza et al. (2012)
	Reverse: TACACCAGGCCTCACTGACA	

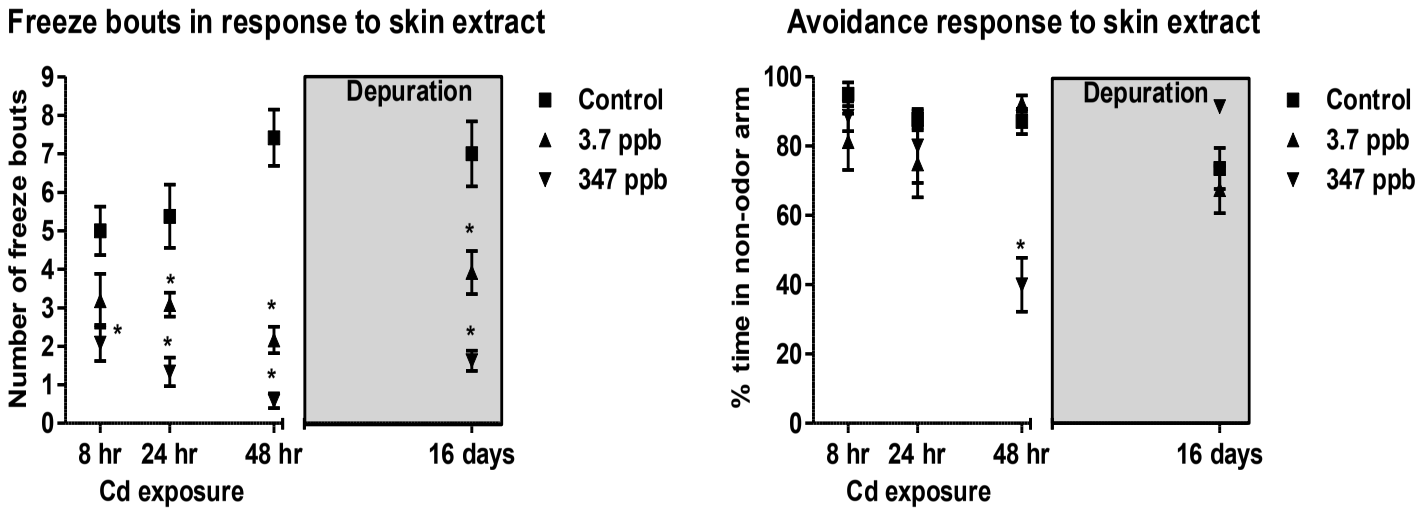

Figure. 1. Behavioral responses to a skin extract following acute exposures to 3.7 and 347 ppb Cd and after a depuration (grey box). (A) Freeze responses. (B) Analysis of avoidance to the skin extract. The 0, 3.7 and 347 ppb datasets are represented as squares, triangles and upside-down triangles, respectively, in the figures. All data represent the mean ± SEM of n=12 individuals. * indicates statistically significant differences relative to controls (p <0.05).

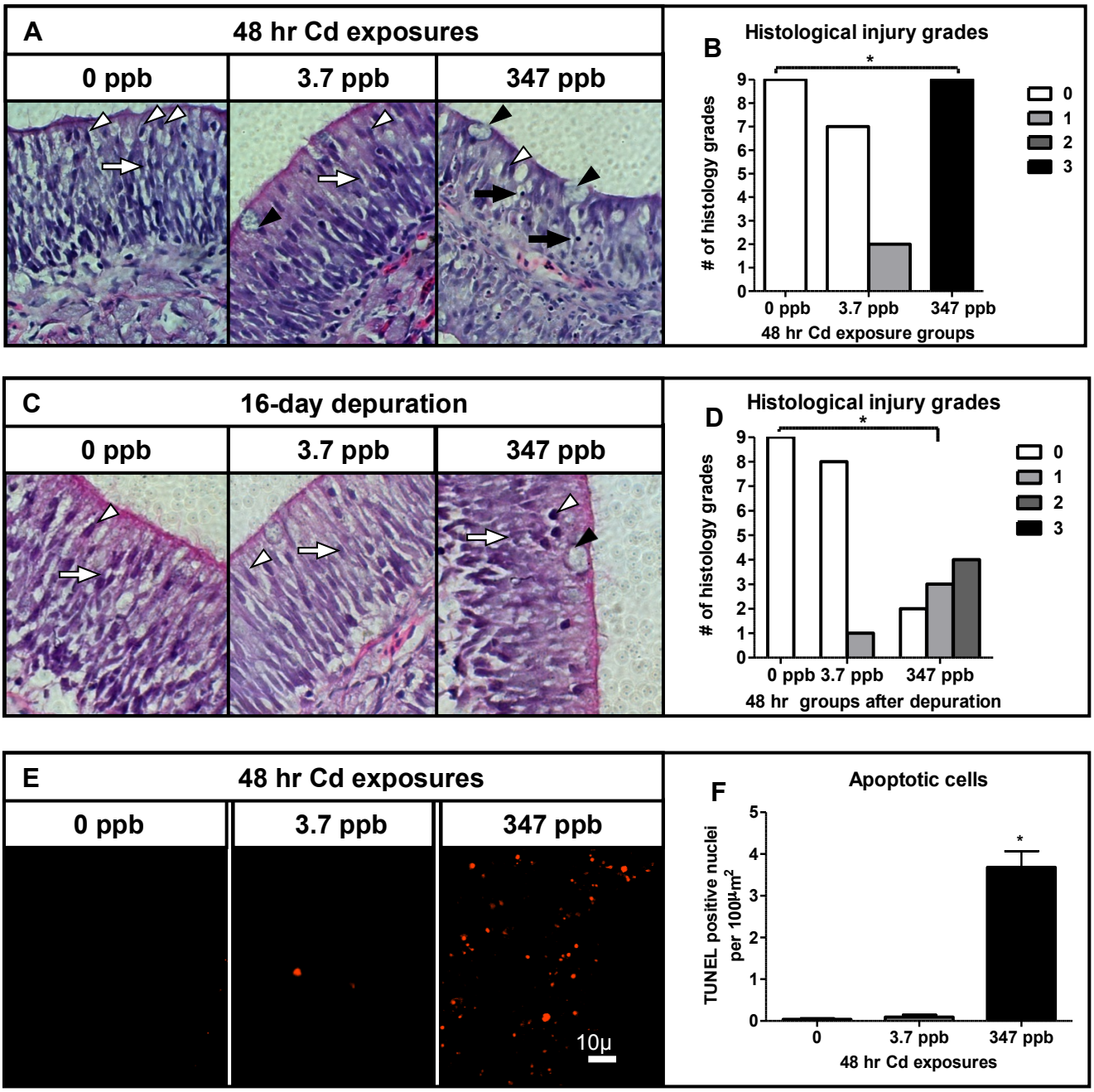

Figure 2. Histological effects of acute Cd exposure on the coho olfactory epithelium. (A and C) Light microphotograph (40x magnification) of cross-sectioned olfactory epithelium of coho exposed to 0, 3.7 and 347 ppb Cd for 48 hrs and following a 16-day depuration. SUS cells (white arrow heads), ORNs (white arrows), goblet cells (black arrowheads) and condensed nuclei (black arrows). (B and D) Histology injury grades of olfactory tissue sections following acute Cd exposures as represented in A and C. (E) Apoptosis in cross-sectioned olfactory epithelium of coho exposed to 0, 3.7 and 347 ppb Cd for 48 hrs (20x magnification). (F) Quantification of TUNEL positive nuclei per 100µm^2 of olfactory epithelium. All data represent the mean ± SEM of n = 3 individuals. * indicates statistically significant differences in histological injury relative to controls (p <0.05).

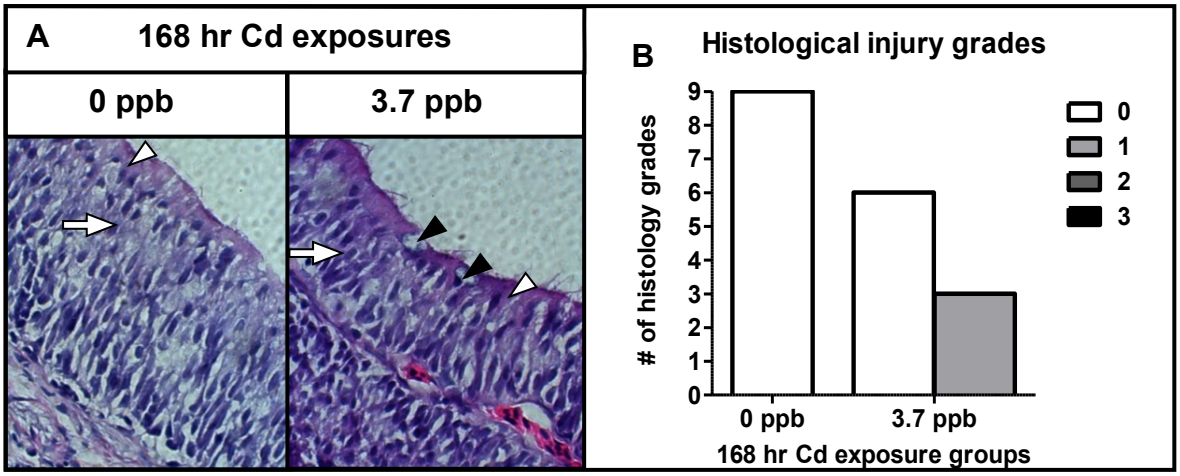

Figure 3. Histological effects of sub-chronic Cd exposure on the olfactory epithelium. (A) Light micrograph (40x magnification) of cross-sectioned olfactory epithelium of coho exposed to 0 and 3.7 ppb Cd for 168 hrs. SUS cells (white arrows), ORNs (white arrows), and goblet cells (black arrowheads). (B) Histology injury grades of olfactory tissue sections following sub-chronic Cd exposures represented in (A). All data represent the mean ± SEM of n = 3 individuals. * indicates statistically significant differences in histological injury relative to controls (p <0.05). (A).

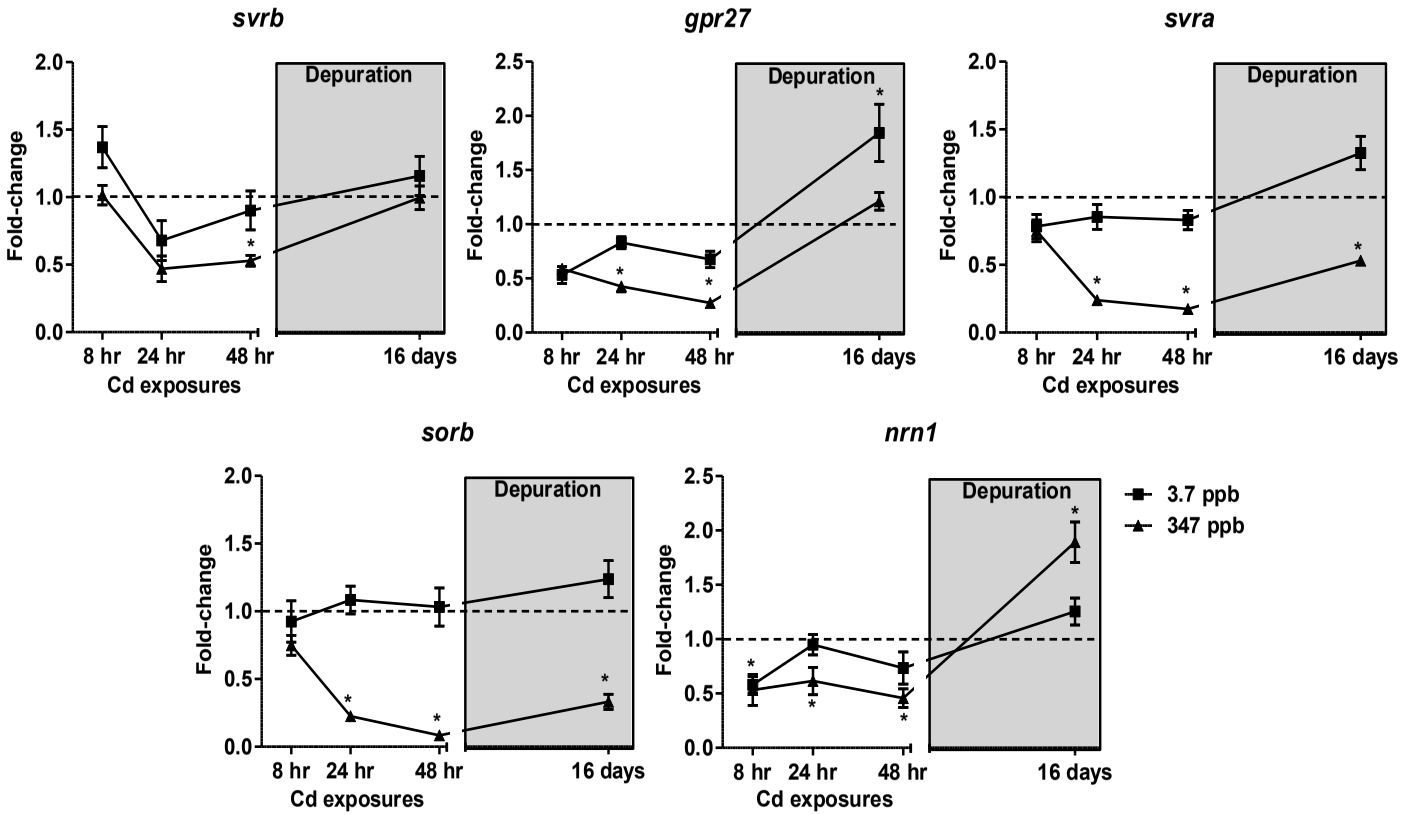

Figure 4. Fold-change in mRNA expression of the olfactory GPCRs and *nrn1* following acute Cd exposures and a depuration (light grey box). The 3.7 ppb and 347 ppb datasets are represented as squares and triangles, respectively. Data represent the mean ± SEM of n=8 individuals normalized to the expression of β-actin mRNA, and expressed as fold-change to control levels. * indicates statistically significant differences in gene expression relative to controls (p <0.05).

Table 2. Fold-change in mRNA expression of the olfactory biomarkers following 168 hr exposures to 3.7 ppb Cd

mRNA biomarker	Fold-change ± SEM[1]
svra	1.0 ± 0.07
svrb	1.3 ± 0.16
sorb	1.1 ± 0.12
gpr27	1.2 ± 0.15
nrn1	1.2 ± 0.17
hmox1	1.0 ± 0.09
prdx1	1.0 ± 0.15
mt1a	7.1 ± 0.60*

[1] values indicate changes in expression relative to controls. mRNA expression data normalized to that of β-actin

* indicates statistically significant differences relative to controls ($p<0.05$).

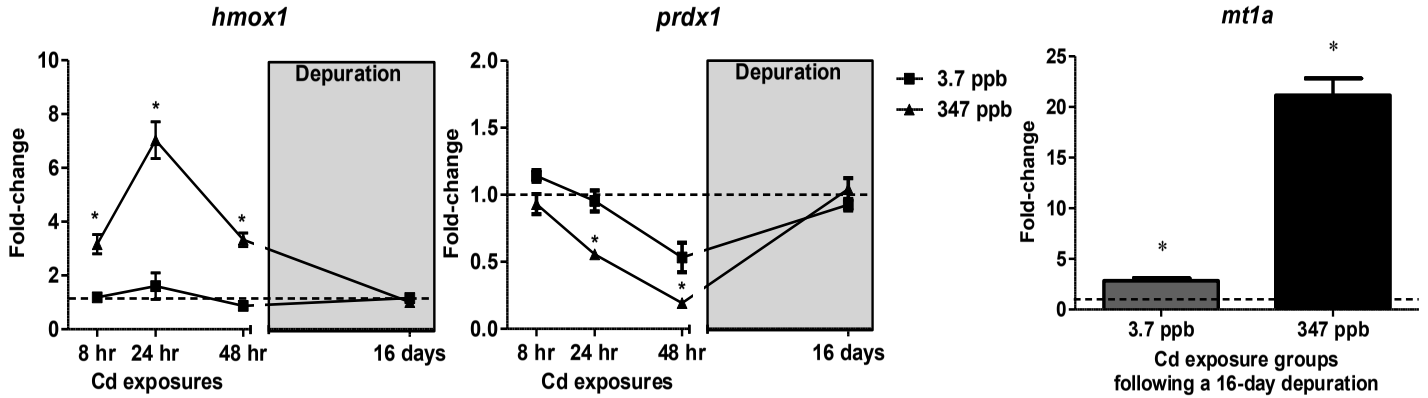

Figure 5. Fold-change in mRNA expression of biomarkers of oxidative stress following acute Cd exposure and depuration. The *hmox1* and *prdx1* data for the: 3.7 ppb and 347 ppb exposure groups are represented as squares and triangles, respectively with depuration data in the grey box. *mt1a* mRNA expression in fish following a 16-day depuration after to 3.7 and 347 ppb Cd, presented as grey and black bars, respectively. All data represent the mean ± SEM of n=8 individuals normalized to the expression of β-actin mRNA, and expressed as fold-change to control levels. * indicates statistically significant differences in gene expression relative to controls (p <0.05).

Chapter 2:

Cadmium exposure differentially alters odorant driven behaviors and expression of olfactory receptors in juvenile coho salmon (*Oncorhynchus kisutch*).

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Abstract

Salmon exposed to waterborne metals can experience rapid olfactory impairment leading to a loss of neurobehavioral function, a major concern as salmon rely on their olfactory system for survival. In the current study, we investigated the effects of cadmium (Cd) on salmon olfactory function in a laboratory based study modelling an exposure scenario where juvenile coho salmon transiently migrate through a polluted waterway. We exposed coho to environmentally relevant concentrations of waterborne Cd (0.3, 2, and 30 μg/L) for 48hrs and 16 days, followed by a 16-day depuration associated with outmigration. All Cd exposures significantly altered behavioral responses towards L-cysteine and conspecific odorants, with effects persisting following the 16-day depuration. Behavioral alterations following exposure to 30 μg/L Cd were associated with increased olfactory epithelial gene expression of stress markers metallothionein (*mt1a*) and heme oxygenase, altered protein expression of olfactory signal transduction (OST) molecules critical in maintaining olfactory function, and loss of expression of mRNAs encoding major coho odorant receptors (ORs). Salmon OR array analysis indicated that Cd predominantly decreased

expression of OST and OR markers for ciliated olfactory sensory neurons (OSNs) relative to markers for microvillus OSNs, suggesting a differential sensitivity of these two major OSN populations. Behavioral alterations on exposure to 0.3 and 2 μg/L Cd were associated with increased *mt1a* expression, but did not coincide with major histological or molecular changes, likely indicating disrupted signal transduction as the major mechanism underlying the olfactory behavioral dysfunction at the low-level Cd exposures. Laser-ablation mass spectrometry analysis revealed that the olfactory cellular injury and behavioral dysfunction was associated with Cd bioaccumulation within the olfactory sensory epithelium. In summary, low-level Cd exposures associated with those encountered in polluted waterways can induce differential and persistent olfactory dysfunction in juvenile coho salmon.

1.0 Introduction

Olfaction is a critical sensory system to fish species (Hara 1992). For salmon, the olfactory system serves a central role in their lifecycle (Quinn 2011). Salmon rely on their exquisitely sensitive sense of smell for critical tasks, such as locating prey, avoiding predators, and long-distance migration (Cooper *et al.,* 1976; Dittman and Quinn 1996; Hara 1992; Quinn 2011; Sutterlin and Gray 1973). However, the sensitivity of the salmon olfactory system renders it a potential target for waterborne pollutants (Tierney *et al.,* 2010). The olfactory sensory epithelium is in close contact with the surrounding water, allowing for interaction between the olfactory sensory epithelium and dissolved pollutants (Hamdani el and Doving 2007), which can lead to a loss of olfactory function. Numerous Pacific salmon populations are threatened or endangered,

and a factor implicated in the declining salmon populations is pollutant-based disruption of olfactory driven neurobehavioral function (Sandahl *et al.,* 2007; Tierney *et al.,* 2008).

Dissolved metals, such as Cd and copper, are common pollutants in urban and agricultural waterways, and are among the most potent fish olfactory toxicants (Atchison *et al.,* 1987; Baldwin *et al.,* 2011; Sandahl *et al.,* 2007; Sloman 2007; Tierney *et al.,* 2010). Important cellular processes that modulate olfactory function and signaling can be disrupted by Cd (Choong *et al.,* 2014; Thévenod 2009). Exposures to Cd lead to rapid and persistent disruption of salmon olfactory function and critical olfactory driven behaviors (Scott *et al.,* 2003; Sloman *et al.,* 2003; Williams and Gallagher 2013). Even brief disruptions in olfactory function can lead to increased mortality in salmon (McIntyre *et al.,* 2012). Juvenile salmon can spend weeks to months in freshwater systems prior to outmigration, highlighting the need for understanding Cd induced effects on behavioral responses to ecologically relevant odorant cues under these conditions and how these perturbations affect survival.

At the cellular level, odorant detection is driven by olfactory sensory neurons (OSNs) located within the olfactory sensory epithelium (Hamdani el and Doving 2007). The OSNs are divided into two main categories, microvillus and ciliated OSNs (Hamdani el and Doving 2007). These two categories are sub-divided into four major odorant receptor (OR) classes: Trace Amine Associated Receptor (TAAR) and Major Olfactory Receptors (MOR) in the ciliated OSN category, and Olfactory receptor class A-related (ora) and Vomeronasal type 2 receptor-like (OlfC) in the microvillus OSN category (Johnstone *et al.,* 2009; Johnstone *et al.,* 2008; Johnstone *et al.,* 2012; Tessarolo *et al.,* 2014). Ciliated and microvillus OSNs differ in morphology and location within the olfactory epithelium, and also in utilization of signal transduction molecules (Hansen *et al.,* 2004; Hansen *et al.,* 2003; Zippel *et al.,* 1997). There are

currently 81 identified putative salmonid ORs, and odorant perception is encoded by combinatorial activation of these different ORs (DeMaria and Ngai 2010; Johnstone *et al.,* 2009; Johnstone *et al.,* 2008; Johnstone *et al.,* 2012; Malnic *et al.,* 1999; Tessarolo *et al.,* 2014). Alteration of an odorant's unique OR activation (i.e. "fingerprint") can lead to altered odorant perception and behavioral responses (DeMaria and Ngai 2010; Troemel *et al.,* 1997). Although there is some evidence that certain waterborne metals can differentially affect these two classes of OSNs (Dew *et al.,* 2014; Kolmakov *et al.,* 2009), the functional and behavioral effects of this differential toxicity has not been investigated in detail.

To date, studies have predominantly focused on acute Cd exposure scenarios and Cd induced effects on responses to single odorants (McIntyre *et al.,* 2012; Tierney *et al.,* 2010). In the current study, we investigated the effects of different Cd concentrations over multiple time periods on responses to a diverse set of waterborne odorants for the purpose of better understanding the physiological mechanisms of Cd olfactory injury. We hypothesized that acute and sub-chronic Cd exposures would induce differential and long-lasting impairment of behavioral responses to a suite of different odorants. Our approach was to analyze changes in olfactory driven behaviors in response to environmentally relevant odorants, as well as changes in histology and gene expression of a diverse set of salmon ORs spanning all four salmon OR families (Johnstone *et al.,* 2012). Our laboratory exposure paradigm modeled the scenario in which juvenile salmon out-migrating through contaminated waterways could be transiently exposed to metals followed by transition to unpolluted water (Ruggerone 2003).

2.0 Materials and Methods

Chemicals. MS-222 (Tricaine methanesulfonate) was purchased from Argent Chemical Laboratories (Redmond, WA). Analytical grade cadmium chloride was purchased from Mallinckrodt Baker (Phillipsburg, NJ). Bradford reagent was purchased from Bio-Rad (Hercules, CA). L-cysteine, Harris Hematoxylin solution-modified, bovine serum albumin and Alcian blue 8GX were purchased from Sigma-Aldrich (St. Louis, MO). Eosin stain was purchased from VWR (Radnor, PA). Triton X-100 was purchased from MP Biomedicals (Cleveland, OH). Tween-20 was purchased from Fisher Chemicals (Pittsburg, PA).

Animals. Juvenile coho salmon (1 yr. of age, 15.0 g ± 5.7 mg) were housed at the University of Washington salmon hatchery in 8-12 °C flow-through freshwater from Lake Washington, Seattle under a natural photoperiod. Fish were fed BioVita Fry Feed (Bio-Oregon) and water quality conditions were typically 80-120 mg/L total hardness as calcium carbonate, pH 7.2, 2.5mg/L DOC, and 9.1 mg/L dissolved oxygen content. Water chemistry was similar to those of other salmon-bearing rivers and streams in the Pacific Northwest (McIntyre *et al.,* 2008).

Cd exposures. All animal welfare and experimental procedures were carried out in accordance with the University of Washington Institutional Animal Care and Use Committee (IACUC) guidelines, protocol# 4096-1. Tracking of individual fish over the course of the experiments was accomplished by implantation of Biomark HPT12-12mm PIT tags. Coho were placed within 6 designated exposure groups (0, 2 and 30ppb Cd for 48hrs; 0, 0.3 and 2ppb Cd for 16 days), with n=30 juvenile coho per exposure group (24 PIT-tagged fish and 6 non-tagged fish). The concentrations of waterborne Cd used, 0.3 ppb and 2 ppb, are concentrations well below the 5 ppb EPA drinking water standard (EPA, water.epa.gov/drink/contaminants) and representative of

urban and agricultural waterways (WDOE, 2008), whereas the 30 ppb exposure is representative of heavily polluted waterways (Srinivasa Gowd and Govil 2008). Total waterborne Cd concentrations were analyzed prior and post Cd exposures by ICP-MS (Williams and Gallagher 2013). All background levels of total Cd in lake water were <0.01 ppb.

Food was withheld prior to initiation of the exposures, and 24hrs prior to the initiation of the exposures all fish were transferred from holding tanks to the 120 L glass exposure aquaria containing control water. Tank water was maintained at ambient lake water temperatures (10^{O}C) via a large chilled water bath. A static renewal approach was implemented in all exposure tanks, with full water renewal after a 24-hr period to avoid ammonia buildup. Coho in all exposures were fed 2% W/W daily for a 20 min period prior to the daily water changes, after which any remaining food was removed. Cadmium exposures were staggered for each experimental period and exposure group so that equal numbers of fish were continually exposed for testing logistics. Nitrate and nitrite levels were below detection for all treatment groups, and ammonia levels were $\leq$ 0.25 ppm during the exposures.

To control for potential handling and housing effects on behaviors, all exposures were conducted in three stages, with behavioral testing conducted following each of the exposure stages on PIT-tagged fish. The first exposure stage included a pre-exposure for all groups (i.e. all fish exposed to control water) to allow for individual PIT-tagged fish to act as their own controls. Following the first exposure stage and behavioral testing of PIT-tagged fish, all fish in each exposure group were transferred back into the glass aquaria containing control water and allowed to re-acclimate for 24hrs before the second exposure stage. The second exposure stage consisted of the Cd exposures for all groups (i.e. *Cd exposure groups*). Following the Cd exposures, non-

PIT tagged fish (n=6 per group) were euthanized and used for gene expression, and histological analysis. All PIT-tagged fish underwent behavioral testing then transferred back into the glass aquaria containing control water and allowed to re-acclimate for 24hrs before the third exposure stage. The third exposure stage consisted of the 16-day depuration in control water for all fish. Following the third exposure stage, the PIT-tagged coho (n=24 per group) underwent behavioral testing and were then euthanized for tissue collection.

Preparation of odorants and neurobehavioral analysis of olfactory function. Coho skin extract was prepared as previously described (Williams and Gallagher 2013). Protein content of the skin extract was determined via the Bradford assay and all stocks were adjusted to final protein stock concentration of 3mg/ml using DI water. Stocks were kept frozen until just before use. Conspecific odorant was prepared by placing 8 juvenile coho (~15g) in a 35L glass tank filled with lake water overnight. The next morning, the fish were removed and the water was used as the conspecific odor stock (3.4mg of fish/L). Aliquots of L-cysteine stocks (each 10^{-2}M) were pre-weighed before use. Stocks were prepared on the day of use.

Olfactory-mediated behavioral analysis was conducted using a two-choice maze. The two-choice maze was constructed out of acrylic and measured 100 x 40 x 25 cm and consisted of two arms, each of which measured 50 cm long and 20 cm wide. The arms terminated at a holding chamber, which was 40 x 40 cm. A transparent screen separated the maze arms from the holding chamber. A black curtain surrounded the entire maze to minimize outside disturbances to fish within the maze, and illumination for the video cameras (JVC Everio camcorders) was provided by a low-level red light. The maze received a continuous flow of fresh lake water, and no mixing of water occurred between the arms. The odorant concentrations used for the behavioral analyses

were 10^{-6}M L-cysteine, 6mg/L conspecific odor and 300µg protein/L of the skin extract. PIT tagged coho from each exposure group (n=24) were tested individually and were acclimated for 15 minutes in the holding chamber prior to odorant addition.

Conspecific odor and L-cysteine were delivered via a peristaltic pump at 10 ml/min with the flow of lake water into each arm at 5L/min. The skin extract was delivered via a slow injection directly into the holding chamber with no lake water inflow. Video recordings for behavioral responses were initiated 10 minutes prior to the odorant addition to establish baseline behavior, and 5 minutes after odorant addition to establish the behavioral response. The maze was flushed with clean water for 20 minutes between behavioral trials. The order of L-cysteine and conspecific delivery and arm scented was randomized each behavioral trial. The skin extract was introduced last in all behavioral tests as the fright response could have affected subsequent odorant responses. Fish odorant responses were tracked and quantified from the video recordings using EthoVision-XT® (Noldus, Leesburg, VA). The behavioral response measured for L-cysteine and the conspecific odorant was arm preference, while reduced swimming activity (fear response) was measured for the skin extract.

Histological analysis of the olfactory sensory epithelium. Histological analysis of the olfactory epithelium was conducted using a subset of fish (n=6) from pre-, post- and depurated exposure groups. Briefly, coho head tissues were fixed in 4% paraformaldehyde (PFA) at 4^{o}C. Olfactory rosettes were excised and embedded in a 2:1 mixture of 20% sucrose to Tissue-Tek® OCT™ and stored at -80^{o}C. Tissue sections (6 µm) were mounted on VWR® glass Superfrost Plus micro-slides.

Hematoxylin and eosin staining. H&E staining of olfactory epithelial tissue sections was conducted using a modification of our previously established method (Williams and Gallagher 2013) with one minor addition: Goblet cell populations (a measure of mucus production) were assessed using alcian blue 8GX staining (Sigma-Aldrich). All images were captured using Nuance 3.0 imaging software. Histological measurements and scoring of injury was conducted using ImageJ software (NIH, USA). Tissue sections were measured and scored blinded using previously published methods (Williams and Gallagher 2013).

Immunohistochemistry. Fluorescent immunohistological analysis of olfactory epithelial tissue sections was performed as follows. Antibodies used included: Adenylyl cyclase-III (ACIII; sc-588 Santa Cruz), a major signal transduction molecule used only by ciliated OSNs; G alpha subunit ($G_{\alpha o}$; sc-387 Santa Cruz), a major G-protein coupled receptor subunit used by microvillus OSNs; sex determining region Y-box 2 (sox2; AB5603 Millipore), a transcription factor only expressed by the olfactory stem cells in the olfactory epithelium. In addition to ACIII, the marker of ciliated OSNs, we investigated the effects of Cd on microvillus OSNs using $G_{\alpha o}$, an important g-alpha subunit involved in inositol-triphosphate (IP3) olfactory signal transduction in microvillus OSNs (Ferrando *et al.,* 2009; Hamdani el and Doving 2007). The olfactory stem cell population (HBCs and GBCs) are important for regenerating lost olfactory cells during natural turn over and following injuries (Huard *et al.,* 1998; Jang *et al.,* 2003). On day of use, slides were thawed at room temperature and rehydrated through serial methanol washes finalizing with PBS. Antigen retrieval was performed on a target specific basis, Sodium citrate (pH 6.0) with 0.05% Tween-20 @ 95^{o}C for 20 minutes was used for sox2 antigen retrieval; 0.9% Triton X-100 @ 27^{o}C for 30 minutes was used for ACIII and $G_{\alpha o}$ antigen

retrieval. Sections were incubated with the primary antibody overnight at 4^{o}C. Secondary antibodies used were Cy3 and Alexa-Fluor 488 (Invitrogen). Nuclei were stained using DAPI in Prolong Diamond anti-fade media (Invitrogen). All slides were visualized using a Nikon LABOPHOT-2 microscope using Nuance 3.0 imaging software. The fluorescent intensity values obtained from the negative controls (e.g. absence of primary antibody) were used for normalization when analyzing fluorescent intensity from primary antibody treated samples using MetaMorph image analysis software (Molecular Devices).

Laser ablation inductively coupled mass spectrometry. Tissues used for Laser ablation inductively coupled mass spectrometry (LA-ICP MS) were prepared as described above, with minor modifications. Briefly, tissues used for metal analysis were sectioned at 20 µm thickness and mounted on 1 x 0.5inch glass slides. Tissue sections were dried overnight in a desiccator before analysis. Tissue sections used for imaging were 6 µm thick. LA-ICP-MS analysis was conducted using an NWR213 laser ablation system (ESI, Portland, OR) connected to an Agilent 7500ce ICP-MS (Agilent Technologies, Santa Clara, CA). The Nd:YAG laser operated at a wavelength of 213 nm. Quantitative measurements of Cd were made with a laser energy output of 35%, firing frequency of 20 Hz, laser beam spot size of 110 µm, and translation speed of 75 µm second-1. Line scans were programmed to ablate around the borders of the olfactory rosette lamellae and included lines between the lamellae to measure background Cd from the tissue preparation and the glass mounting slide. Data for the heat map image were collected using a laser energy output of 40%, firing frequency of 20 Hz, laser beam spot size of 25 µm, and translation speed of 25 µm second-1 following a raster pattern. All ICP-MS measurement were

performed with rf power 1500 W, argon carrier gas flow 0.5 L min-1, helium reaction cell gas flow 1.3 mL min-1, helium gas flow from the laser cell 0.8 L min-1 and dwell time 400 ms. Quantification of Cd was conducted using dried, spiked gelatin films as external calibration standards. A linear relationship (R^2 >0.99) was obtained from 0.5 to 250 µg g-1, using the same LA-ICP-MS parameters as described above for the quantitative measurements. Bioimages were prepared using Igor Pro 6.37 (WaveMetrics, Inc., Lake Oswego, OR) and Iolite 3.32 (Iolite, Victoria, Australia) software applications.

Quantitative PCR analysis of olfactory gene expression. Eight individual coho were collected for qPCR analysis of molecular biomarkers. The fish were euthanized with MS-222 prior to cervical dislocation, and the olfactory rosettes were quickly excised, transferred to Qiazol® reagent, frozen in liquid nitrogen and stored at -80 ˚C. Total RNA isolation was performed using Qiagen RNeasy kit (Venlo, Limburg). Isolated RNA was DNase treated and cDNA synthesis was conducted using BioRad iscript cDNA synthesis kit (Hercules, CA). Candidate olfactory biomarker gene primers were generated by multiple sequence alignment of rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), and zebrafish (*Danio rerio*) target sequences. To assess the effects of Cd on the mature OSN cell population we analyzed gene expression of *omp* (marker of mature olfactory neurons) and *nrn1* (a marker of neuronal regrowth and axonal growth) in olfactory rosettes (Kudo *et al.,* 2009; Naeve *et al.,* 1997). Additionally, to assess the effects of Cd on markers of cellular stress and metal exposures we analyzed gene expression of metallothionein 1a (*mt1a* - a sensitive metal response marker)m and heme oxygenase-1 (*hmox1*- a sensitive marker of oxidative and cellular stress) (Coyle *et al.,* 2002; Ryter and Choi 2002; Tallkvist *et al.,* 2002; Wang *et al.,* 2008). Primers for *hmox1*, *nrn1,*

omp and mt1a (Table 1) were designed using Primer 3 software program (San Diego Biology Workbench 3.2, http://workbench.sdsc.edu/). Primers used for PCR are listed in Table 1. All PCR products were confirmed by sequencing. Quantitative PCR analysis was conducted in a 96-well format with BioRad SsoAdvanced SYBR Green supermix using the relative standard curve method.(Espinoza *et al.,* 2012) ß-actin expression did not differ between treatments and was used for normalization purposes.

Multiplex PCR Specific Target Amplification and real-time PCR of salmon olfactory receptors. Initially, a target pool of coho salmon olfactory receptors was identified based upon previously identified putative Atlantic salmon ORs.(Johnson and Banks 2011; Johnstone *et al.,* 2009; Johnstone *et al.,* 2008; Johnstone *et al.,* 2011; Johnstone *et al.,* 2012) Out of the total pool of putative ORs, a small subset of 6-8 ORs from each of the four major receptor classes (MOR, TAAR, OlfC and ora) were chosen based on sequence similarity (e.g. genes with more sequence dissimilarity within the same major class), relative expression levels and ability to reliably amplify the target OR mRNA. Primers were designed using the Primer 3 software program (San Diego Biology Workbench 3.2, http://workbench.sdsc.edu/) and are shown in Table 1. All PCR products were sequenced and used for the design of the TaqMan probes. cDNA samples (synthesis described above, 2.12) were pre-amplified following the Fluidigm (South San Francisco, CA) Specific Target Amplification (STA) protocol to increase target gene template cDNA for gene expression reactions.

Following design and validation of OR probes, the pool of PCR primers and TaqMan probes (Table 1) were multiplexed in a single PCR for each diluted aliquot of DNA. Reactions were conducted according to the manufacturer's TaqMan protocol (Applied Biosystems Inc.,

Foster City, CA, USA) on an Applied Biosystems 2720. Pre-amplified samples were then diluted 1:5 with DNA Suspension Buffer. Fluidigm GE Dynamic Array 96.96 plates were primed on the Fluidigm IFC Controller prior to loading. The assays were prepared by adding equal volumes of Assay Loading Reagent (Fluidigm) to TaqMan Gene Expression Assays (Applied Biosystems). Each 10X assay mix was then loaded onto each assay well on the GE Dynamic Array (Fluidigm). Sample Pre-Mix was prepared by combining TaqMan Universal PCR Master Mix with GE Sample Loading Reagent (Fluidigm) and STA cDNA. Each Sample of Pre-Mix was then loaded into each sample well on the GE Dynamic Array. Assays were conducted in triplicate. The GE Dynamic Array was then loaded onto the IFC Controller before thermocycling following the manufacturer's established protocol. Data was collected using the Fluidigm BioMark Data Collection Software and analyzed using the Fluidigm Real-Time PCR Analysis Software 4.1.2.

Statistical analysis. Fluorescence data from the immunohistological analysis were inspected for homogeneity of variances using D'Agostino and Pearson omnibus normality testing. Data conformed to a non-parametric distribution, and these datasets were assessed using Kruskal-Wallis one-way ANOVA test followed by Dunn's non-parametric post-hoc test. Gene expression data were inspected for homogeneity of variances using D'Agostino and Pearson omnibus normality testing, and treatment-related effects were assessed using Kruskal-Wallis one-way ANOVA test followed by Dunn's non-parametric post-hoc test. Statistical testing was conducted using Prism GraphPad software. Behavioral data consisted of repeated measures on the same set of fish, within Cd exposure. These data were analyzed within exposure levels using conventional ANOVA, with a blocking factor for each fish to account for fish-specific behavioral effects.

3.0 Results

Behavioral effects of waterborne Cd exposure

Coho showed a preference for the arm receiving the conspecific odorant prior to Cd exposure (Figure 1). Coho spent between 60-90% of their time in the arm receiving the conspecific odorant compared to the non-scented arm (Figure 1A, C). Following the 48-hr exposure to 2 ppb Cd, coho spent 30% of the time in the conspecific scented arm (a 30% decrease compared to pre-exposure behavior), while coho exposed to 30 ppb Cd for 48hrs spent 60% of the time in the conspecific arm (a 10% decrease compared to pre-exposure behavior; Figure 1a). As observed in Fig. 1a this trend continued following depuration. The coho actively avoided the conspecific odor, marking a reversal of pre-exposure behavior. Similarly, coho exposed to 0.3 and 2 ppb Cd for 16 days actively avoided the conspecific scented arm. Coho exposed to 0.3 and 2 ppb Cd for 16 days spent ~35% of the time in the conspecific scented arm compared to their pre-exposure behavior of ~85% in the conspecific scented arm (Figure 1C). This behavioral reversal persisted following the 16-day depuration. Coho previously exposed to 0.3 and 2 ppb Cd for 16 days spent ~45% and ~30% of the time in the conspecific scented arm, respectively (Figure 1C). Coho in the control groups showed no significant changes in odorant attraction over any of the exposure periods (Figure 1A, C).

Similar behavioral changes were observed for L-cysteine. As observed in Fig. 1, all coho showed an aversion towards the arm receiving the L-cysteine odorant prior to Cd exposure. Coho spent between 30 to 45% of the time in the arm receiving L-cysteine (Figure 1B, D). However, following exposure to either 2 or 30 ppb Cd for 48hrs, coho were indifferent to L-cysteine

(Figure 1B). Following the depuration, coho in the 2 and 30 ppb exposure groups exhibited an increase in attraction behavior towards L-cysteine, with the 30 ppb exposure group spending ~60% of their time in the odorant arm (Figure 1B). Coho exposed to 0.3 and 2ppb Cd for 16 days also showed more of an indifference/attraction to L-cysteine following exposure. Coho in the 0.3 ppb Cd group spent 60% of their time in the odorant arm, compared to 25% prior to exposure ($p< 0.05$, Figure 1D). This trend continued following the depuration; coho previously exposed to 0.3 and 2 ppb Cd exhibited a significant attraction towards L-cysteine compared to pre-exposure behaviors ($p<0.05$, Figure 1D). Coho in the control groups showed no significant changes in odorant response over time (Figure 1B, D).

In contrast to the significant Cd induced alterations in responses to the conspecific and L-cysteine odorants, behavioral responses towards the skin extract were not significantly altered (Figure 2). Coho exposed to 2 and 30 ppb Cd for 48hrs did exhibit a decreased response towards the skin extract, remaining more active compared to their pre-exposure behavior (Figure 2). However, following the depuration, coho previously exposed to 2 and 30 ppb Cd exhibited a skin extract induced response similar to their pre-exposure behaviors (Figure 2). Coho exposed to 0.3 ppb Cd for 16 days exhibited an increased response compared to their pre-exposure behaviors towards the skin extract. The increased response towards the skin extract persisted following Cd depuration (Figure 2). Conversely, coho exposed to 2 ppb Cd for 16 days exhibited a decreased response towards the skin extract (Figure 2). Following depuration, coho previously exposed to 2 ppb Cd still exhibited a reduced response towards the skin extract compared to their pre-exposure behavior (Figure 2). It should be noted that following depuration, coho previously exposed to control water for 48hrs did show a reduced response towards the skin extract, but this

was likely due to habituation. Coho in all Cd exposure groups did not experience a significant impact on general health (basal swimming activity and growth).

Bioaccumulation of Cd within the peripheral olfactory system

LA-ICP-MS analysis found increased levels of Cd within the olfactory epithelium following exposures, compared to background tissue levels (Figure 3). The Cd concentrations within the olfactory sensory epithelium of coho in each of the exposure groups increased in an exposure level and time dependent manner, and the Cd accumulation profile closely followed the observed behavioral deficits (Table 2). The concentration of Cd with the olfactory rosettes of coho exposed to 2 and 30 ppb Cd for 48hrs were 854 and 5737 μg/kg, respectively, compared to 58 μg/kg in control olfactory rosette tissue (Table 2). Cd concentrations within the rosettes of coho following Cd depuration remained elevated relative to controls. Cd concentrations within the rosettes were 308 and 4237 μg/kg in coho previously exposed to 2 and 30 ppb Cd for 48hrs, respectively, compared to 45 μg/kg in control coho (Table 2). Coho exposed to 0.3 and 2 ppb Cd for 16 days had an olfactory rosette tissue Cd concentration of 891 and 3577 μg/kg, respectively, compared to control coho with a Cd concentration of 81 μg/kg (Table 2). Post depuration rosette Cd concentrations were 790 and 2592 μg/kg in coho previously exposed to 0.3 and 2 ppb Cd for 16 days, respectively, compared to control coho with a Cd concentration of 54 μg/kg (Table 2).

Induction of mt1a, an olfactory metal responsive biomarker. Compared to controls, coho exposed to all levels of Cd showed a significant increase in expression of metallothionein 1a (*mt1a*), respectively (Figure 4). Gene expression of *mt1a* mRNA remained significantly elevated following depuration in coho previously exposed to 30 ppb Cd for 48hrs or 2 ppb Cd for 16 days

(Figure 4). The *mt1a* gene expression data had a similar trend to the Cd tissue concentration profile we found within the olfactory rosettes following exposures.

Effects of Cd on the olfactory sensory epithelial morphology and neuronal stress response

Analysis of the olfactory sensory epithelium following Cd exposures revealed evidence of decreased expression of a *omp* (a mature OSN marker) and induction of *hmox1* (a OSN stress marker), while the general histology of the sensory epithelium remained largely unchanged. Coho exposed to Cd for 48hrs and 16 days showed no significant histological differences in their olfactory sensory epithelium when compared to coho from control exposures (Figure 5). Likewise, following the depuration, all coho, regardless of previous Cd exposures, had morphologically similar olfactory epithelium (Figure 5). Expression of *omp* mRNA was significantly decreased in coho exposed to 30 ppb Cd for 48hrs, compared to controls (Figure 6). Following depuration, expression of *omp* mRNA remained significantly low in the olfactory rosettes of coho previously exposed to 30 ppb Cd for 48hrs, compared to controls (Figure 6). All other Cd exposures did not significantly change *omp* gene expression within the olfactory rosettes (Figure 6). Additionally, gene expression of *nrn1* was not altered by Cd exposure or following the depuration (Figure 6).

Immunoreactivity of sox2 expressing olfactory stem cells following the 48hr and 16-day Cd exposures, including the depurations, revealed no differences in the number of stem cells between control and Cd exposures (Figure 7). Gene expression of *hmox1* was significantly elevated in coho exposed to 30 ppb Cd for 48hrs (Figure 8), but did not differ among the other exposure groups relative to controls.

Effects of Cd on ACIII and $G_{\alpha o}$ protein expression

As observed in Fig. 9, immunoreactivity of ACIII in the olfactory sensory epithelium was reduced by 50% and 70%, respectively, following 48-hr exposure to 2 and 30 ppb Cd (Figure 9). Following depuration, analysis of ACIII immunoreactivity in both the 2 and 30 ppb exposure groups showed no difference in staining compared to coho from the control exposure group (data not shown). Immunoreactivity of ACIII in coho from the 16-day 0.3 and 2 ppb exposure groups showed no differences after exposure or depuration compared to controls (data not shown). Immunoreactivity of $G_{\alpha o}$ was not significantly altered following any of the Cd exposures or following the depurations (Figure 9).

Effects of Cd on expression of major ORs

As observed in figure 10, exposure to 30 ppb Cd for 48hrs resulted in a significant reduction in gene expression of 50% of the ciliated OSN-linked ORs and 20% of the microvillus OSN-linked ORs. Following Cd depuration, 28% of the ciliated OSN-linked ORs and 10% of the microvillus OSN-linked ORs remained significantly under-expressed compared to controls (Figure 10). Coho in the other Cd exposure groups did show minor modulation of expression of the OR-encoding genes, however these effects were not statistically significant (Figure 10).

4.0 Discussion

The significant Cd induced effects on olfactory driven behaviors towards the three different odorants tested in the present study were particularly noteworthy, especially given the greater disruption of behavioral responses towards the conspecific odorant following Cd exposures

relative to those for L-cysteine. These results may owe to the complexity of conspecific odor vs. the simplicity of L-cysteine. It's also possible that conspecific odorant signaling is more complex compared to L-cysteine odorant signaling, making the conspecific odorant processing potentially more susceptible to Cd induced disruptions. This could also underlie the reduced Cd driven effect on the observed behavioral responses towards the skin extract. Current scientific data indicates that a single class of compounds within fish skin extract could be responsible for inducing alarm responses (Mathuru *et al.,* 2012), however, the specific compound or compounds that elicit a fear response in salmon has yet to be identified. The transient changes in the behavioral response towards the skin extract observed is somewhat surprising as our previous study found that similar levels of Cd significantly disrupted responses to skin extract (Williams and Gallagher 2013). However, in our previous study, we conducted behavioral tests in small groups while the current study tested individual fish. Nevertheless, the magnitude of the behavioral changes measured for the conspecific and L-cysteine odorants outweigh previously observed behavioral changes towards the skin extract. These results indicate that Cd is differentially impacting odorant processing or perception to these three structurally and compositionally diverse odorants.

To elucidate the factors underlying the Cd-induced behavioral alterations, we measured Cd concentrations within the coho olfactory sensory epithelium, as accumulation of Cd within the olfactory system has been associated with olfactory deficits (Scott *et al.,* 2003). The rapid and persistent accumulation of Cd within the sensory epithelium, at levels as low as 0.3 ppb, was correlated with the significant and persistent behavioral alterations. These findings are consistent with previous studies, demonstrating that the salmon peripheral olfactory system can rapidly accumulate waterborne Cd (Harrison and Klaverkamp 1989; Scott *et al.,* 2003; Tjälve *et al.,*

1986). However, our study was unique in that we directly measured Cd concentrations within the sensory epithelium using LA-ICP-MS. One possible mechanism for the accumulation of Cd within olfactory neurons is the high number of calcium channels in their membrane. It has been shown that Cd can enter cells via calcium channels (Usai *et al.,* 1999). Once inside cells, Cd can disrupt many important cellular processes and induce cellular stress and cellular death, all of which could be deleterious for olfactory function (Cuypers *et al.,* 2010; Ercal *et al.,* 2001). These effects may have been compounded by the persistence of the olfactory Cd concentration following the 16-day depuration, indicating a potentially long depuration period for Cd within the peripheral olfactory system and the potential for long-term functional disruption.

While Cd readily accumulated within the olfactory sensory epithelium, we did not find evidence for substantial morphological changes following any of the Cd exposures. These results are in agreement with our previous study which found that only very high Cd exposures significantly induced morphological changes in the olfactory sensory epithelium (Williams and Gallagher 2013). The findings observed in the current study do not rule out Cd-induced effects on specific olfactory cell populations. Expression of *omp* mRNA, a marker of mature OSNs, was significantly reduced at the 30 ppb Cd exposure level, and remained depressed following the depuration. These results indicate that mature OSNs are likely impacted at the higher Cd exposure levels. Previous studies have shown that exposures to metals can kill mature OSNs without impacting other olfactory cell populations (Julliard *et al.,* 1996). This may be because mature OSNs are in direct contact with the epithelial surface/surrounding water, increasing the likelihood of Cd uptake compared to the more deeply located immature OSNs and stem cell populations. Our immunohistochemistry analysis confirmed that none of the Cd exposures significantly impacted the olfactory stem cell population. The OSCs, HBCs and GBCs, are

robust as they must be able to fully regenerate the olfactory sensory epithelium following significant injury (Jang *et al.,* 2003). The Cd exposures had little effect the overall numbers of OSCs within the coho olfactory sensory epithelium. This indicates that the olfactory sensory epithelium is morphologically intact, but its function is impaired.

Collectively, our data indicate that Cd-induced cellular stress, as opposed to total cellular loss, most likely underlies the observed behavioral deficits. The induction of *mt1a* closely mirrored the increased Cd concentration within the olfactory epithelium, confirming that Cd migrated into the olfactory cells and induced a rapid and persistent cellular response. An increased intracellular Cd load could induce oxidative stress, as well as disrupt cellular transporters and ion channels that act to maintain cellular metal homeostasis for calcium, zinc and copper, metals for which Cd has been shown to interact (Choong *et al.,* 2014; Cuypers *et al.,* 2010; Hartwig 2001; Jacobson and Turner 1980). This potential destabilization of intracellular metals/signaling could negatively affect normal OSN function. Furthermore, the elevated *hmox1* gene expression indicates that oxidative stress was likely a factor in olfactory dysfunction at the higher acute Cd exposure levels. Collectively, these results suggest the underlying mechanisms driving the behavioral alterations were not linked to morphological injury, OSN loss or inhibited regeneration. They are likely related to interruption of signal transduction molecules due to an elevated and persistent intracellular OSN Cd burden.

Olfaction functions on a combinatorial principal in which a diverse array of OSNs must detect a structurally diverse array of odorants (Malnic *et al.,* 1999). Odorants activate a unique combination of ORs that is decoded in the brain, eliciting a behavioral response for that specific odorant. This system is sensitive to disruption, as disruption of certain OSN populations can completely change an odorant's OR activation "fingerprint" and alter the downstream behavioral

response (Del Punta *et al.,* 2002). Based on previous studies (Dew *et al.,* 2014; Kolmakov *et al.,* 2009), it is possible that Cd could induce a differential toxicity on distinct classes of OSNs. We have previously shown that copper significantly decreased the expression of ACIII; inhibition of ACIII blocks olfactory function (Bakalyar and Reed 1990; Chen *et al.,* 2000; Wang *et al.,* 2013). In the current study, the decrease in ACIII and not $G_{\alpha o}$ suggests that coho ciliated OSNs are more sensitive to Cd exposures compared to microvillus OSNs. As Cd did not significantly change protein expression of ACIII or $G_{\alpha o}$ at lower exposure levels, it is possible the impaired olfactory behaviors may be explained by alterations in protein function instead of loss. For example, both ciliated (ACIII) and microvillus (IP3) signal transduction pathways utilize calcium for signaling, although in different ways, and Cd has been shown to disrupt calcium signaling due to its affinity for calcium binding sites (Choong *et al.,* 2014; Lansman *et al.,* 1986). Deactivation of ACIII, following activation by ORs may rely on calcium signaling via activation of CaMKII, which Cd has been shown to directly activate. Although this deactivation pathway is under debate (Cygnar *et al.,* 2012; Wei *et al.,* 1998).

In addition to alteration of signal transduction machinery pathways by Cd, alteration of OR expression patterns appear to contribute to the observed behavioral alterations. Our OR gene expression array provided coverage of approximately 25% of the currently known putative salmon ORs (Johnstone *et al.,* 2012; Tessarolo *et al.,* 2014). We found that mRNA expression of ORs encoding ciliated OSNs were predominantly impacted compared to those associated with microvillus OSNs, in accordance with the ACIII and $G_{\alpha o}$ immunostaining results. However, how a differential decrease in ciliated vs. microvillus OSNs directly relates to a specific behavioral change remains unknown. Salmon ORs expressed within the olfactory sensory epithelium have not been extensively characterized with respect to relationships among their odorant binding

specificities and associated olfactory-driven behaviors. While we measured OR gene expression in the current study, it has been proposed that olfactory GPCRs contain functional metal-binding sites (Wang *et al.,* 2003). It is possible that Cd could interact with these metal-binding sites and alter function independent of gene expression, as Cd has been shown to interact with zinc and copper binding sites on proteins (Hartwig 2001).

In conclusion, while the underlying factors driving these behavioral alterations remain to be elucidated, we provide evidence that a differential toxicity of Cd on ciliated and microvillus OSNs is important. Differences between olfactory signal transduction molecules, potential disruption of GPCR function, and increased intracellular Cd load/stress on the cellular balance of metals could all explain the differential effects of Cd on distinct classes of OSN and downstream behavioral alterations. In the wild, such behavioral alterations could pose a serious risk to juvenile coho salmon, as appropriate behavioral responses towards odorant cues are essential for survival. Our study provides new evidence of differential toxicity of metals on the salmon olfactory system at Cd levels lower than those previously studied. The salmon olfactory system is highly susceptible to rapid and persistent accumulation of waterborne Cd, which could be deleterious for juvenile salmonids migrating through impacted sites.

Figures

Table 1. Primers used for TaqMan and qPCR.

Fluidigm probe sequences				
Gene name	Accession number	Fwd primer	TaqMan probe	Rvs primer
MOR 600-1	FJ613884.1	CGCTTCCCAGCCTTTGC	CGCAAAGTGAGCAGTG	TTGAGACAAGTAGGGACCACACTA
MOR 119.1	JN860039	GCTAAACCTACCCATCAATGACCTT	TCCACGGCACTCTTTA	GGTCCTTGTGTCAAGTAAAATCTCTTTG
MOR 300-1	FJ611229	CGATTCATATACTCAATGCCACTGCAT	ATTGTTGCAAAAGCCTG	GGAAGAGGATAGGCCCTAATTACCA
MOR 112.1	JN860046	CCTGTTCACTGTTATAAGGAGTGGTACT	AGTTGCGCTCGCTGG	CATTGGAACCACAGAAAGACAGAGA
MOR 120.1	JN860040	CATAGCATTATGACCCCTTCTAAAGTGA	GATGTTGGTAGCATTGGT	CCTAGAAGTGATAAATATTTGAAAAGCAAGCAT
MOR 121.1	JN860028	GTGGCTTAATTCTACTGTCCTGCTT	GGCCATCATTATCTCCCTGAG	CAGTCGGTCTAGAACATTACCACAA
MOR 500-1	FJ613878	TTGGTGTTGTCCTTTATTTATAGCACTCA	GTTAGTTTAGCTGTCAGAA	CAATGCGAGATCCACACAAAGAAA
MOR 129.1	JN860042	CACCTCCACCTTCCAGAACAC	CCTGGCAGCTATGTCC	AGAGGGTAGAAGATGGCCACATAG
OlfC 17.1	HM133605	CATGTCAGAGCTGAGAATACCCAATA	CGATTGCACATGCTCTG	GTCTGTGTTATTCTTTATGTCTGTCATTAGG
OlfC 4.9	HM133620	GGGTGTCACGCTTGGTTATCAA	TGACTCCTGAGCCTCGG	GCCACTTTCACGGCCATAG
OlfC 16.1	HM133613	CAGGAGGACCTGAGGGATGT	GGAGACCAACTGTGGG	CGTTATACTCCGGCCTGAGC
OlfC 2.2	HM133630	GTGCTGGCAGAGACTTATCCATT	CCTGGATGGGACTATTG	CTGAGCCCTGGGACTTTCC
OlfC 3.1	HM133631	CACCATCCCCAGCGACTAC	ATCAGGCAAAGGCTTTAG	AGTCCAGCCAAAGTGCTTGA
OlfC 13.1	HM133609	ACGGGCAGCCAGAATGG	GGCAGACTACAGGGGAG	CGGTGCAGTCCGAACCT
OlfC 15.1	HM133612	CTGCAGGCCTGTGTCAATTT	CCAAGAGGAACCATTGCAAT	GCAAACACCAGCGCATGT
Ora 1-2	FJ613844	CCAGCCTGTGGACATAATTCTCT	TCGTTGACCTGCTGCTGC	CAGGCCAAACACAGTCATAGTCT
ora 2	EU143809	GGAGACCATGGCCACCTT	CCTGGTCAACATCTTC	CCTGGCAGCCCGTATCC
ora 3a	AGKD01005870.1	CACAGAGACCCTGATGTTGGT	CTGTGTGTGGAACTTC	GCCAGGCCGTTGTAGCT
ora 4	AGKD01088259.1	GGAGCATGAGAGGAGGAAGGT	AATCTCGCCTTTTCCC	CCTTGGACATGTGTGGTGTAAAC
ora 5a	AGKD01052694.1	AGGTGTTTGCTCGGGATCATG	TTGTCTCCGTTCTCTCCC	GTTGATGAAGAGGACCTCATTGGT
TAAR 530.1	AGKD01103530.1	TGTTGTTCTGATTATGATGCTGGTCA	TCTGGACGGTTTCTGC	GCCCCAAATATTGATCCCCAGAAATA
TAAR 375.1	AGKD01134375.1	TCCTTCTGGGAGTGTTTGTAATGC	GCTCTGTCCAGGGCTG	ACAGAAACCCTCCTAGGTACCAA
TAAR 626.1	AGKD01001626.1	CGATGCTGCTGTTGTAAAAAACGT	AAATGTACAGACACCCGG	CCCAGGTTTTTCCTTCAACAATAAAACA
TAAR 652.1	AGKD01034652.1	GCAATAACGGAATCATGCTGGAAT	CAATTCTACGTCTCTTGTT	ACCAAATTGCCCAGAGATAAAGAAGT
TAAR 407.1	AGKD01006407.1	TGTGTTGTATATTCATTACCTGGTGTTGT	ACCGTGCTGCTATTATAA	CTTTCAAACACCTACTGGGTACCA
TAAR 637.1	AGKD01003637.1	CTGGTACCCAGTAGATGTTTGAAAGA	AAGGGTCAGTCTGGGGTAATAT	AGAGCATGGGACAACCATTGTAAAT
TAAR 415.1	AGKD01115415.1	CCAGTCGGTGTTTGAAAGAATGTTT	GGAATAACCTGGGGAAATATA	AGAGCACGGGACAACCATTG
TAAR 184.1	AGKD01081184.1	TCATCCTCTCTCTGGCTGTGT	CAGATCTCCTGGTGGG	GCTACAGTCGTTACTGGTATCACAA
β-actin	FJ890357.1	CCCACACAGTACCCATCTACGA	CCACGCCATCCTGC	GGCCGGCCAGATCCA
qPCR primer sequences				
β-actin	FJ890357.1	GACCCACACAGTGCCCATCT		GTG CCCATCTCCTGCTCAAA
mt1a	BT059876	CAAGTGCTCCAACTGTGCAT		TACACCAGGCCTCACTGACA
hmox1	BT046987	GATGCTGGCCTACCAGAGAG		GACTCCAGCCGTGCTAGTTC
nrn1	BT058878	GCTCTCACCGCCTGTAGCAG		TGCCCAGACCTCAACATCGTT
omp	AB490250	GACCCCTGACCTCACACACT		GTACATGACCTTGCGGACCT

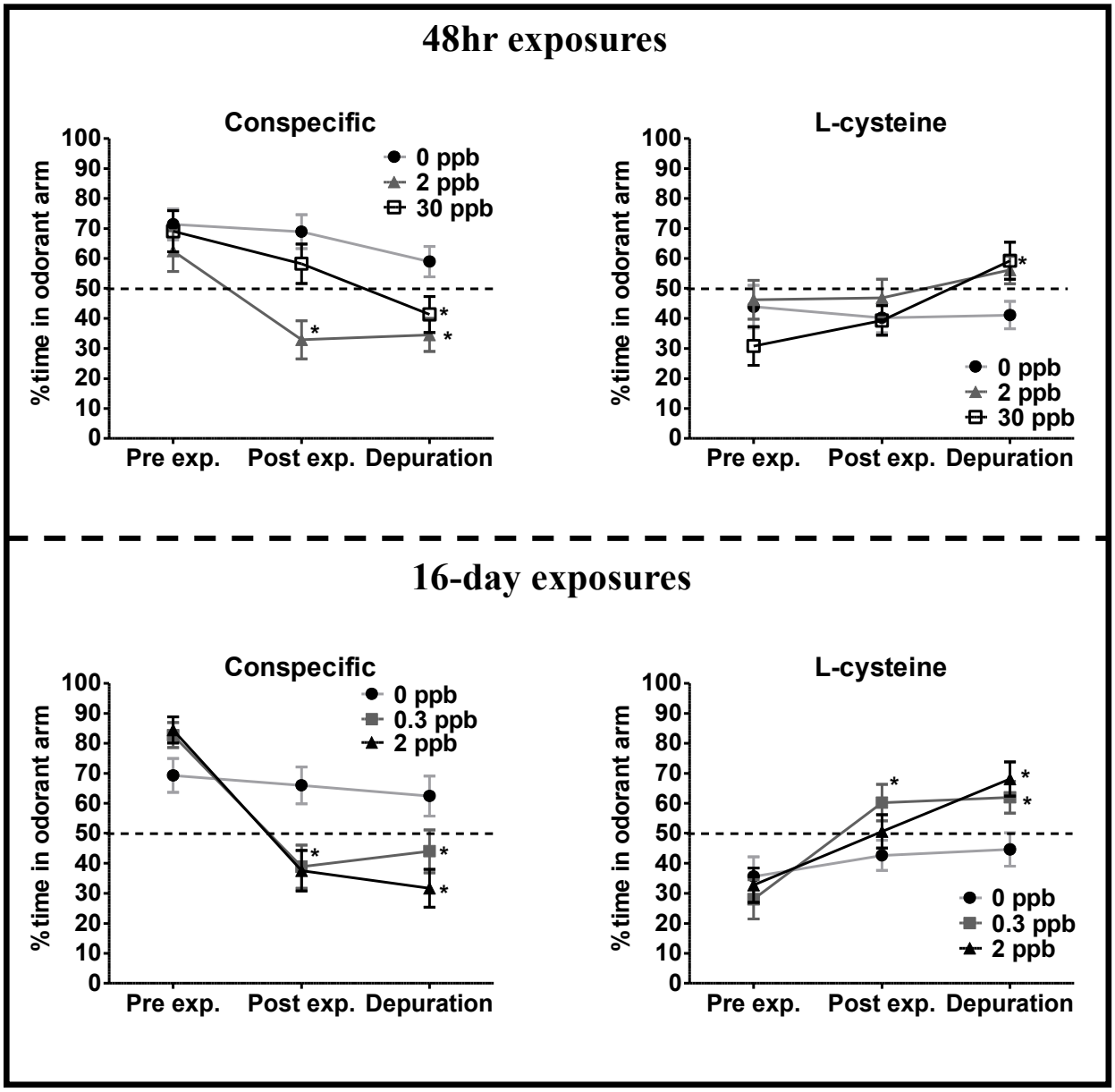

Figure 1. Behavioral responses to L-cysteine and conspecific odorants following Cd exposures. Percent time juvenile coho spent in the arm of a two-choice maze receiving conspecific odorant or L-cysteine odorant before exposures (Pre exp.), after exposure (Post exp.), and after a 16-day depuration (Depuration). All data represent mean ± SEM of n=24 individuals. Asterisks indicates statistically significant differences between pre-exposure and post-exposure/depuration behaviors within exposure groups (*P <0.05).

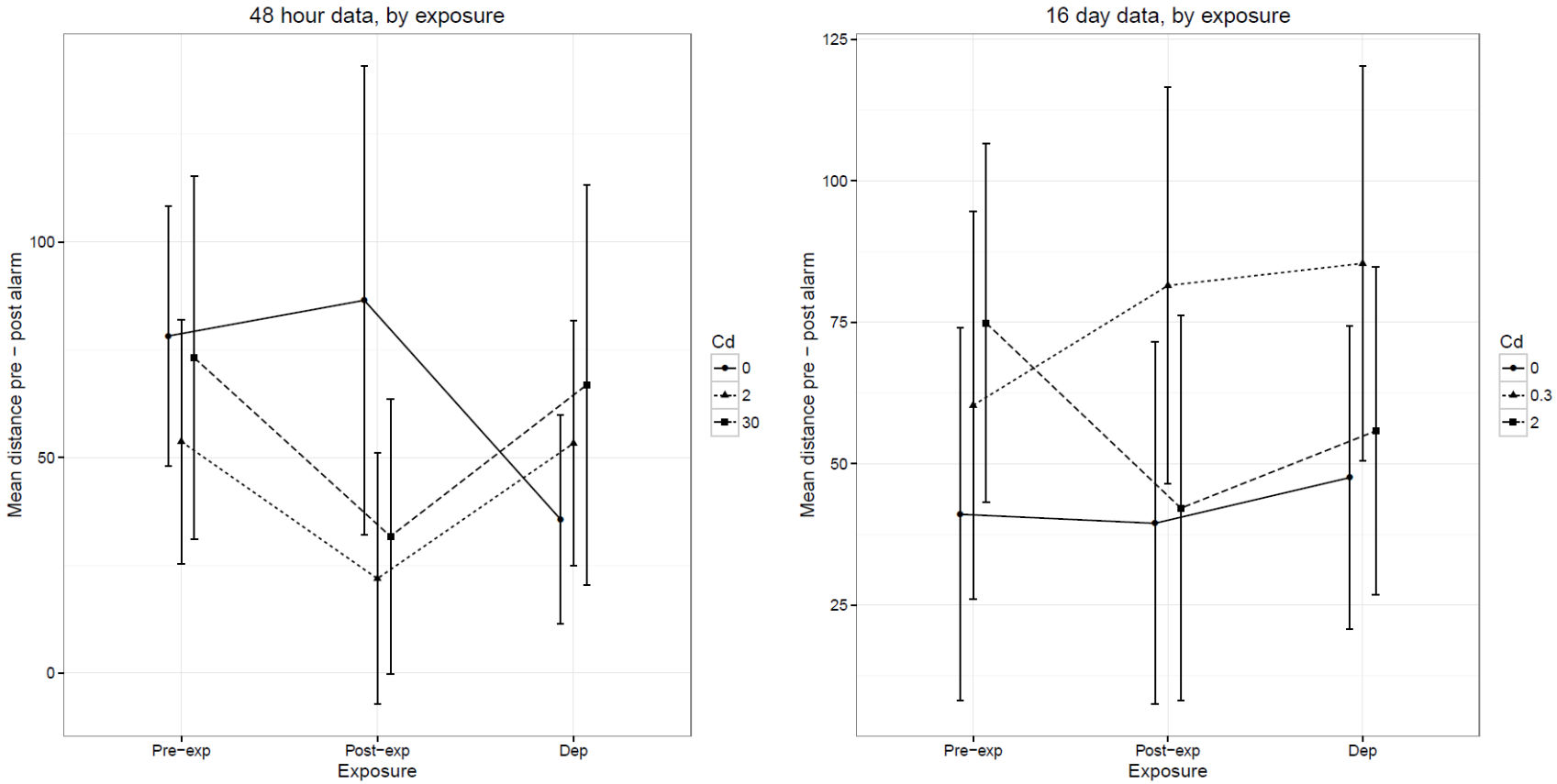

Figure 2. Behavioral responses to skin extract following Cd exposures. Mean difference of distance swam before addition of skin extract compared to the distance swam after the addition of the skin extract. All data represent mean ± SEM of n=24 individuals.

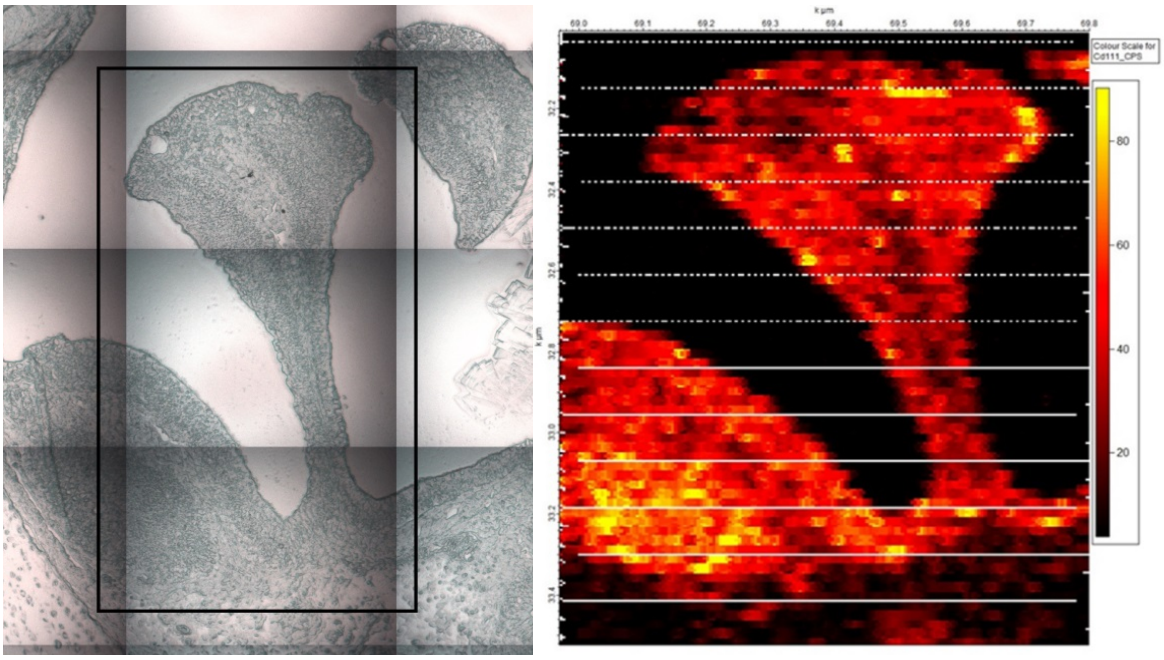

Figure 3. Heat map of Cd accumulation within a representative coho olfactory rosette lamella following exposure to 310 ppb Cd for 48hrs.

Exposure time	**Water conc. (ppb)**	**Tissue conc. (µg/kg)**	**Tissue conc. following depuration**
48 hour	0	116	45.5
48 hour	2	854	308
48 hour	30	5737	4237
16 day	0	81	54.5
16 day	0.3	891	790
16 day	2	3577	2592

Table 2. Concentrations of Cd within the olfactory sensory epithelium following the 48hr and 16-day Cd exposures and following the 16-day depuration. Data represents n=1.

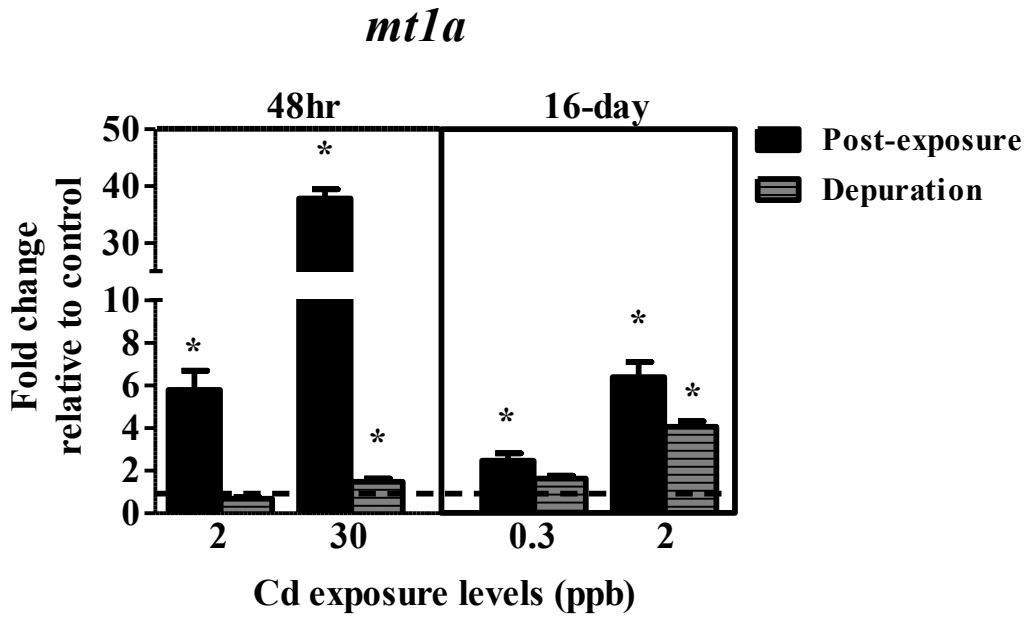

Figure 4. Effects of Cd on olfactory expression of mt1a. Fold-change in mRNA expression of the olfactory *mt1a* and *hmox1* following acute (48hr) and sub-chronic (16-day) Cd exposures and a depuration. Data represent the mean ± SEM of n=8 individuals normalized to the expression of β-actin mRNA, and expressed as fold-change relative to control levels. Asterisks indicates statistically significant differences in gene expression relative to controls (*P <0.05).

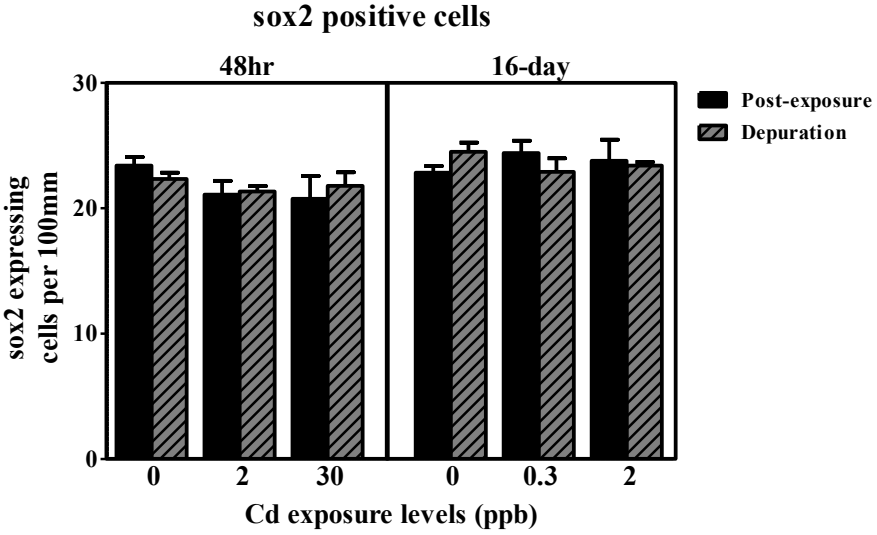

Figure 5. Effects of Cd on sox2 expressing multipotent olfactory stem cells in the coho olfactory epithelium following Cd exposures (48hrs: 0, 2 and 30 ppb Cd; 16 days: 0, 0.3 and 2 ppb Cd) and depuration. Data represents mean ± SEM of n=6 individuals per exposure group.

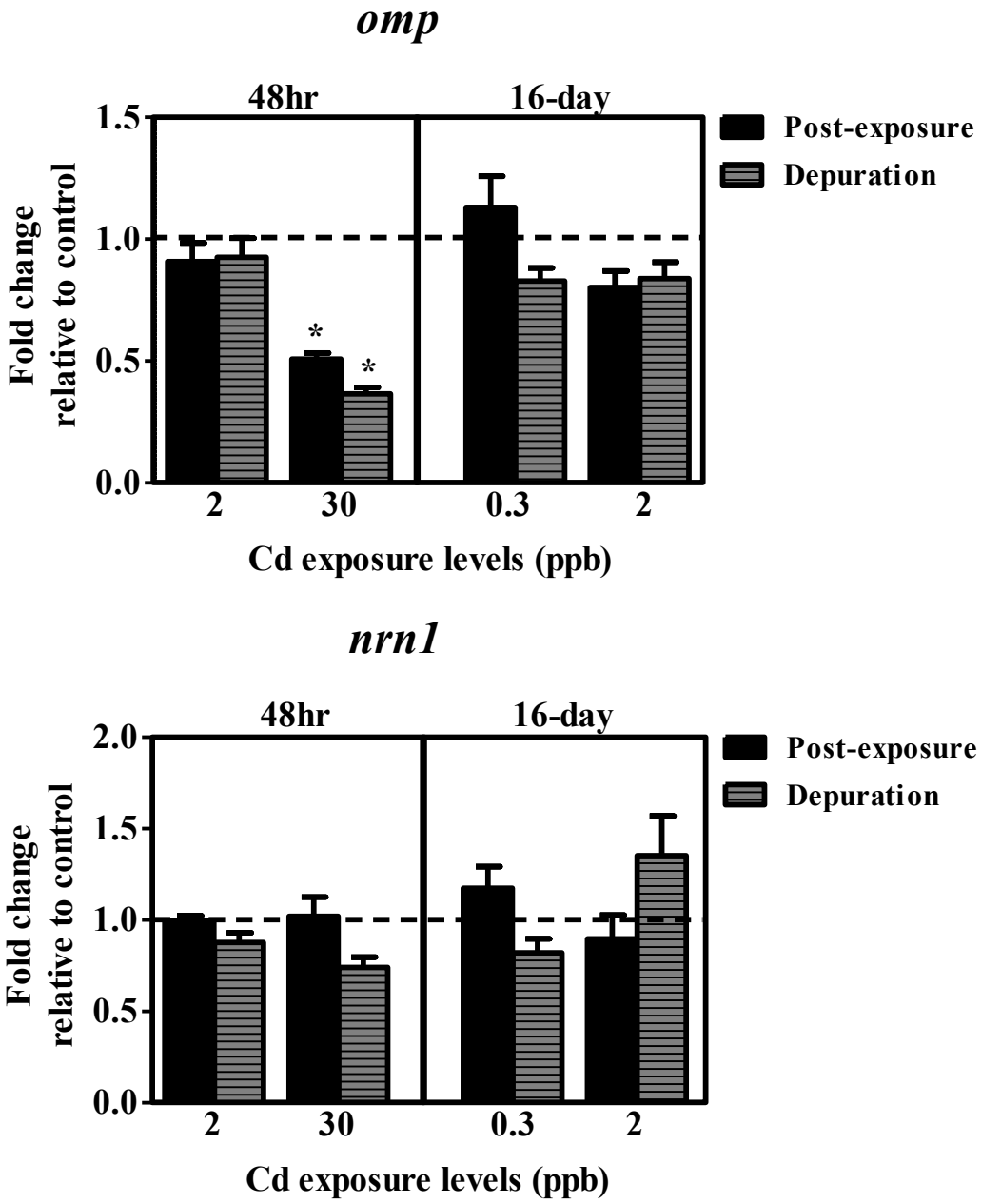

Figure 6. Effects of Cd on olfactory expression of omp and nrn1. Fold-change in mRNA expression of the olfactory *omp* and *nrn1* following acute (48hr) and sub-chronic (16-day) Cd exposures and a depuration. Data represent the mean ± SEM of n=8 individuals normalized to the expression of β-actin mRNA, and expressed as fold-change relative to control levels. Asterisks indicates statistically significant differences in gene expression relative to controls (*P <0.05).

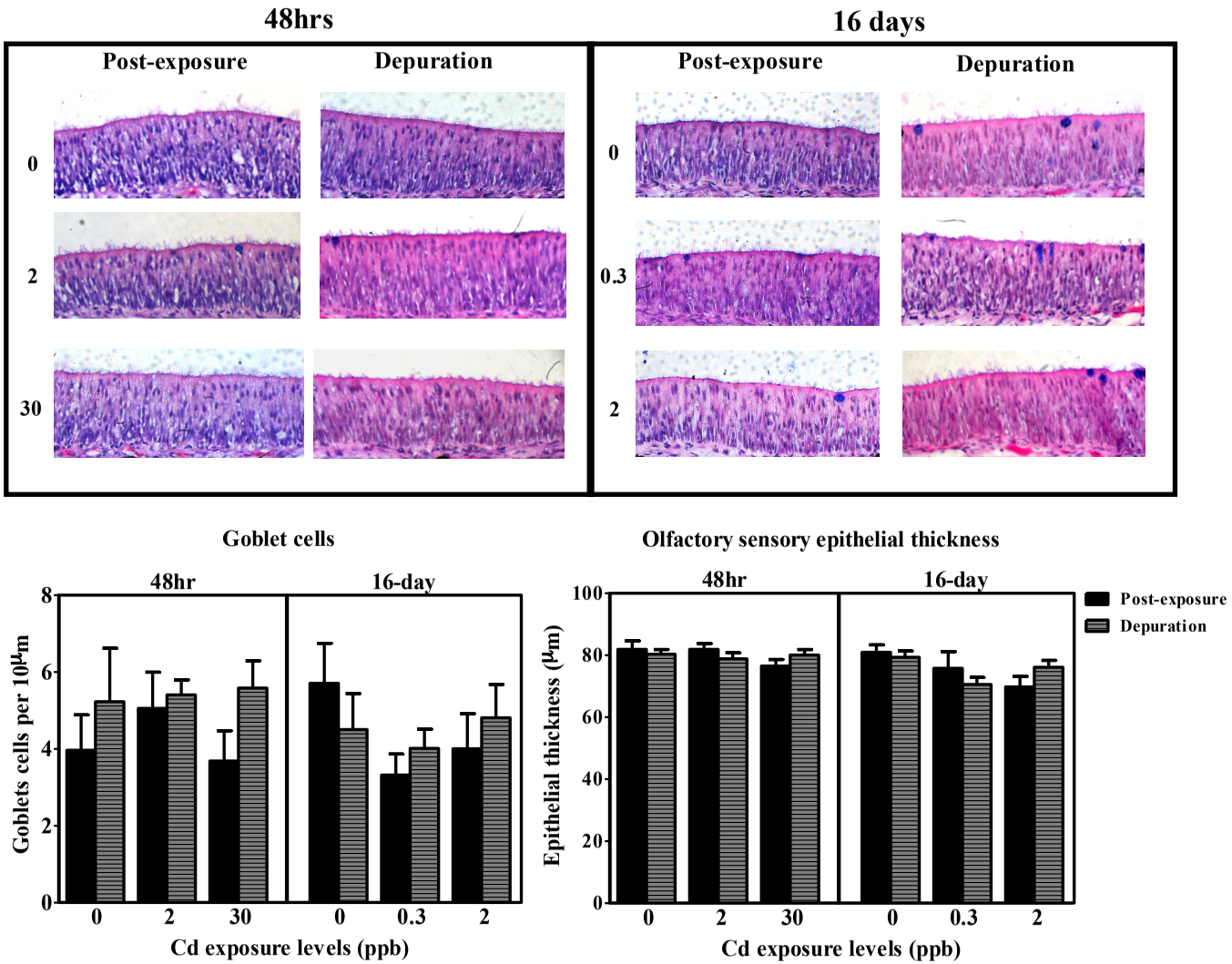

Figure 7. Effects of Cd on olfactory sensory epithelial histology. H&E staining of coho olfactory sensory epithelium following Cd exposures (48hrs: 0, 2 and 30 ppb Cd; 16 days: 0, 0.3 and 2 ppb Cd) and following depuration. Goblet cells (mucus producing cells) are stained dark blue using Alcian blue dye. Goblet cell population and thickness of coho olfactory sensory epithelium following Cd exposures (48hrs: 0, 2 and 30 ppb Cd; 16 days: 0, 0.3 and 2 ppb Cd) and following depuration. Data represents mean ± SEM of n=6 individuals.

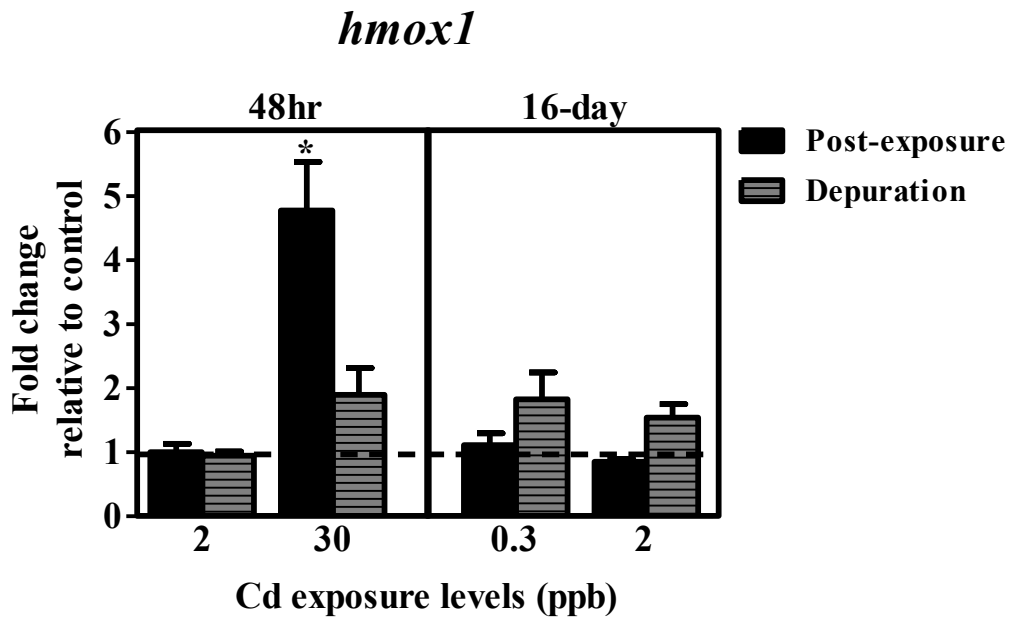

Figure 8. Effects of Cd on olfactory expression of hmox1. Fold-change in mRNA expression of the olfactory *mt1a* and *hmox1* following acute (48hr) and sub-chronic (16-day) Cd exposures and a depuration. Data represent the mean ± SEM of n=8 individuals normalized to the expression of β-actin mRNA, and expressed as fold-change relative to control levels. Asterisks indicates statistically significant differences in gene expression relative to controls (*P <0.05).

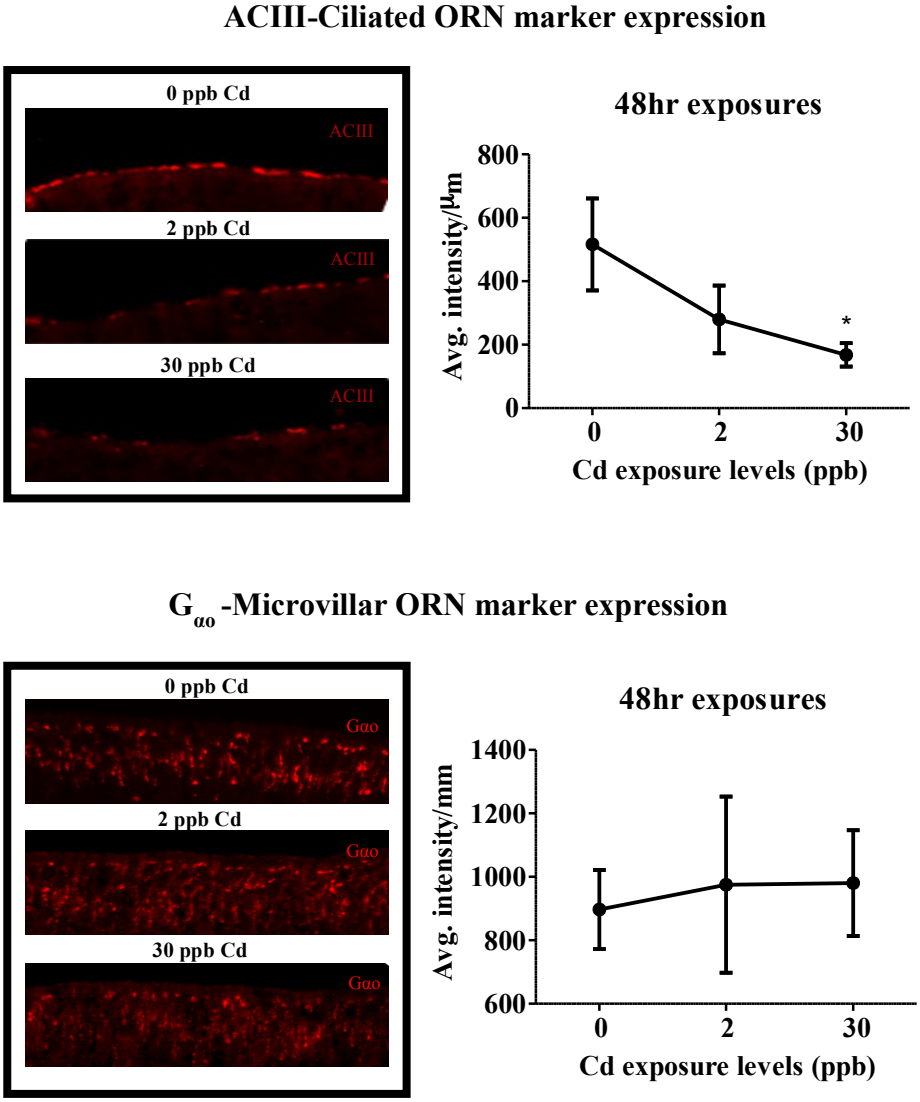

Figure 9. Effects of Cd on ACIII and $G_{\alpha o}$ protein expression in the olfactory epithelium. ACIII and $G_{\alpha o}$ immunolabeling as percent control levels following exposure to 2 and 30 ppb Cd for 48hrs. Data represent the mean ± SEM of n=6 individuals normalized to background fluorescence. Asterisks indicates statistically significant differences in signal relative to control (*P <0.05).

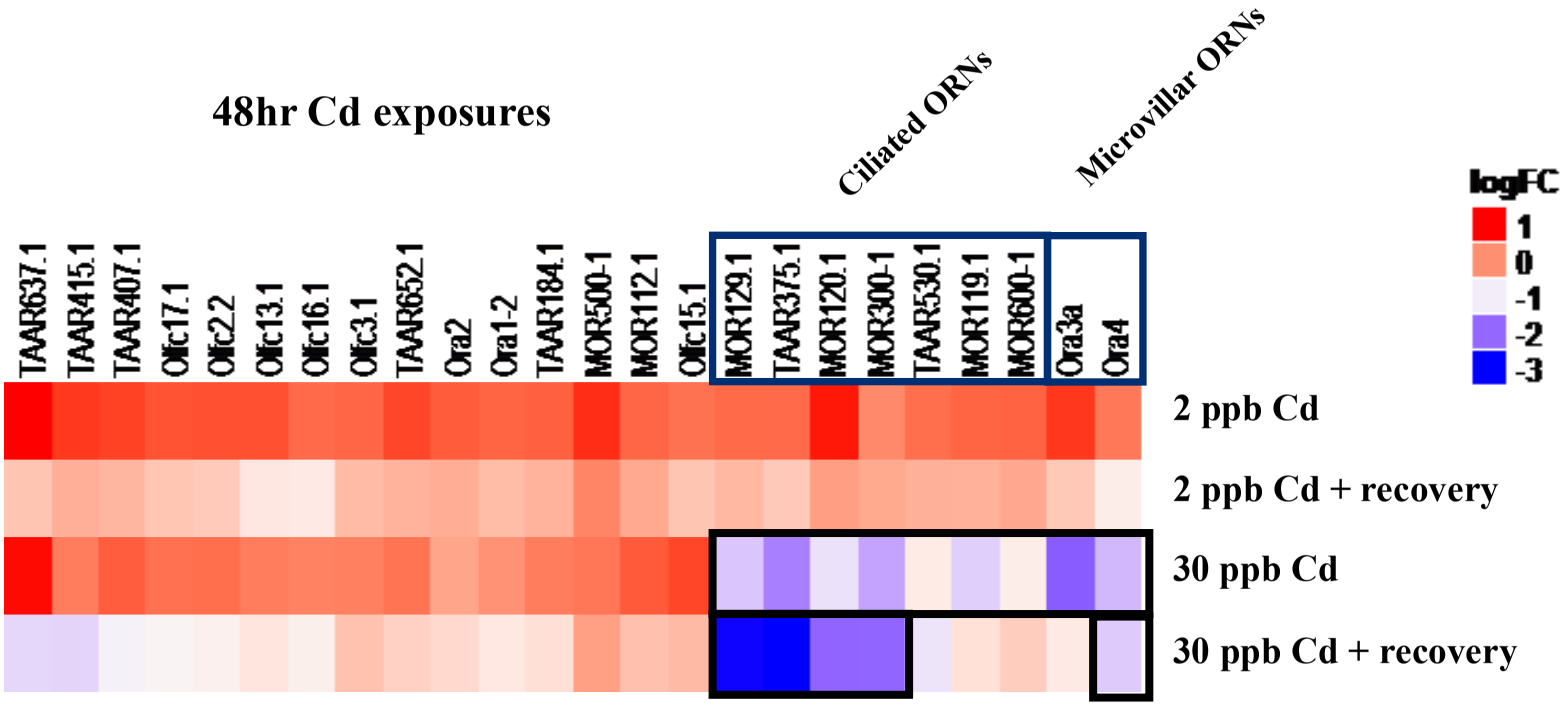

Figure 10. Effects of Cd on expression of major olfactory receptors. Heat map of coho olfactory receptor gene expression changes in the olfactory epithelium following exposures to Cd for 48hrs and following a 16-day depuration. Gene expression is represented as log-fold change compared to controls. Data represents n=8 individuals with OR gene expression normalized to the mean expression of elf1a and elf1b. Boxes indicate expression significantly different from control levels, $P \leq 0.05$.

Conclusions

The salmon olfactory system is a highly complex, sensitive and robust sensory system with an ability to detect extremely low-levels of an odorant in the water column, and to regenerate following severe morphological injury. While the functional complexity, sensitivity and regenerative properties of the salmon olfactory system allow it to function in many aquatic environments, it provides potential targets for disruption by waterborne metal exposures. The use of heavy metals in many anthropogenic activities has exponentially increased the amount of heavy metals entering salmon bearing waters, which presents a significant danger for wild salmon populations. While the cellular toxicity of Cd and the reliance of salmon on their olfactory system for survival are well known, there has been a significant lack of research on the effects of Cd on salmon olfactory function.

My research endeavored to address some of these research gaps. I found that exposure to environmentally relevant concentrations of Cd disrupt salmon olfactory function. Additionally, there is a partial, but incomplete, recovery of olfaction following termination of Cd exposures. The disruption to salmon olfaction is manifested by differential responses to prototypical odorants and olfactory sensory neuron populations. My data suggests different mechanisms of olfactory injury occur at different Cd exposure concentrations. Specifically, high Cd exposures are associated with frank epithelial cellular injury, whereas lower Cd exposure levels are likely negatively impacting olfactory signal transduction. Molecular markers of mature and immature neurons, as well as antioxidant response markers, appear to be robust biomarkers of Cd olfactory injury. These responses are strongly associated with significant Cd bioaccumulation in the salmon olfactory system. In conclusion, the results of my study indicate that juvenile coho

migrating through waterways impacted by high levels of Cd (and potentially those impacted by other metals) may experience a rapid and persistent loss of olfaction whereas juvenile coho exposed to low-levels of waterborne Cd could experience differential disruption of olfactory function, leading to altered odorant perception. Both outcomes could ultimately result in increased mortality. Many Pacific salmon populations are currently listed as threatened or endangered, and increased mortality events induced by waterborne metal exposures during sensitive periods in their lifecycles could push their population numbers even lower.

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