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Tolerance of the lamprey olfactory system to copper concentrations found in roadway runoff waters to the Great Lakes

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Tolerance of the lamprey olfactory system to copper concentrations found in roadway runoff waters to the Great Lakes

By

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Tolerance of the lamprey olfactory system to copper concentrations found in roadway runoff waters to the Great Lakes

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Declaration of Co-authorship

I hereby certify that this thesis incorporates material that is the result of joint research as follows: My third chapter was co-authored with my committee member Dr. Daniel Heath, as well as Kyle Wellband. My collaborators contributed to experimental design, provided technical support that directly contributed to the dataset, and provided editorial input. I am the primary contributor to each chapter of this thesis. No part of this thesis has been published or submitted for publication.

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Abstract

In fishes, olfaction mediates a variety of behaviours necessary for survival and reproduction. Environmental contaminants, specifically copper, are able to disrupt a broad range of olfaction-mediated behaviour, and can cause long-lasting damage due to physiological and genetic changes at low concentrations. To investigate effects of copper on olfaction, the sea lamprey (*Petromyzon marinus)*, an invasive fish species in the Laurentian Great Lakes, was exposed to environmentally relevant copper concentrations of 0, 5, 10 and 30 µg Cu-L. Three techniques were used to measure the effects of exposure of contamination: (i) immunohistochemical analysis of olfactory epithelium, (ii) local field potential recordings in odor-evoked responses, and (iii) gene transcription response to copper toxicity. Impairment in olfactory epithelium was demonstrated through loss of dendritic extension of olfactory sensory neurons, as well as a dose-dependent reduction in olfactory response to male sex pheromone odors. Differentially expressed genes were identified following exposure in each concentration. Low levels of copper measured in urban waterways were found to impair olfactory senses of the sea lamprey.

Dedicated to my parents, Mike and Debbie Jones, and my sister, Kara Jones.
Thank-you for your constant love and support in every aspect of my life.

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List of Abbreviations

3kPZS	3- keto-petromyzonal sulfate
BLAST	Basic local alignment search tool
Cu	Copper
GLM	Generalized linear model
GO	Gene ontology
GPCR	G-protein coupled receptor
ICP-MS	Inductively coupled plasma-mass spectometry
LFP	Local field potential
LRPCP	Little River Pollution Control Plant
LRWRP	Lou Romano Water Reclamation Plant
mRNA	Messenger ribonucleic acid
OB	Olfactory bulb
ON	Ontario
OR	Olfactory receptor
OSN	Olfactory sensory neuron
PBS	Phosphate-buffered saline
RNA	Ribonucleic acid
TAAR	Trace-amine receptors
V1R	Vomeronasal receptor

Chapter 1: General Introduction

In fishes, olfaction mediates social behaviours, such as reproduction, homing, schooling, as well as food-search, and predator avoidance (Kotrschal, 2000). Contaminants are able to disrupt a broad range of olfaction-mediated behaviour in fishes, and can cause long-lasting damage to physiological and olfactory epithelial structures (Wang et al., 2013). One contaminant that has received great attention in the literature with respect to olfaction is copper (Cu). Cu contamination is typically observed through anthropogenic sources, including those in urban areas near roadways. Disc brake utilization in motor vehicles causes particles to be abraded onto the roads and subsequently drained into waterways during snowmelt and rain events (Boulanger, 2003).

Copper is a ubiquitously distributed olfactory toxicant and a pervasive contaminant in urban runoff at concentrations that vary from 1 to 64 μg Cu-L (Soller et al., 2005). In a concentration-dependent manner, Cu concentrations within this range can inhibit physiological responsiveness of olfactory sensory neurons during short-term exposures in salmon (Sandahl et al., 2007), as well as lead to inhibition of genes in the olfactory system pathway of teleost fish (Tilton et al., 2008; Wang et al., 2013). Since salmon rely on olfaction for behaviours necessary for survival, including spawning, they may be threatened by living within areas of urban development (Baldwin, 2003; Sandahl, 2007). The adverse effects of copper on the fish olfactory system have been found even at levels below legally established concentrations (Sandahl et al., 2007).

Whereas the effects of copper on olfaction in salmon and a variety of other teleost have been investigated, responses of basal vertebrates were not tested. Lamprey rely heavily on olfaction for migration and spawning events (Vrieze et al., 2011), yet the lamprey olfactory system has not been investigated for the potential effects of copper

toxicity. Being an invasive fish species to the Great Lakes, the sea lamprey has received increasing attention in terms of population abatement strategies (Johnson et al., 2009). Current abatement strategies under development utilize a male sex-pheromone 3- keto-petromyzonol sulfate (3kPZS) application to streams, in attempts to lure and capture ovulating females (Johnson et al., 2009). The potential of copper toxicity effects on the olfactory systems may hinder the success of these strategies. In comparison, populations of lamprey are seen as a delicacy and threatened in parts of Europe, such as Portugal, as well as species in Asia, where conservation methods are widely employed to address declines in native sea lamprey populations (Pereira et al., 2010). Both scenarios present important implications for analyzing the effect of copper toxicity; for species that are targets for abatement management, as well as those whose populations are considered critically endangered (Cabral et al., 2005).

The goals of this thesis are to first explore the levels of copper in an urban environment proximal to sea lamprey populations. This includes independent copper analysis of roadway runoff and a study of copper values assessed by the City of Windsor (through a collaborative internship). The effects of copper levels entering through roadway runoff into urban watersheds were examined for olfactory sensory impact by testing neural responses to pheromones using electroencephalogram (EEG) local field potential recordings. A technique widely used for studies on olfaction (Byrd and Caprio, 1982), the EEG measures activity of olfactory sensory neuron responses to an odour in the olfactory bulb. Based on reduced responses during copper exposure, the cellular integrity of the peripheral olfactory organ was examined using immunohistochemical techniques, to address physical damage to the olfactory epithelium during increasing

toxicity. Lastly, the underlying molecular mechanisms of toxicity responses were further analyzed for genetic modifications to olfactory tissue that result during exposure. The recent annotation of the sea lamprey genome (Smith et al, 2013) presents the opportunity for investigating the effect of environmental copper on the RNA (transcriptome) of the olfactory system in the sea lamprey, a process previously unexplored. By analyzing RNA transcriptome data, the analysis of genes being up-regulated or down-regulated in response to copper can be identified, exploring the potential for adaptive responses in the sea lamprey. This study will provide a quantification of the lamprey's tolerance effects to copper, and the changes that result from exposure. It is hypothesized that low levels of heavy metals in urban waterways will impair sea lamprey olfactory function and alter their gene expression pattern in a number of biological processes.

Chapter 2: Copper levels in urban roadway runoff in an urban area and effects on neural activity in the olfactory system of the sea lamprey

2.1 Introduction

Chemical communication in fishes is managed by olfactory responses, mediating social behaviours and processes necessary for survival (Kotrschal, 2000). It is hypothesized that functions associated with reproduction, recognition, foraging, and predator avoidance are behaviours mediated by conspecific odours in a variety of organisms (Wyatt, 2014). While much of our understanding of chemical communication is based upon invertebrate species, pheromone communication utilized by the sea lamprey (*Petromyzon marinus)* has been well documented (Pietrzakowski et al. 2013; Buchinger et al. 2015).

The sea lamprey is a basal vertebrate, with a complex life history involving a variety of life stages throughout its life cycle. As an invasive fish species to the Great Lakes, the sea lamprey has inflicted considerable damage to the ecosystem, as well as the economy, with severe damages including those to populations of native fish species. Current abatement strategies implement the development of a synthesized pheromone based trapping (Johnson 2008), a strategy relying heavily on the olfactory biology of the sea lamprey. Previous research has demonstrated that traps baited with the natural migratory cue and male sex pheromone (3kPZS) successfully capture more sea lamprey than unbaited traps (Johnson et al., 2006; Johnson et al., 2009). Trapping efficiencies increased by 10% when 3kPZS was applied as bait (Johnson et al., 2013); however, these methods explore trapping in pristine, odour-controlled environments. In contrast, some populations of lamprey such as the brook lampreys (*Lampetra planeri*) of Portuguese streams and the northern hemisphere lampreys (*Petromyzontidae)* are of high economic

value. Conservation methods have been implemented in attempt to suppress the threat of survival of these species (Pereira et al, 2010; Almeida et al. 2002; Renaud, 2007).

One contaminant that has received great attention in the literature with respect to olfaction is copper (Cu). Cu contamination is typically observed through anthropogenic sources, including those in urban areas near roadways. Disc brake utilization in motor vehicles causes particles to be abraded onto the roads and subsequently drained into waterways during snowmelt and rain events (Boulanger 2003). Cu is a ubiquitously distributed olfactory toxicant and a pervasive contaminant in urban runoff at concentrations that vary from 1 to 64 μg Cu-L (Soller et al., 2005). Concentrations within this range can inhibit physiological responsiveness of olfactory sensory neurons during short-term exposures in salmon (Sandahl et al., 2007). Based on this information, recent studies have since been successful in contributing to the banning of brake pads containing copper in the states of Washington, New York, Rhode Island, Oregon and California (Lee and Lin, 2013; Barlow et al., 2014). Adverse effects of Cu in fishes include suppression of pheromone detection, reproduction and predation (Sandahl et al., 2007; Kolmakov et al., 2009).

Here we explored the levels and the effects of copper concentrations present in an urban roadway runoff waterway, as well as passage through a rural region, and compare these to waterways that inhabit and are migrating/spawning streams of the sea lamprey. Water samples were collected seasonally in urban waterways adjacent to high traffic streets, and also those in low traffic areas. It was hypothesized that there would be an increased copper concentration during the spring snow melt and rain events, where the highest amount of copper particles from brake pads will be washed into waterways.

Based on the measurement of environmentally relevant levels of copper found in local urban roadway runoff, we tested to determine the effects of both short term (rapid onset) and 24-hour exposures of copper on the olfactory system of the sea lamprey. Odour responses to pheromones were tested for short exposure to Cu. For histological effects on the olfactory epithelium, immunohistochemical labeling of acetylated tubulin (present in the dendrites, cell bodies, and axons of olfactory sensory neurons) was conducted to explore olfactory odour responses at various copper concentrations.

2.2 Materials and Methods

2.2.1 Copper levels in urban roadway runoff analysis

Urban and rural streams were sampled to compare copper concentrations in streams that exhibit high traffic roadway runoff, as well as streams that inhabit the sea lamprey in a less urbanized setting. A series of eight sites were chosen in Windsor, ON, and the surrounding area to cover highly urbanized streams surrounded by high traffic roadways in downtown Windsor. Samples were strategically chosen along a single water source, the Grand Marais Drain, to represent the range of effluent levels from roadways in areas of high traffic across the City (Figure 2.1). Indicative of an urban environment, each site was documented to have consistently high traffic based on information obtained from the City of Windsor. Water samples were obtained for water quality analysis from summer (July 4 2014)- spring (April 28 2015) at all 8 locations. Both unfiltered and filtered 50 mL water samples were taken at each site to account for total suspended copper concentrations. Sites included major roadways in Windsor, including: Outfall at the Detroit River, West Grand Blvd, Huron Church Rd, Walker Rd, Dougall Storm Drain. Areas in Lasalle, ON were also sampled for areas with less traffic flow than inner city,

which included: Turkey Creek, Grand Marais Drain and Basin Drain. Water samples were tested seasonally following major rain events in the area.

To compare urban water copper concentrations to pristine locations with inhabit sea lamprey, 5 rural streams in Millersburg, Michigan known for migration and spawning locations for the sea lamprey were sampled in the spring of 2015. These locations are also where current abatement strategies utilizing the male sex pheromone 3kPZS are implemented. Sites in Michigan included Black Mallard Creek, Ocqueoc River, Trout River, Cheboygan River and Schmidt Creek. All 5 sites exhibited low traffic year-round.

Copper concentration analysis from water samples was conducted at the Great Lakes Institute of Environment Research using inductively- coupled plasma mass spectrometry (Agilent Technologies 7900 ICP-MS, method detection limit of 0.0612 µg Cu-L).

2.2.2 Effects of copper on neural activity in the olfactory system

2.2.2.1 Experimental fish

Petromyzon marinus were obtained from United States Geological Survey Hammond Bay Biological Station in Millersburg in May 2015, Michigan. All animals were then transferred to the University of Windsor and held in the Central Animal Care Facility at University of Windsor, according to UWindsor Animal Care Guidelines (AUPP#14-05). Animals were housed in an 8°C cold room in tanks with recirculated dechlorinated water. Temperature and pH were measured using a standardized pH meter. Copper levels were reported for each tank to be at or below 0.4 µg Cu-L. Immunohistochemistry analysis of 24-h Cu exposures using transformer phase lamprey at 0 µg Cu-L (N=3), 5 µg Cu-L (N=3), 10 µg Cu-L (N=3) and 30 µg Cu-L (N=3).

Electrophysiology test procedures used adult phase sea lamprey for a similar range of copper concentration, not including 30 µg Cu-L; of 0 µg Cu-L (N=5), 5 µg Cu-L (N=5) and 10 µg Cu-L (N=5) for exposures of 30 min.

2.2.2.2 Changes to the olfactory epithelium

To investigate the potential effects of copper on olfactory epithelial tissues, cresyl violet staining, as well as labeling of acetylated tubulin, were performed. Olfactory epithelium was collected from transformer phase sea lamprey. Animals were placed in individual 10 L tanks, with appropriate amounts of copper chloride of 0, 5, 10 and 30 µg Cu-L. Immediately following each 30 minute Cu-exposure, each fish was deeply anesthetized with 150 mg/L MS-222, and then decapitated. For histological and immunohistochemical analysis, the olfactory epithelium tissue was placed in paraformaldehyde fixative containing 4% paraformaldehyde in 0.1 M PBS for 24-h. Olfactory epithelium tissue was then placed in 30% sucrose gradient in 0.1 M PBS, and cryosectioned in 18 µm slices and rinsed in phosphate-buffered saline (PBS) with Triton to rehydrate cells, for 2-h. Cresyl violet staining was also completed on sections of olfactory epithelium from the same animals to outline structural components of lamellae in the peripheral olfactory organ. Additionally, tissue was labeled with primary antibody, monoclonal mouse anti-acetylated tubulin (1:1000; Sigma- Aldrich, Oakville ON, T7451) overnight. Labeling of α-acetylated tubulin is a novel marker of olfactory sensory neurons, ciliary processes and microtubules labeling in olfactory epithelium. Each olfactory epithelial tissue was then mounted, coverslipped with VectaShield Hardset with DAPI (fluorescence anti-fade mounting medium; Vector Laboratories Burlingame, CA, USA) and stored in 4°C until analyzed.

Low power micrographs (20x objective) were captured using LAS AF (Leica DM1600B) following cresyl violet staining. High power micrographs (60x oil immersion objective) were captured using confocal (Olympus fluoview FV1000) microscopy following anti-acetylated tubulin labeling.

2.2.2.3 Electrophysiology test procedure

Stimulus water contained 10^{-8} M 3KPZS (3-Keto Petromyzonal Sulfate), a sea lamprey male sex pheromone known to elicit consistent responses in adult lampreys (Li et al., 1995). Copper-exposure water was produced with the appropriate additions of 0, 5, and 10 Cu-L, to chilled lamprey Ringer solution (130 mM NaCl, 2.1 mM, KCl, 2.6 mM $CaCl_2$, 1.8 mM $MgCl_2$, 4 mM HEPES, 4mM dextrose, 1 mM $NaHCO_3$). Fresh Ringer's was prepared prior to each experiment, and pH adjusted to 7.4 with 5 mM NaOH. Ringer's were chilled and oxygenated (95% oxygen and 5% carbon dioxide) throughout the duration of experiments.

Following Ringer's solution preparation, a lamprey was obtained from Animal Quarters and anaesthetized using a 0.15 g-L dose of tricaine mesylate (MS-222, Sigma-Aldrich, Oakville, ON, Canada). The individual lamprey was then decapitated at the third branchiopore and tissue placed in a dissection dish containing Ringer's solution. Tissue was rotated onto the ventral side, so that a cut could be made along the gill pores and removed down to the buccal cavity and nasopharyngeal pouch. On the dorsal side, the skin, musculature and cartilage were removed to expose the brain, olfactory epithelium, nasal cavity and olfactory bulbs. Dura matter was removed from the surface of the olfactory bulbs and nerves (Green 2012).

The exposed dissected tissue was placed into a recording chamber. During the

experiments, chilled and oxygenated Ringer's solution was continuously perfused at a flow rate of 1mL/min, from the top of the dish and into the nasal cavity, and at the bottom of the dish (Figure 2.3). A 1 h recovery period between the dissection and recording was allowed (Green 2012, Boyes 2014).

Copper concentrations were created using copper chloride ($CuCl_2$), and added to the Ringer's solution during the copper exposure experimental duration. Copper concentrations of 0 µg Cu-L (N=5), 5 µg Cu-L (N=5) and 10 µg Cu-L (N=5) were tested.

The same procedural outline was performed for all sets of experimental copper concentrations. The lamprey pheromone 10^{-8}M 3kPZS was delivered to the nasal cavity in three phases: i) a 9 minute pre-copper control period, (ii) a 30 minute copper exposure period and (iii) a 30 minute post-copper recovery period. During the pre-copper control period, solely Ringer's solution was perfused through the nasal cavity. At 3 min intervals, an odour delivery system applied 3kPZS above the nasal cavity, causing stimulation of olfactory sensory neurons. During administration of 3kPZS, an electrode recorded local field potential responses from the dorsal area of the olfactory bulb. Local field potentials are electrophysiological signals generated from the sum of the electric currents from a group of neurons found on nervous tissue. At the 12 min mark, once a steady baseline of pre-copper exposure was established, the copper exposure period began. Appropriate copper concentrations were added to the Ringer's solution, and were perfused through the nasal cavity. 3kPZS was then administered in 5-minute intervals for a total of 6 deliveries. At the 42 min mark, the recovery period began and the copper/Ringer's mixture was replaced with initial Ringer's solution. 3kPZS was administered in 5-minute intervals for a total of 6 deliveries (Green et al., 2010).

2.2.3 Electrophysiology data analysis and statistical evaluations

Local field potential recordings (LFP) were digitally filtered through a low pass filter, cut off at 100Hz, to reduce random noise using LabChart software (version 6.1.3, ADInstruments). The mean peak amplitude was calculated for each delivery during each odour response. Averages of mean peak amplitudes were used as a reference point, for the pre-copper control period. The mean peak amplitudes for the remaining deliveries were then divided by the reference value, to produce individual response magnitudes for the deliveries corresponding to each individual animal. Individual response magnitudes were then averaged across all animals to produce a total olfactory bulb (OB) response magnitudes value.

Repeated measures two-way ANOVA was conducted using JMP (SAS Institute), in order to determine if there any significant differences between the pre-copper control values and the values during the copper exposure and the post-copper recovery period.

2.3 Results

2.3.1 Chemical analysis of local waterways in Windsor, ON and surrounding area

Frequency distributions of copper concentrations of Windsor sampling sites, both urban and rural, are presented in Figure 1. Values ranged from 0.54 - 7.42 µg Cu-L over all time points, with the majority of the values falling between 1- 3 µg Cu-L. Spring, summer, autumn and winter samples all show similar distribution patterns, with a significant increase in copper concentration in spring 2015, compared to all other seasonal time points. Summer 2014 sampling of copper concentrations ranged between 0.54 – 3.1 µg Cu-L, Fall 2014 between 1.21- 4.79 µg Cu-L, Winter 2014 between 1.36 – 3.54 µg Cu-L and Spring 2015 between 1.59 – 7.42 µg Cu-L (Table 2.1).

Water sampling from sea lamprey migratory streams in Hammond Bay, Michigan, in Spring May 28, 2015 demonstrated relatively low levels of copper (0.34-2.45 µg Cu-L) in all 5 streams after a significant rain event.

2.3.2 Effects of 24 hour copper exposure on the olfactory epithelium

The olfactory epithelium was examined in views of cresyl violet stained preparations (Figure 2.5) after 24 copper exposures of 0, 5, 10 and 30 µg Cu-L. In negative control sections of 0 µg Cu-L, the lamellae in the peripheral olfactory organ show uniform and intact structure of the epithelium (Figure 2.5A). Differences were evident with increasing copper concentrations, as tips of lamellae begin to stain more darkly and appear more pointed (Figure 2.5B). With increasing copper concentrations of 10 and 30 µg Cu-L, debris begins to accumulate in the lumen of the nasal cavity, as well as surface of the olfactory lamellae appear shortened (Figure 2.5 C, D).

Acetylated α-tubulin immunoreactivity indicated the presence of cilia, as well as microtubules in the dendrites (Figure 2.6). In lampreys not exposed to copper, intense labeling was seen in the cilia mucociliary complex above of the olfactory epithelium, as well as in dendrites as far as the apical surface of the epithelium. This labeling in the apical dendritic regions of the olfactory sensory neurons treated with increasing copper concentration, as some acetylated tubulin labeling of the dendrites did not reach the surface of the olfactory epithelium starting in the lowest copper concentration (5 µg Cu-L) (Figure 2.6B). Loss of apical extension of dendrites become more prominent as copper concentration increased and the olfactory sensory neurons appeared swollen at higher doses of copper (30 µg Cu-L) (Figure 2.6 D).

2.3.3 Effects of copper on olfactory bulb responsiveness to male sex pheromone odour

Following a 5 µg Cu-L exposure to the olfactory epithelium, 2 out of 5 animals showed a significant decrease in peak amplitudes of LFP response to 10^{-8}M 3kPZS, during copper exposure. The remaining 3 individuals did not show significant reduction in peak amplitude values. Following exposure to 10 µg Cu-L, 4 out of 5 animals showed a significant decrease in LFP peak amplitude response to 10^{-8}M 3kPZS, during copper exposure. The remaining individual showed a small decrease in peak amplitude value (Figure 2.3).

Individual olfactory bulb response to 10^{-8}M 3kPZS was also observed following 5 and 10 µg Cu-L copper exposures (Figure 2.4). In the 5 µg Cu-L experiment, pre-copper control period showed a small OB response magnitude variation. During the 5 µg Cu-L exposure, 2 out of 5 animals had reduced OB response magnitude values. The responses to 5 µg Cu-L were highly variable, resulting in a high standard error value. During post-copper recovery period, OB response magnitudes for all 5 animals showed an even greater variation between animals, resulting in large standard error. During the increased copper exposure concentration of 10 µg Cu-L, variation in OB response magnitude was small in the pre-copper period. During copper exposure period, 4 out of 5 animals were observed to have reduced OB response magnitudes, with less variation during exposure than the 5 µg Cu-L exposure. Variation in post-copper recovery was also large, resulting in large standard error.

Relative to the control exposure, OB response to 10^{-8}M 3kPZS was reduced during both copper concentrations of 5 and 10 µg Cu-L (Figure 2.4). Individuals exposed to control concentrations did not show a reduced in OB response magnitude. The

variation between animals during the control exposure was also large, resulting in large standard error.

Animals subjected to the 5 µg Cu-L showed a decrease of 20% in OB response magnitude during copper exposure periods. In comparison, animals subjected to the 10 µg Cu-L exposure showed approximately 40% reduction in OB response magnitude. However, during the 5 µg Cu-L copper exposure, only the 26 min delivery mark showed a significant reduction in OB response magnitude relative to the pre-copper exposure values. Following the 5 µg Cu-L exposure, OB response magnitude returned to pre-copper exposure values within 20 min of recovery period. Exposure to the higher concentration of 10 µg Cu-L caused a greater reduction in OB response magnitude during copper exposure periods, with a significant decrease during each time point during exposure with respect to pre-copper period values. During the post-copper recovery period, OB response magnitude showed a slight increase in recovery, however these values did not return to pre-copper exposure levels.

2.4 Discussion

Understanding the functional and mechanistic relationships between aquatic habitats and organisms presents major challenges. The olfactory system of fish has been recognized to be particularly vulnerable to the toxic effects of dissolved pollutants, specifically copper, in aquatic environments (Baldwin et al., 2003). This study correlates levels present in the environment to effects on the olfactory system of the sea lamprey. It is shown that copper suppresses odour responses to sex pheromone odour 3kPZS in the olfactory bulb of the sea lamprey OSNs that respond. Moreover, this inhibitory effect is

increased at higher concentrations of Cu. As consequence, these low levels of copper exposures may affect the potential of olfactory-mediated behaviours, such as mate attraction mediated by pheromones.

Copper exposures used in this study were of environmental relevance, and designed to mimic copper pollution in local urban habitats, such as those where the sea lamprey inhabits. Low levels of copper (1-3 µg Cu-L) (Sandhal et al., 2007) known to cause impairment in fish are found to be present in local urban waterways sampled in Windsor, ON. Additionally, higher values of 1.59- 7.42 µg Cu-L were present during the Spring of 2015, consistent with the hypothesis of the spring snow and rain melt events presenting highest copper concentration values. Roadways that are known to exhibit higher traffic levels (Huron Church) also experienced higher values of copper contamination consistently through the season.

Indicative of a typical rural environment, Hammond Bay streams in Michigan were sampled and experienced relatively low values of copper in all sample locations, compared to urban sites tested in this study. Similar values of copper were reported for urban sites in historical water quality data from the Ministry of the Environment (2003), as well as previous studies (Davis 2003; Prestes 2006). These findings highlight the importance of water quality analysis during pheromone abatement strategies, which may have the potential to be unsuccessful in streams with heavy metal contamination. Although streams in Windsor do not contain sea lamprey, they do allow for a comparison to urban environments in lamprey may be found. Some streams in Toronto, Ontario inhabit sea lamprey, as well as use pheromone abatement strategies to control sea lamprey populations. Based on historic Provincial Water Quality Monitoring Network

Data, Cu values for streams in Toronto streams range from values of 3.77- 7.46 µg Cu-L. Based on findings in the current study, values as low as 5 µg Cu-L can inhibit olfactory neural activity in the sea lamprey. This should be considered during future pheromone based applications into urban streams when attempting to control increasing lamprey populations in the Great Lakes.

Dosages of low (5 µg Cu-L) to high (30 µg Cu-L) copper exposure were used to measure potential effects on olfactory epithelial tissues. Olfactory sensory neuronal effects were present starting at the lowest dosage of 5 µg Cu-L, through the depletion of dendritic extension to the apical surface of the epithelium, as well as lamellar irregularity. Previous studies have also found similar changes in olfactory epithelial tissues in response to copper, reporting the number of olfactory receptors significantly reduced in Chinook salmon exposed to ≥ 0 µg Cu-L (Kolmakov et al., 2009). Based on the changes indicated by immunohistochemistry techniques, doses of 5 and 10 µg Cu-L were used to analyze odour response changes in response to the male sex pheromone 3KPZS. As reported in previous research in teleost fish, changes in odour response were also present in doses as low as 5 and 10 µg Cu-L, with increasing inhibition present in the higher dose of 10 µg Cu-L (Green, 2012; Hansen et al., 1999).

In summary, we have shown exposure to copper significantly impairs the olfactory bulb responsiveness of the sea lamprey, as well as causes impairment to the olfactory epithelial tissues. These exposures were typical of copper concentrations found in urban water runoff. Therefore, copper may interfere with a variety of olfactory mediated behaviours, as well as the potential success of implemented abatement strategies of the invasive fish species, as well as those species targeted for conservation

methods. More work is needed to understand the behavioural consequences, as well as the potential for transcriptomic analysis on the biological processes affected by this neurotoxicity.

Tables

Table 2.1 Total dissolved copper values (µg Cu-L) reported for urban sampling sites in Windsor and surrounding area.

Sampling Location in Windsor, ON	Copper Concentration (µg Cu-L) Summer July 4/ 14	Fall Oct 17/ 14	Winter Mar 10/15	Spring April 28/15
Outfall at River 42°31`N, 83°07`W	0.54	1.21	1.36	1.59
West Grand Blvd 42°27`N, 83°01`W	0.85	2.67	2.33	4.13
Dougall Storm Drain 42°27`N, 83°01`W	3.10	4.79	2.56	5.97
Huron Church Rd 42°26`N, 83°04`W	1.60	2.89	3.54	7.42
Walker Rd 42°29`N, 82°98`W	0.54	1.21	3.04	4.72
Grand Marais Drain 42°25`N, 83°06`W	1.17	2.38	2.19	5.20
Basin Drain 42°25`N, 83°06`W	1.18	2.05	1.79	3.11
Turkey Creek 42°24`N, 83°09`W	1.31	2.00	2.04	3.38

Figures 2

Figure 2.1 Water sampling values for total dissolved copper chloride found in urban waterway runoff sites in Windsor, ON for all sampling time points.

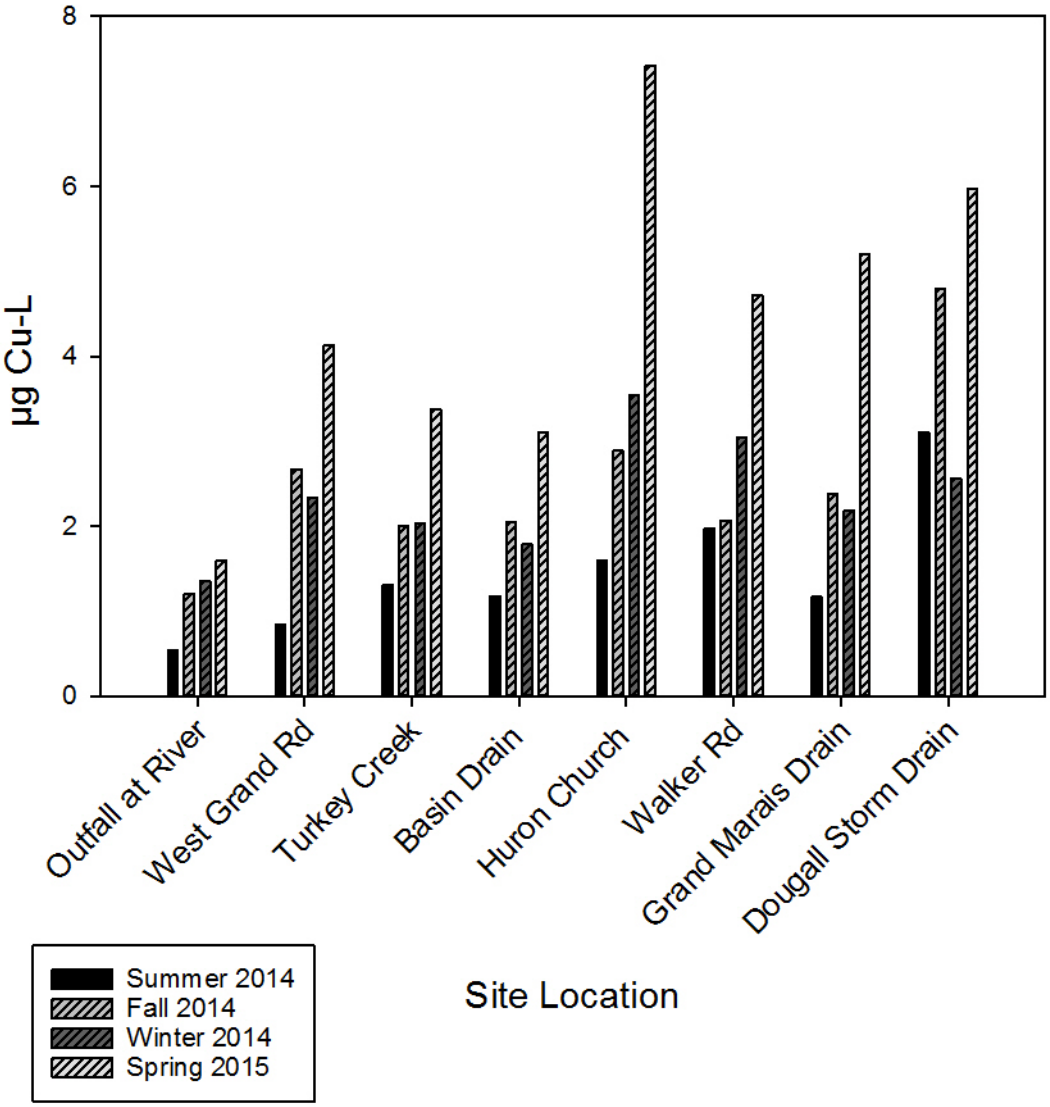


Figure 2.2 Map of locations of water sampling sites in Windsor, ON and surrounding areas. Water quality data for Winter March 10, 2015 copper values appear in order from lowest to highest copper concentrations at each of the 8 site locations.

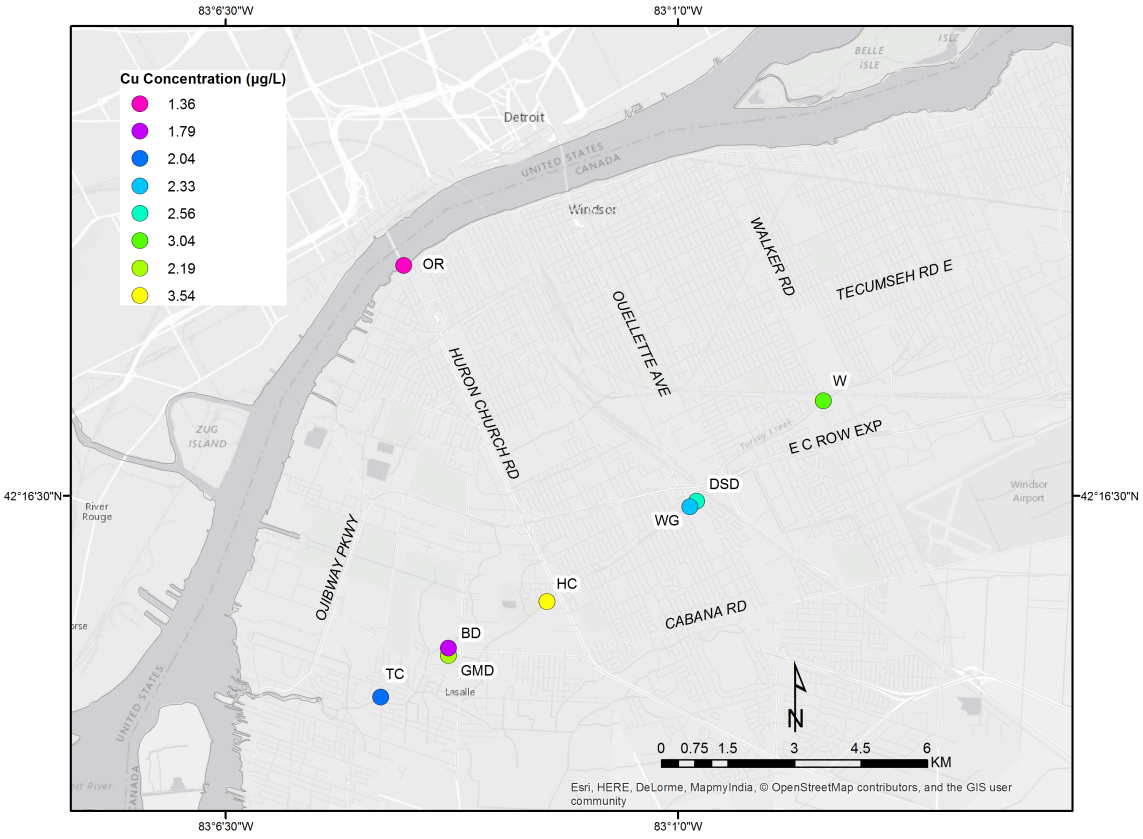


Figure 2.3. The effect of copper chloride on olfactory bulb responses to the pheromone 3KPZS applied to the olfactory epithelium. The nose-brain preparation was utilized to examine the effect of applying copper chloride to the peripheral olfactory organ, on olfactory bulb responses to 10^{-8} M 3KPZS applied to olfactory epithelium. Local field potentials were recorded from the dorsal region of the olfactory bulb. The amplitude of each peak in response is shown in microvolts (μV) and duration in milliseconds (ms). The center trace in each panel indicates the copper exposure to the peripheral olfactory organ for 30 min periods for (**A**) 0 μg copper (Cu)-L (**B**) 5 μg copper (Cu)-L and (**C**) 10 μg copper (Cu)-L. For each treatment, the control responses, depicted by the top trace in each panel, were collected prior to 30 min copper exposure. Recovery responses, depicted by the bottom trace in each panel, were recorded 30 min following each copper treatment.

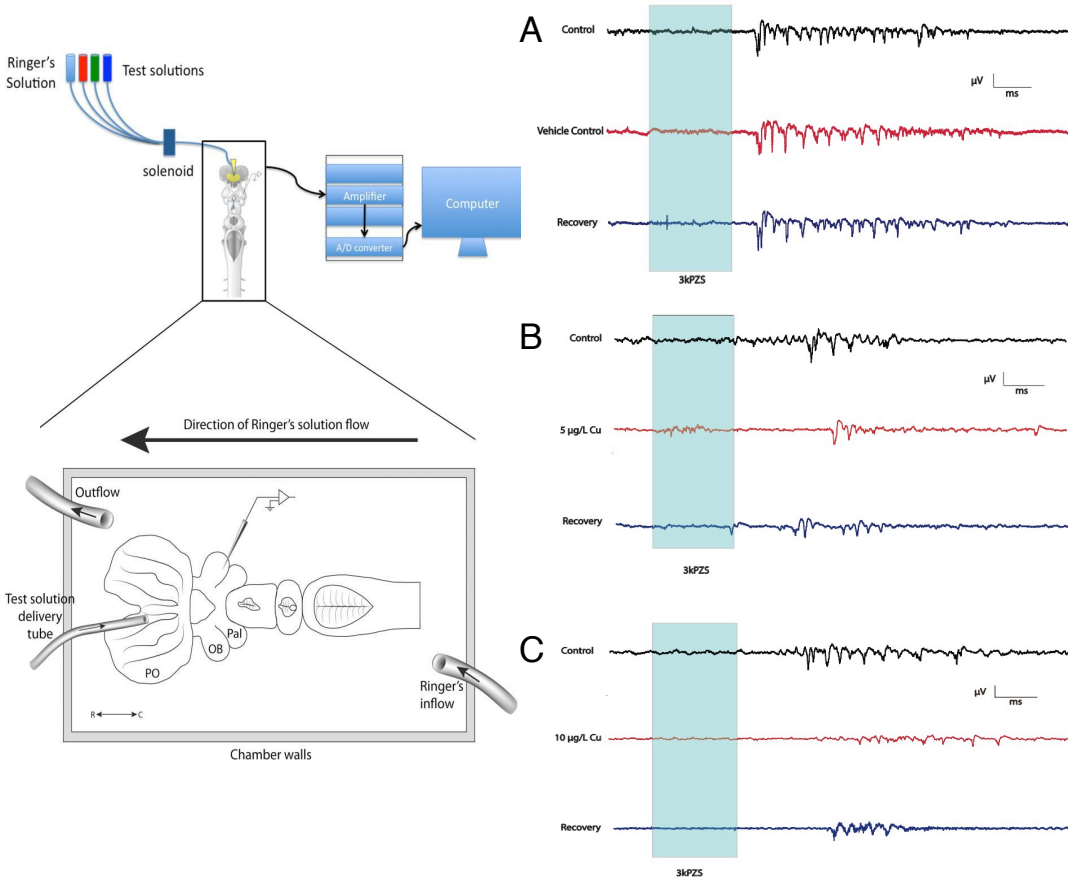


Figure 2.4. The effect of dissolved copper chloride applied to the sea lamprey olfactory olfactory epithelium. Mean (*n*=5) relative LFP response were recorded from the olfactory bulb to 10^{-8} M 3KPZS delivered to the olfactory epithelium for the precopper, copper and postcopper exposure periods of concentrations 0, 5 and 10 µg copper (Cu)-L. Relative responses were determined by dividing each response value for a given fish by the respective premetal exposure response average. Plotted means were determined by averaging the response to 10^{-8} M 3KPZS of all five fish for a given time point. Repeated measures ANOVA was conducted for each Cu concentration and asterisks indicate significant differences from respective premetal exposures ($p \leq 0.05$). Solid bar indicates copper exposure period.

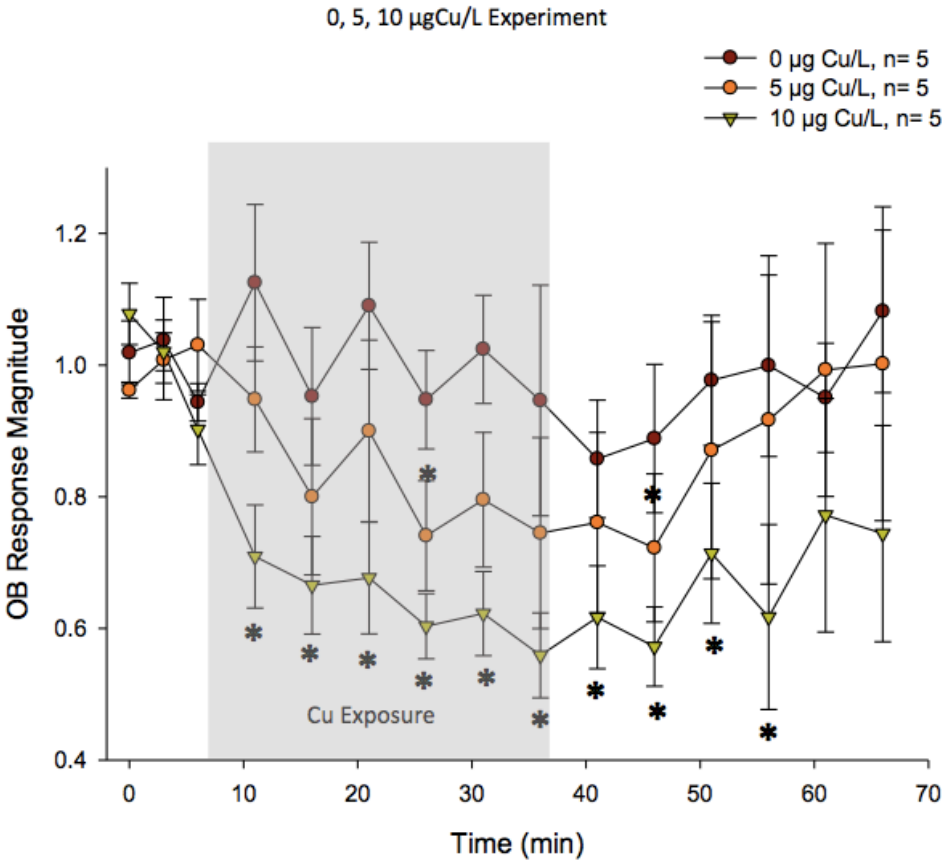


Figure 2.5. Cresyl violet staining of lamellae in the peripheral olfactory organ of sea lampreys exposed to dissolved copper chloride for 24 h.
(**A**) Negative control. Staining of the lamellae in the peripheral olfactory organ shows uniform and intact structure of the olfactory epithelium.
(**B**) 5 µg Cu-L. The tips of the olfactory lamellae (indicated by asterisks) appear pointed and are more darkly stained than the surrounding epithelium.
(**C**) 10 µg Cu-L. The arrows point to debris in the lumen of the nasal cavity and adjacent to the olfactory epithelium. Lamellae appear shriveled, and the epithelial thickness is irregular
(**D**) 30 µg Cu-L. The surface of the olfactory lamellae is irregular. Arrows point to debris in the lumen of the nasal cavity, adjacent to the olfactory epithelium.
Bar shown in (**B**) indicates 200 µm.

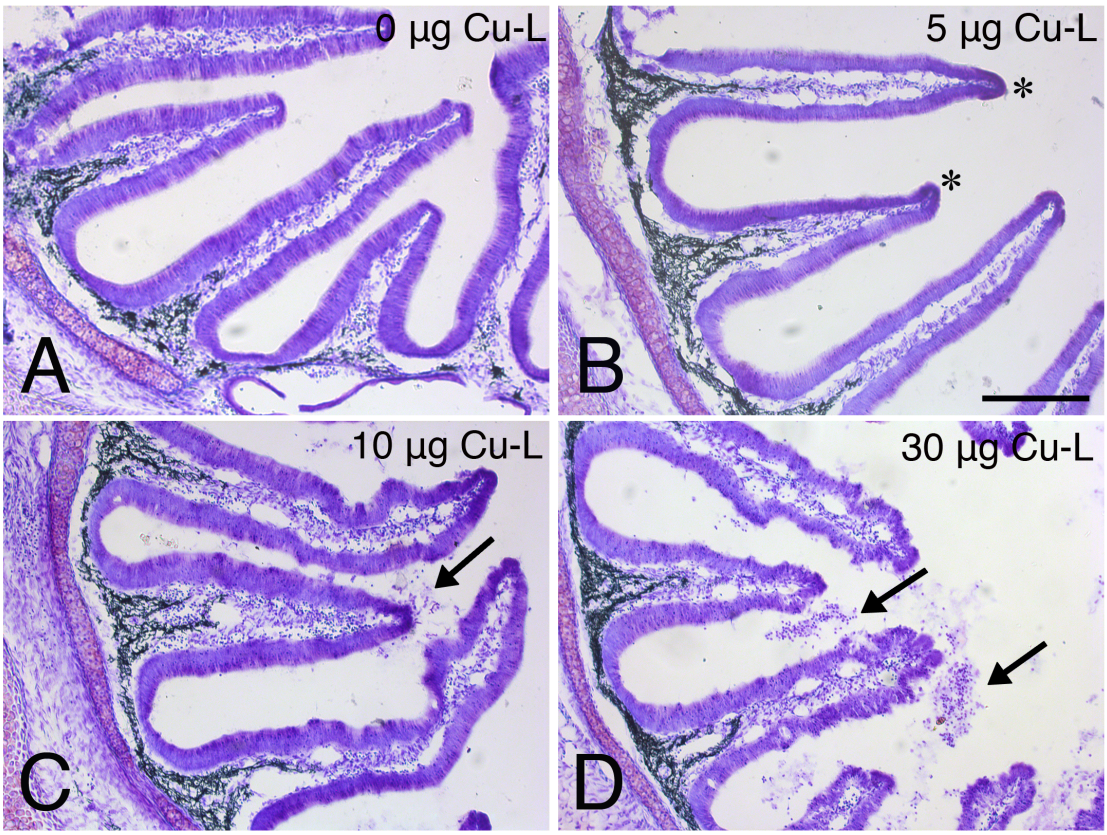
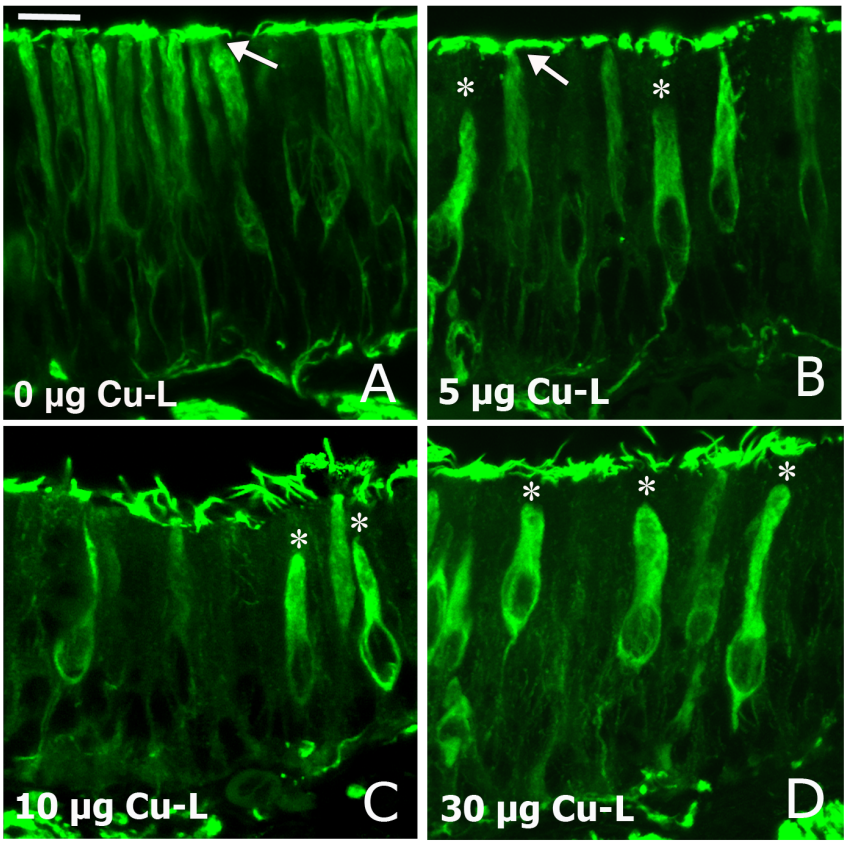


Figure 2.6. Acetylated α- tubulin immunoreactivity in the olfactory epithelium after sea lamprey were exposed to copper chloride for 24 hours. Confocal laser scanning microscope Z stacks are projections of 11 sections per image.
(A) Negative control. Acetylated tubulin immunoreactivity surrounds the cell bodies of olfactory sensory neurons and is localized in the dendrites, including the apical regions of the dendrites (arrows). Intense acetylated tubulin labeling is seen in the mucociliary complex on the surface of the olfactory epithelium.
(**B**) 5 µg Cu-L. Acetylated tubulin labeling is dense in the mucociliary complex. Arrows point to the immune-labeled apical dendritic regions of the olfactory sensory neurons. Labeling is seen in the dendrites, cytoplasm around the cell body, and in the axons. In some olfactory sensory neurons, dendritic acetylated tubulin immunoreactivity does not reach the surface of the olfactory epithelium (asterisks).
(**C**) 10 µg Cu-L. Prominent cilia extend from the apical surface. While some acetylated tubulin-immunoreactive olfactory sensory neuron dendrites reach the apical surface of the olfactory epithelium, others do not (asterisks).
(**D**) 30 µg Cu-L. Cilia are prominent on the surface of the olfactory epithelium. The acetylated tubulin immunoreactivity does not reach the apical surface in most olfactory sensory neurons. Overall, the dendrites of the olfactory sensory neurons appear swollen.
Bar in **(D)** indicated 10 µm.



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Chapter 3: Transcriptomic effects of copper on molecular activity in the olfactory system of the sea lamprey

3.1 Introduction

Olfaction in fish is necessary for reproduction, schooling, homing, pheromone detection, as well as predator avoidance (Tierney et al., 2009). Odours within the aquatic environment that facilitate these behaviour in fish are hypothesized to mediate chemical communication (McIntyre, 2012); however, chemical communication based on olfaction can be disrupted by a variety of environmental pollutants, leading to the suppression of these behaviours and a loss of fitness (Baatrup, 1991; Atchison et al., 1987; Sandhal et al., 2007). The majority of studies have focused on the effect of metals on behaviors (Atchison et al., 1987; Scott and Sloman 2004, McIntyre 2012). However, very few of them explore the underlying molecular mechanisms of the effects of exposure. New molecular genetic technologies such as of next-generation sequencing (NGS) are increasingly applied to issues in human health, yet few studies have employed these methods to study ecotoxicogenomics in fish (Mehinto et al., 2012). With the development of NGS whole- genome gene transcription analyses, environmental stressor effects can be studied on a molecular level.

One contaminant receiving increasing attention in the aquatic environment literature is copper, which is highly toxic to aquatic organisms that come in contact with it through water (Vieira et al., 2009; Grosell et al., 2003). Copper is known to be an active olfactory toxicant at environmentally relevant concentrations, that is, from 2.0 to 64 μg Cu/L, levels reported in urban waterways (Sandhal et al., 2007; Soller et al., 2005). Common sources of copper include disc brake pad dust from vehicles, as well as a variety of other anthropogenic sources, such as roof and downspout corrosion (He et al., 2001). During rain and snow melt events, dissolved copper is washed off roadways and urban

landscapes, and enter drainage systems leading to aquatic habitat contamination (Boulanger, 2003). Copper has been shown to disrupt broad ranges of fish behaviour and physiology; however, copper is known to specifically target the olfactory system of fish (McIntyre et al, 2012; Baldwin et al., 2003). Long lasting damage of olfactory epithelial structures result following copper exposure, which is associated with inhibition of olfactory responses to odours (Wang et al., 2013; Dew et al, 2012). Recent studies on zebrafish (*Danio rerio)* have shown that copper contamination leads to downregulation of genes within the olfactory signal transduction pathway (Tilton et al., 2008). Additionally, a study on Chinook salmon (*Oncorhynchus tshawytscha)* found olfactory receptors undergo necrosis following copper exposure (Hansen et al., 1999), while brown trout (*Salmo trutta)* exposed to copper exhibit rapid apoptosis of nearly half the olfactory neurons (Moran et al., 1992). Consequently, the olfactory system is especially susceptible to copper toxicity, and thus copper has the potential to impact olfactory-related fish behaviors and ultimately fitness.

The sea lamprey (*Petromyzon marinus)* is a native fish species to the northern Atlantic Ocean and Mediterranean Sea, where today they are a threatened fish species, with conservation efforts implemented to address their decline in some populations (Pereira et al., 2010). Since their invasion into the Great Lakes, the sea lamprey has caused significant damage to native fish stocks, leading to population declines and loss of economic opportunities for the commercial and recreational fisheries (Lawrie, 2011). Lamprey rely on pheromones to locate spawning streams, an olfactory based behavior (Vrieze et al., 2011), and is the basis for lamprey control efforts in the Great Lakes. Proposed abatement strategies in the Great Lakes include a male-sex pheromone odor

application into streams to lure ovulating female into traps or streams ill suited for offspring survival (Johnson et al., 2008). Copper exposure may affect the olfactory system (Tierney et al., 2009) and thus migration and homing, reducing the success of current abatement strategies that rely on olfaction. Due to the recent sequencing of the lamprey genome (Smith et al., 2013), transcriptomic approaches characterizing the effect of copper on sea lamprey olfaction offer a powerful model for predicting the effects of copper exposure on lamprey behaviour. It is important to use new molecular genetic methods to determine the mechanism of contaminant effects on olfaction for management of this invasive species, but also to augment our fundamental understanding of the olfactory system in this primitive vertebrate.

Here, we investigate the mechanisms of olfactory sensory loss resulting from copper exposure in sea lamprey, to aid in conservation where they are native, as well as containment where invasive. Transcriptomic RNA sequencing ("RNA seq") analysis of copper-induced olfactory impairment in the olfactory organ was implemented on sea lamprey exposed to environmentally relevant levels of copper. This approach provides a detailed overview of the mechanisms of biological response to copper in the olfactory system. It is hypothesized that copper will cause two types of response, involved in adaptive repair or compensation, and non-adaptive damage leading to downregulation of genes involved in olfactory signaling, coupled with up-regulation of apoptotic processes within olfactory sensory neurons. The current study uses a novel approach to study the potential effects of copper toxicity at a molecular level in the olfactory system, providing insight into the sea lamprey transcriptomic response to a common aquatic environmental contaminant.

3.2 Materials and Methods

3.2.1 Animal care and maintenance

Transformer sea lamprey were obtained from United States Geological Survey Hammond Bay Biological Station in Millersburg, Michigan. Animals were transferred to the University of Windsor, and held in the Central Animal Care Facility, according to UWindsor Animal Care Guidelines (AUPP#14-05). Animals were maintained in aquaria with re-circulating filtration and water quality was recorded daily.

Copper exposures

Groups of 4 transformer sea lamprey were exposed for 24 hours to 3 concentrations of copper ($CuCl_2$) and one control group were handled identically; however, no copper was added. Copper chloride was weighed out based on dilution into 10L tanks, at concentrations of 5 µg/L, 10 µg/L, and 30 µg/L. Water samples were taken after the exposure trial for later copper concentration determination to confirm dose. Exposure trials were conducted in 10 L tanks with recirculated water, while temperature was maintained at 9 °C.

3.2.2 Collection of olfactory tissue and RNA isolation

Immediately following the 24-hour copper exposure, each fish was deeply anesthetized with 150 mg/L MS-222, and decapitated. Heads were placed under a dissecting microscope, where the nasal cavity was cut to expose the olfactory rosettes, olfactory bulb, as well as portions of the brain. Tissue containing the olfactory rosettes, olfactory bulb, as well as portions of the brain were removed and immediately placed in RNAlater™ and incubated at 4°C for 24 hours, after which they were held at -80°C until total RNA extraction.

Tissue samples were thawed and mechanically homogenized with glass beads in 0.75 mL Trizol (Ambion) and total RNA extraction was carried out following protocols established by Chomczynski & Sacchi (1987). Total RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). RNA samples were also tested for contamination using the absorbance ratio at A260/A280.

3.2.3 RNA Sequencing quantification

For each of the 16 olfactory RNA samples, 15 µg of total RNA was aliquoted for library preparation. Total RNA samples were shipped on dry ice to BGI Americas (Cambridge, MA, USA) where RNA sequencing libraries were prepared and sequenced using 100 bp pair-end sequencing on the Illumina-HiSeq 2000 platform.

Sequences were assembled using a reference-guided transcriptome assembly with the sea lamprey genome (Smith et al. 2013). Sequences from all samples were pooled and mapped onto the lamprey genome using Tophat (Trapnell et al. 2009). Putative gene transcripts were assembled from the mapped sequences using the reference-guided approach implemented in Trinity v3.0.3 (Grabherr et al. 2011).

3.2.4 Whole transcriptome analysis

Gene transcription patterns in the lamprey olfactory system were investigated first on a whole transcriptome basis, investigating broad processes that may be affected following copper challenges. To compare gene transcription at various copper concentrations, individual lamprey samples were aligned to the assembled transcriptome using Bowtie2 (Langmead and Salzberg 2012), thus producing an estimate of transcript abundance for each “gene” for each sample using RSEM (Li and Dewey, 2011).

To characterize patterns of gene transcription response for the three copper challenge trials across the whole transcriptome, differentially expressed gene transcripts (relative to control samples) were identified using generalized linear models (GLMs) with likelihood ratio rests of significance in the edgeR v3.8.6 package (Robinson et al. 2010) in R v3.1.3 (R Core Team 2015). A false discovery rate of 0.05 was used to correct p-values for multiple comparisons (Benjamini and Hochberg 1997). GLMs implemented were negative binomial approximations of RNAseq count data, which first normalize trimmed mean of M-values, accounting for individual variation. Using an empirical Bayes approach (McCarthy et al. 2012), the negative binomial models are then fit to normalized count data for individuals gene by gene, and significance tested using a likelihood ratio test. We tested for significant responses between the control and 5 µg/L Cu treatment animals, between the control and 10 µg/L Cu exposure, and finally between the control and 30 µg/L Cu exposure. Gene ontology (GO; Ashburner et al., 2000) terms describing gene function were assigned to each unique differentially expressed gene based on annotation in Blast2GO v3.1 software (Conesa et al., 2005).

3.2.5 Dose response patterns analysis

Our copper exposure trials were designed to detect dose-response for genes affected by copper exposure. To identify genes showing a consistent dose response to the challenge of increasing copper concentrations, we used a Venn diagram analysis to identify genes that showed consistent and significant transcription response to two or more copper doses. It was hypothesized that few genes would show a strong response to 5 µg/L, where more would in the 10 µg/L and the most within the 30 µg/L exposure. The greatest overlap of response among exposure concentrations were hypothesized between

the medium and high doses of copper. Additionally, single dose response genes should be most common at the 30 µg/L, and the least in the lower dose of 5 µg/L. To further elucidate copper toxicity on the olfactory transcriptome, genes differentially expressed in at least two treatment groups were investigated in greater detail. To visualize the patterns of dose response, we plotted relative transcription against dose for all 3 categories of consistently regulated genes using R v3.1.3 (R Core Team 2015) (Figure 3.1- 3.2).

3.2.6 Candidate gene response

Although whole-transcriptome response to copper exposure in lamprey is important for our understanding of the process, a candidate gene approach also was included to test for dose-response patterns in genes known or suspected to have a role in olfaction and that may be sensitive to copper. Previous published studies in fish have shown that copper causes impairment to olfactory sensory responses (Green, 2012; Hansen et al., 1999), as well as a reduction in the number of olfactory receptors in exposed fish (Kolmakov et al., 2009; Julliard et al., 1996). Twenty-four genes of interest were thus selected based on previous studies on chemosensory receptors that interact with odorant molecules in olfactory epithelia in fish (Libants et al., 2009; see Appendix B). Specific olfactory sensory receptor genes in the sea lamprey (Libants et al., 2009) were analyzed for an expected down-regulation with increasing copper concentrations. We included G-protein coupled receptors (GPCRs), expressed in olfactory sensory neurons, as they are hypothesized to be a target for copper toxicity and show a downregulation of expression in a dose dependent manner (Sandhal et al., 2006). The G-protein coupled receptors included in this study are homologous to chemosensory receptors including: olfactory receptors (ORs; N=12), trace amine-associated receptors (TAARs; N=4), as

well as vomeronasal pheromone receptors (V1Rs; N=7). Individual gene sequences were selected using sequence homology through BLAST to identify candidate genes. All 24 transcripts identified using BLAST had a minimum e-value of $1x10^{-5}$, indicating significant matches between candidate gene and transcripts in our transcriptomic data. We tested for copper effects on the transcription level for these candidate specific genes using GLMs to examine overall dose effects across all four trials and subsequently tested for specific differences between the control and each copper concentration.

Lastly, anonymous function candidate genes were identified based on the magnitude of transcriptional response to copper exposure (fold change) within our transcriptome data. Transcriptional responses to copper exposure were ranked in order of fold change for up- and down-regulation at each of the three exposures, and then compared to the other two concentrations to analyze potential dose-response patterns. The five highest responding genes (of either up- or down-regulation) for each individual copper challenge were selected for analysis as genes experiencing the greatest change of expression during copper exposure, and hence are candidate genes for physiological response to copper toxicity in the olfactory regions of the lamprey. GO functional annotations were then assigned to the selected candidate genes to identify biological processes associated with each gene.

3.3 Results

All of the RNA samples were of high quality (RIN great than: 7.0, 28S: 18S rRNA ratio greater than: 1.0) with little contamination with DNA (A260:A280>1.9). A range of 15.9- 24.7-million paired-end reads were produced per sample. Following assembly using Trinity v3.0.3 software, a total of 286 011 transcripts were reconstructed.

The number of final assembled transcripts analyzed based on a minimum level of expression of 1 count per million in one treatment group (over 4 individuals) was 29 167 putative genes (transcripts).

3.3.1 Differentially expressed transcripts involved in copper toxicity

Transcripts responsive to copper exposure were identified as statistically significantly (FDR≤0.05) differentially expressed based on read number relative to the control samples (Figure 3.3). A total of 48 differentially expressed genes were identified at the lowest copper concentration of 5 µg/L. At 10 µg/L copper exposure, 179 genes were differentially expressed. Lastly, in the highest dose of Cu exposure (30 µg/L), 279 differentially expressed genes were identified. As predicted, more genes showed a significant transcriptional response as copper exposure dose increased (Figure 3.4). Of those total 536 genes, 266 genes were successfully blasted to putative known function, while the remaining 270 were unannotated (see Appendices 1, 2 &3).

3.3.2 Dose response patterns in differentially expressed transcripts

One gene showed differential expression in all treatment groups (Figure 3.1 A), reflecting decreased (down-regulated) response processes associated with the protein proteolysis with peptidase activity as identified by Blast2GO mapping. In addition, one gene showed significant increase in expression in both the 5 µg/L and 30 µg/L dose treatments (Figure 3.1 B) but did not have a known annotated function.

The highest number of genes that showed a significant transcriptional response in at least two dose treatments was between the 10 µg/L and 30 µg/L dose, where 84 common differentially expressed genes were identified (Figure 3.2). Among the 84 common differentially expressed genes, 10 exhibited down-regulation (negative fold

change value) with copper exposure and of these 10 genes, all were unannotated (no known function). The remaining 74 genes exhibited up-regulation (positive fold change value) in response to copper exposure, and of those genes, 37 were unannotated. All genes showed consistent response of either upregulation or downregulation for the 10 µg/L and 30 µg/L doses of copper. Of the 37 annotated transcripts, a few genes of particular significance emerged, such as: *cAMP-responsive element modulator, cytochrome c, Rho-related GTP-binding protein,* and *metalloproteinase*.

3.3.3 Candidate gene comparison

Sequences for candidate genes identified in published literature were obtained from the public database for the sea lamprey genome, and blasted to identify them in our whole transcriptome data. Of the 24 candidate genes, all were successfully identified within our sea lamprey transcriptome data allowing us to test for the effect of copper on their transcription levels. Our overall GLMs were (used to test whether there was a significant effect between overall effect of dose compared to control) showed no significant effect for any of the candidate genes. Response at individual dose concentrations were compared to control to explore variation between concentrations and potential threshold effects. Five genes were significantly down-regulated in the 10 µg/L dose. Of these 5 genes, 3 represented olfactory receptors (OR 330, OR 23O, OR 424), 1 trace-amine receptor (TAAR 345), and 1 pheromone odor receptors (V1R 320), all showing significant decrease in transcription at the highest copper exposure (Figure 3.5).

Lastly, the five genes that experienced the greatest fold change (up- or down-regulated) in transcription at each copper concentration were analyzed. These five genes represent the greatest alteration in expression following copper exposure. Expression of

these genes for all copper exposures was then compared for dose-response patterns (Figure 3.6- 3.8). Thirteen of the 15 selected genes did not have annotation information, while one gene in the 10 and 30 µg/L dose successfully annotated as *prostaglandin g h synthase 2*, displaying functions associated with response to oxidative stress, oxidative-reduction process and peroxidase activation. Patterns within individual genes in both the 10 µg/L and 30 µg/L doses of copper showed generally consistent up- or down-regulation of gene expression (Figure 3.7- 3.8). However, all genes showing either up- or down-regulation in the Cu-L dose of copper, changed direction of transcription response with increasing copper concentrations (Figure 3.6).

3.4 Discussion

Transcriptome-wide RNA seq analyses of sea lamprey olfactory sensory loss resulting from copper exposure were investigated to aid in abatement where the lamprey are invaders, and conservation in their native range. Transcriptomic analysis showed a general trend of increasing copper exposure driving dose dependent down-regulation in transcription of genes in the olfactory system, as well as downregulation in some candidate genes of chemosensory receptor expression. This study supports our predictions for the effect of copper exposure on the olfactory system transcriptome, as well as provides insight into other potentially novel mechanisms involved in copper toxicity through whole-transcriptome analysis.

Differentially expressed transcription following copper exposure was examined first through transcriptome-wide analyses. Of the genes that were identified in our whole-transcriptome analysis, the greatest biological functional groups affected were related to; animal organ development, response to external stimulus, intracellular signal

transduction, and cell proliferation. It has been previously shown in fish that copper exposure disrupts olfactory responses by blocking voltage-gated ion channels and G-protein coupled receptors, which may explain the differential expression of intracellular signal transduction processes in the current data (Chapter 2; Green et al., 2010; Mazon et al., 2002; Sandhal et al., 2006). Additionally, previous studies have identified rapid proliferative processes of olfactory sensory neurons following copper exposure, suggesting a recovery mechanism in the olfactory system following exposure (Julliard et al, 1996; Kolmakov et al, 2009).

Using transcriptome-wide responses, we identified specific genes showing dramatic responses to copper exposure. One gene in particular showed consistent downregulation in response to all copper exposures. The transcript was characterized as producing a protein involved in proteolysis and peptidase activity. This decrease in production and thus reduced proteolysis is associated with the accumulation of oxidized proteins, oxidative damage, and impairment of proteasome functionality, ultimately leading to cellular dysfunction, such as those found in previous studies on sunflower (*Helianthus annuus L)* leaves (Pena et al., 2007). Additionally, a decrease in proteasome activity is associated with the accumulation of carbonylated proteins, promoted by reactive oxygen species in mammalian cells (Grune et al., 2003). A previous study on cadmium exposure in carp (*Cyprinus carpio)* also reported a decrease in the activity of proteases in the gill at lethal concentration exposures (Smet and Blust, 2001). In our study at the highest copper dose, genes of interest include those involved in the G-protein olfactory sensory neuron messaging cascades, such as *cAMP-responsive element modulator,* and *Rho-related GTP-binding protein,* showing up-regulation of response.

This suggests the disruption of signaling, and ion and voltage gated channels within the olfactory system (Hansen et al., 1999; Green et al., 2010; Wang et al., 2013). Additionally, transcripts associated with downregulation of *cytochrome c* suggest processes involved in oxidative damage, often linked to copper overload, which initiates cell death signaling (Gaetke and Chow, 2003; Craig et al., 2007; Hosseini et al., 2014), and *metalloproteinase*, a metal binding protein, suggests a detoxifying transcriptomic response to increased copper toxicity through metallothioneins, of which copper has the highest affinity (Craig et al., 2007; Dameron and Harrison, 1998; Daniel et al., 2004; van Heerden et al., 2004). The olfactory processes that we show are impacted by copper exposure in the sea lamprey may also be affected in other organisms during copper exposure. Such a community level loss of chemosensory communication among fishes could contribute to ecosystem-wide decrease in response to chemical cues and pheromones in the aquatic environment.

We know copper causes impairments to olfactory epithelial tissues reflected in structural irregularity of the lamella, as well as depletion in the number of olfactory sensory neuronal dendrites (Hansen 1999; Julliard et al., 1996; Chapter 2). Specifically, copper causes inhibition in olfactory bulb responses to a male sex pheromone odor (3kPZS) in the sea lamprey (Chapter 2), similar to previous studies on fish in response to odours during copper exposure (Green et al., 2012; Sandahl et al., 2007). Five of the 24-selected candidate genes exhibited a dose dependent down-regulation response to copper exposure, with the response becoming significant following the highest dose of copper. This supports previous findings that identified copper induced olfactory injury through the inhibition of olfactory sensory G-protein coupled receptors, associated with olfactory

sensory neurons (Tilton et al., 2008). It is also important to note that the exposure concentrations used in previous studies on ranged as high as 50-1000 µg/L (Hansen et al., 1999; Johnson et al., 2007; et al., Wang et al., 2013), while the current study utilized concentrations that were relevant to urban environment waterways (5- 30 µg/L). Our use of environmentally relevant copper exposure concentrations may thus account for the lack of transcriptional responses for some of the candidate genes for which we expected a significant response.

The single gene identified in BLAST2Go in the highest responding candidate gene analysis (an upregulated response to copper exposure) was identified as *prostaglandin g h synthase 2,* associated with oxidative stress and peroxidase activation. Previous studies have identified necrosis of olfactory receptors to be mediated by lipid peroxidation by oxidizing plasma membranes and causing them to rupture (Hansen et al., 1999; Matta et al., 1999; Hosseini et al., 2014). In addition, oxidative stress has been identified as playing a role in copper toxicity in fish (Vieira et al., 2009). The majority of the highest responding gene transcripts were unannotated, resulting in unknown biological processes. However, at the lowest dose of copper, genes initially showing one direction of regulation (either up- or down) changed direction with increasing doses of copper in each of the five genes. This suggests that some genes may display different types of responses resulting from copper exposure. One type of response may result due to simple damage, expected to down-regulate and inhibit function of processes in response to cellular damage. An alternative response to copper exposure may be an adaptive response (up- or down-regulated), driving either repair or compensatory response for damaged pathways. Thus, although the majority of these processes have

unknown function, all show dramatic response to copper. It is possible that these highest responding genes at each copper exposure are associated with adaptive repair mechanisms in the olfactory system, showing upregulated dose responses with increasing copper exposure, but have yet to be characterized at a molecular level.

3.5 Conclusion

In summary, our findings suggest that acute exposures to copper does have rapid and profound impacts of the transcriptome, that are likely directly connected with olfactory sensory loss in the sea lamprey. Similar effects from copper exposure have been linked to impairments in odor detection and chemical communication, ultimately leading to suppression of processes necessary for survival and successful reproduction in a variety of other fish species (Baldwin et al., 2003; McIntyre et al., 2012; Saucier et al., 1991). Our molecular examination of olfactory responses following copper exposure indicates impairment of processes involved in G-protein coupled receptor olfactory receptors, as well as up and down regulation of processes previously identified as targets of olfactory injury. In the context of pheromone abatement strategies applied to the Great Lakes, it is important to measure levels of copper present in waterways, prior to implementation of such programs. Additionally, it would be useful for conservation purposes to measure and perhaps regulate potential heavy metal pollution entering waterways where they are threatened. Our study is important for both conservation and management issues around contaminate exposure in aquatic ecosystems.

Figures

Figure 3.1. **(A)** Dose response curves for the single differentially expressed gene (associated with proteolysis and peptidase activity) common for all copper treatments (Cu-L (5 µg/L)), Cu-M (10 µg/L) and Cu-H (30 µg/L)). **(B)** Dose response curve for the single differentially expressed gene common for copper treatments (Cu-L (5 µg/L)), and Cu-H (30 µg/L)). Log2expression is based on read count data. Error bars depict 95% confidence interval and the asterisk denotes a significant difference in expression between control and 30 µg/L.

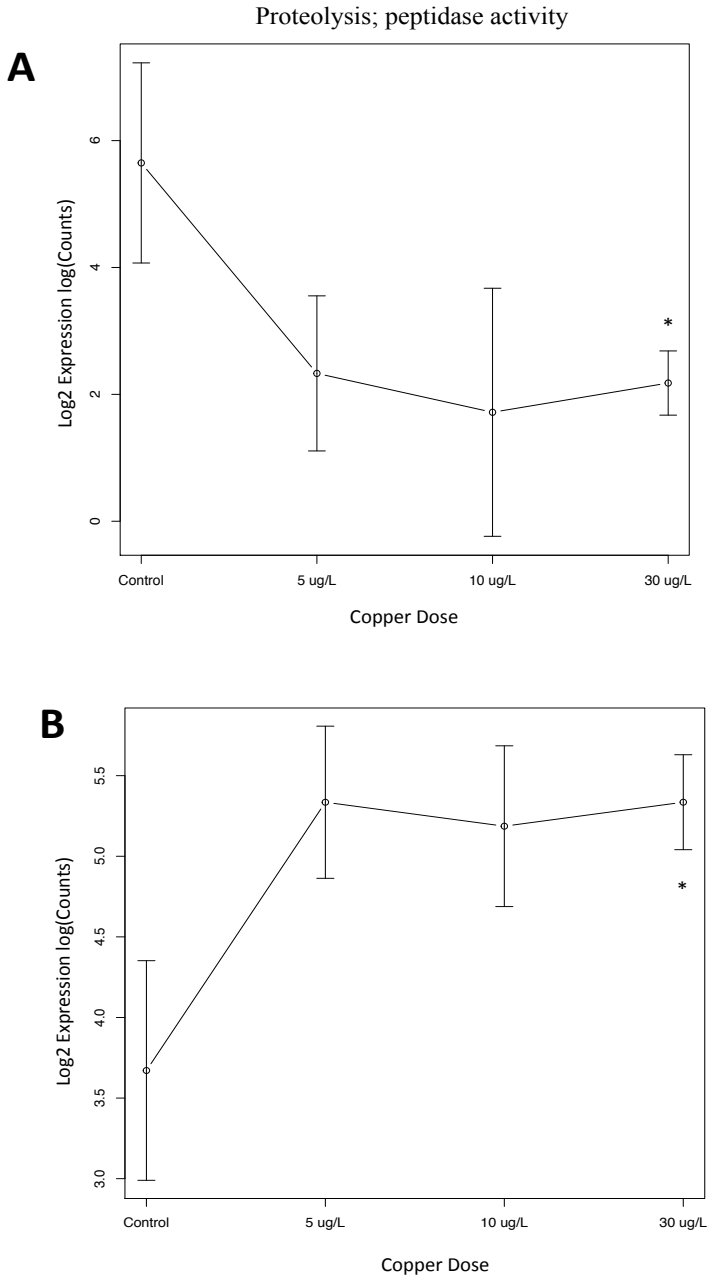


Figure 3.2. Dose response curve for the single differentially expressed genes common for copper treatments (Cu-M (10 µg/L) and Cu-H (30 µg/L)). Log2expression is based on read count data. The asterisk denotes a significant difference in expression between control and 10 µg/L, and control and 30 µg/L.

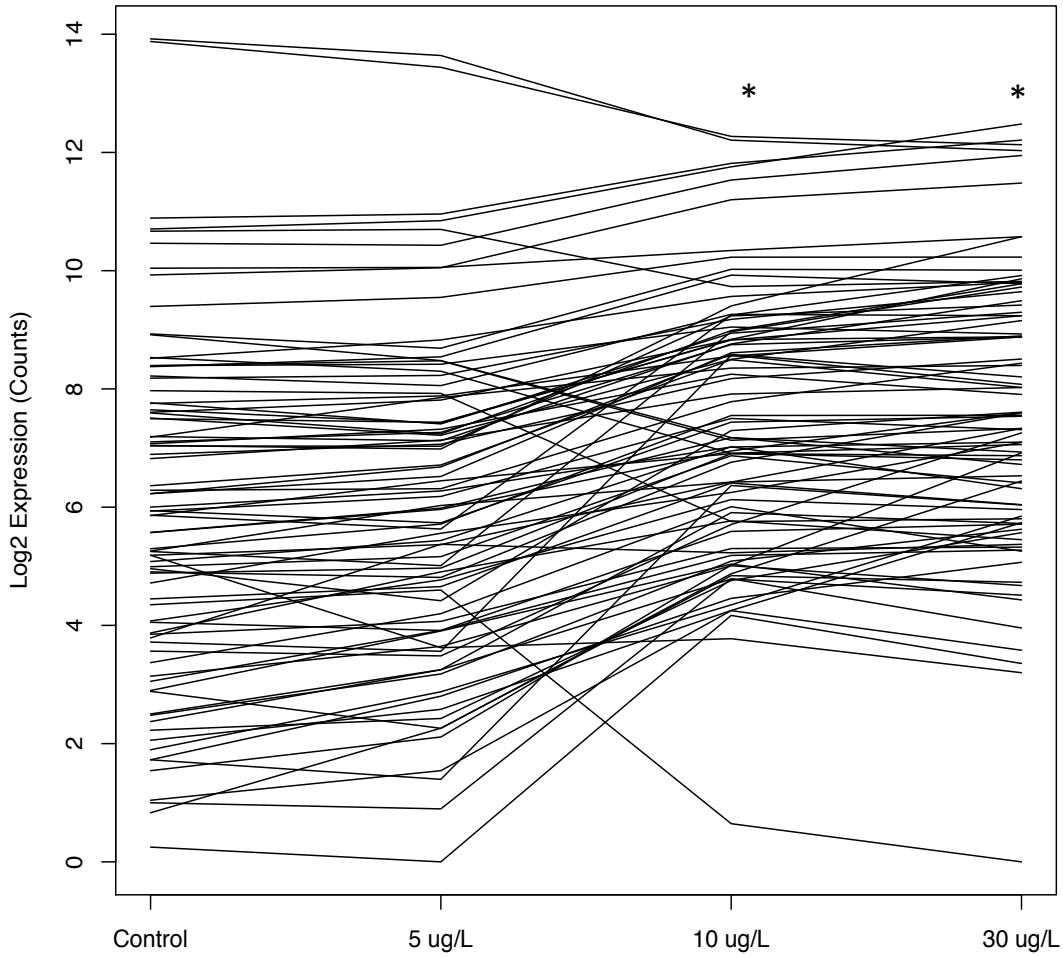


Figure 3.3. Functional assignment of all genes significantly responding to copper exposure (FDR≤0.05) identified using Blast2Go. Gene ontology (GO) groups have similar functions and are graphed according to the number of genes assigned to that functional group. Functional groupings suggest copper affects processes likely related to intracellular signal transduction, response to external stimulus, and organ development. A total of 270 out of 419 genes did not have GO descriptions, and are not represented. Colours repeat from 12 o'clock clockwise in the order listed on the top of image.

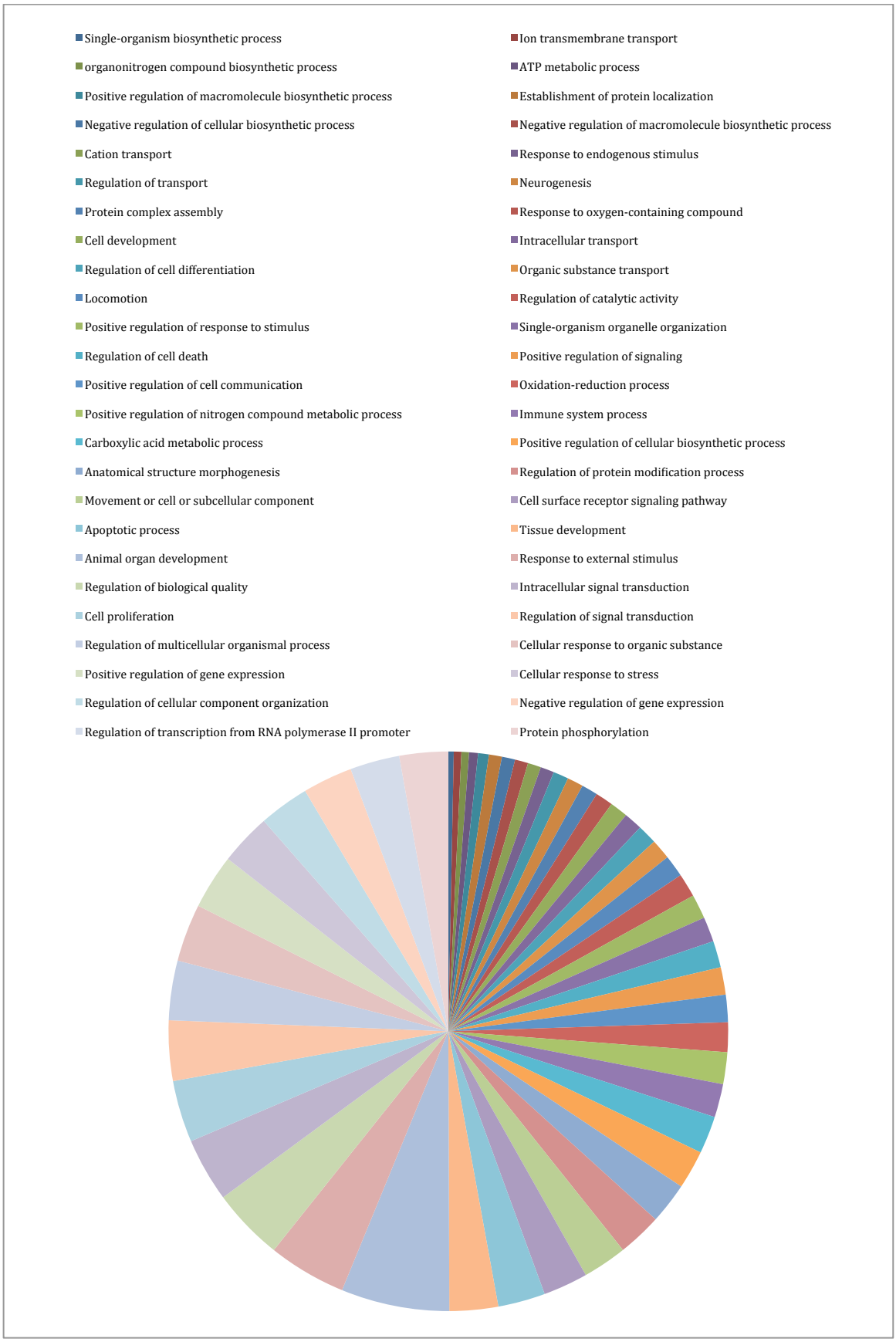

Single-organism biosynthetic process
organonitrogen compound biosynthetic process
Positive regulation of macromolecule biosynthetic process
Negative regulation of cellular biosynthetic process
Cation transport
Regulation of transport
Protein complex assembly
Cell development
Regulation of cell differentiation
Locomotion
Positive regulation of response to stimulus
Regulation of cell death
Positive regulation of cell communication
Positive regulation of nitrogen compound metabolic process
Carboxylic acid metabolic process
Anatomical structure morphogenesis
Movement or cell or subcellular component
Apoptotic process
Animal organ development
Regulation of biological quality
Cell proliferation
Regulation of multicellular organismal process
Positive regulation of gene expression
Regulation of cellular component organization
Regulation of transcription from RNA polymerase II promoter
Ion transmembrane transport
ATP metabolic process
Establishment of protein localization
Negative regulation of macromolecule biosynthetic process
Response to endogenous stimulus
Neurogenesis
Response to oxygen-containing compound
Intracellular transport
Organic substance transport
Regulation of catalytic activity
Single-organism organelle organization
Positive regulation of signaling
Oxidation-reduction process
Immune system process
Positive regulation of cellular biosynthetic process
Regulation of protein modification process
Cell surface receptor signaling pathway
Tissue development
Response to external stimulus
Intracellular signal transduction
Regulation of signal transduction
Cellular response to organic substance
Cellular response to stress
Negative regulation of gene expression
Protein phosphorylation

Figure 3.4. Venn diagram showing the pattern of the differentially transcribed genes in response to copper exposure shared among the 3 copper exposure concentrations. The number of transcribed genes (percent in parentheses) is displayed for all unique and intersecting groups based on different concentrations

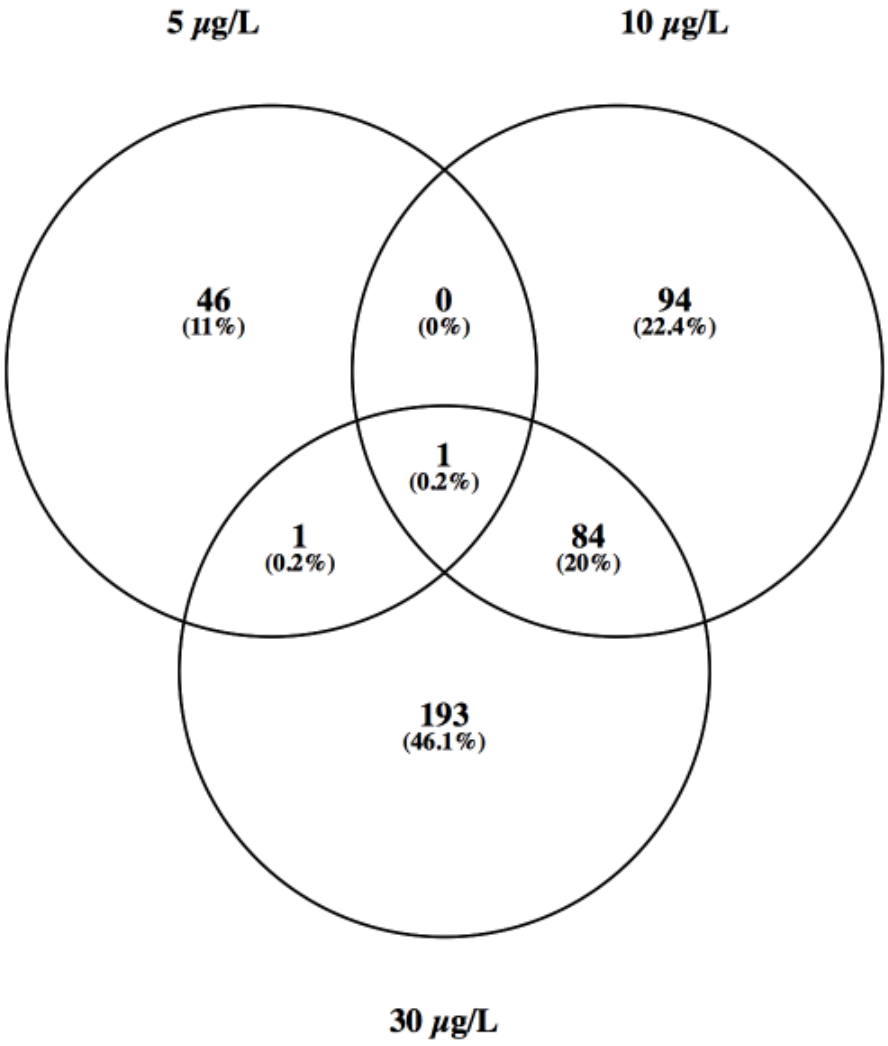


Figure 3.5. Dose response plot for candidate genes that showed a significant change in expression compared to control at the 30 µg/L Cu dose. Olfactory receptors OR 330, OR 230 and OR 424 show a down-regulation of transcription, as does the trace amine-receptor TAAR 345, and vomeronasal receptor V1R 320. The asterisk denotes a significant difference in expression between control and copper exposed for each candidate gene based on FDR. Confidence intervals reflect p-values for individual tests.

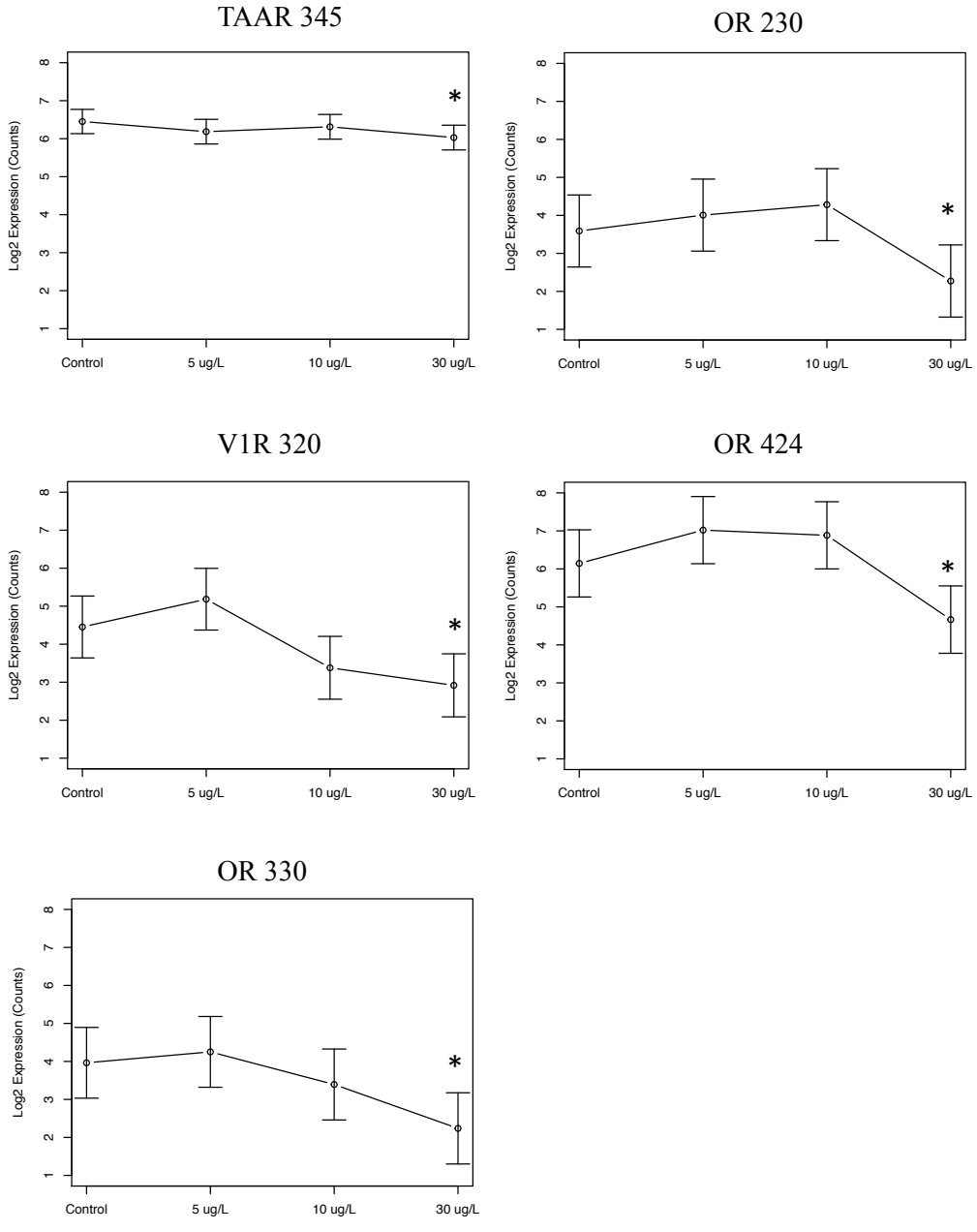


Figure 3.6. Expression of top five genes showing greatest fold change in the Cu-L (5 µg/L). Log2expression is based on read count data. X axis represents dose of copper. Confidence intervals reflect p-values for individual tests.

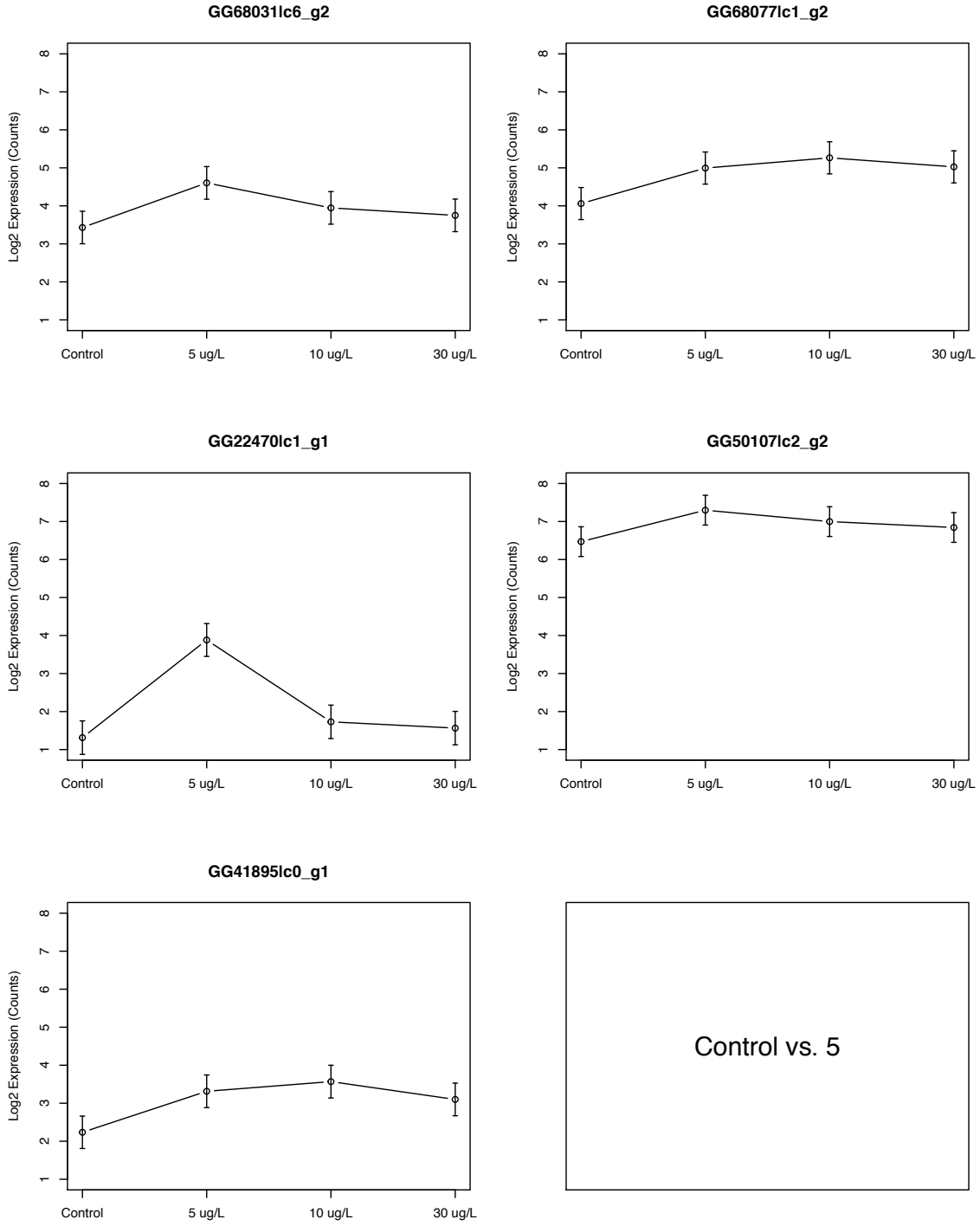


Figure 3.7. Expression of top five genes showing highest fold change in the Cu-L (10 µg/L). Log2expression is based on read count data. X axis represents dose of copper. Confidence intervals reflect p-values for individual tests.

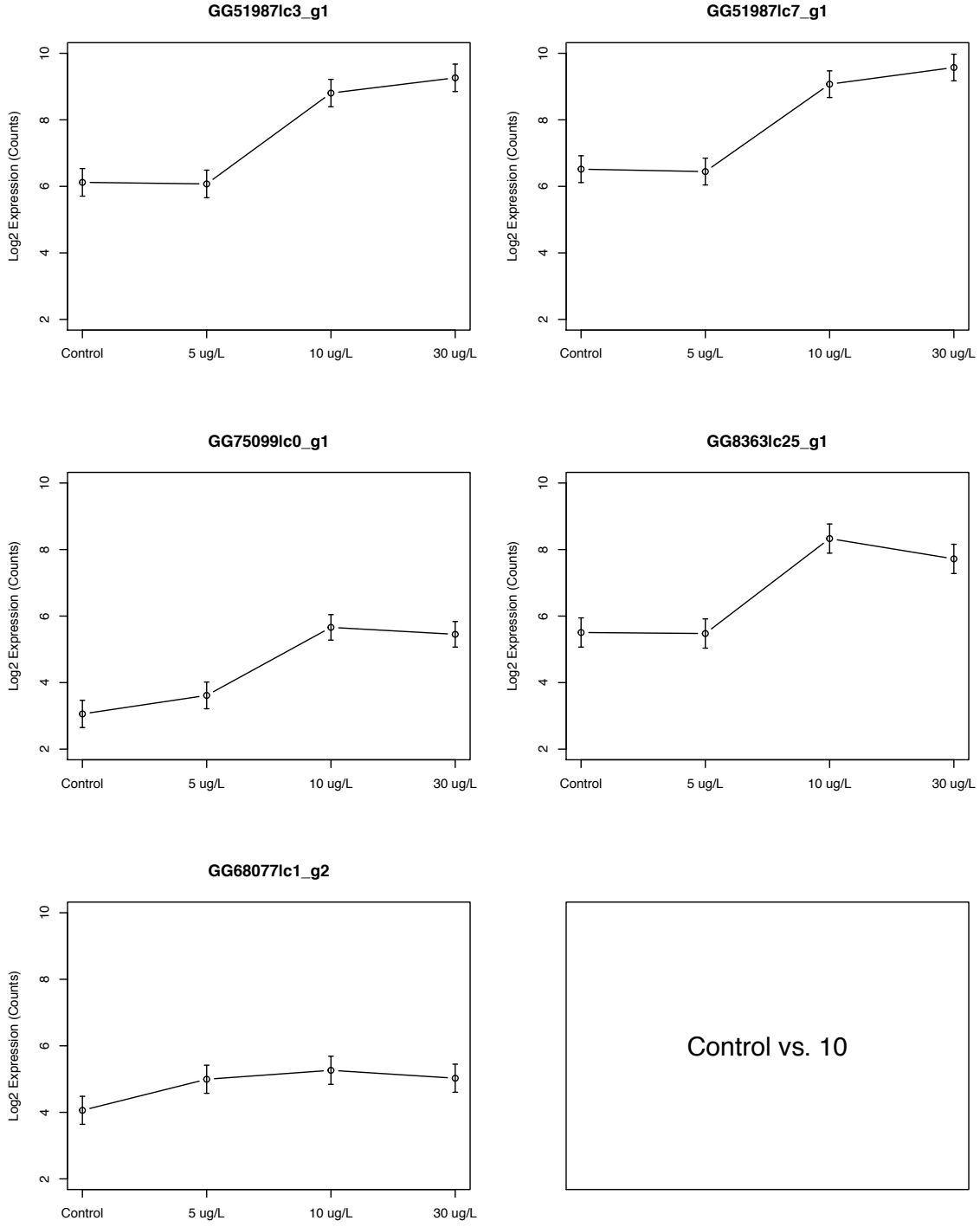
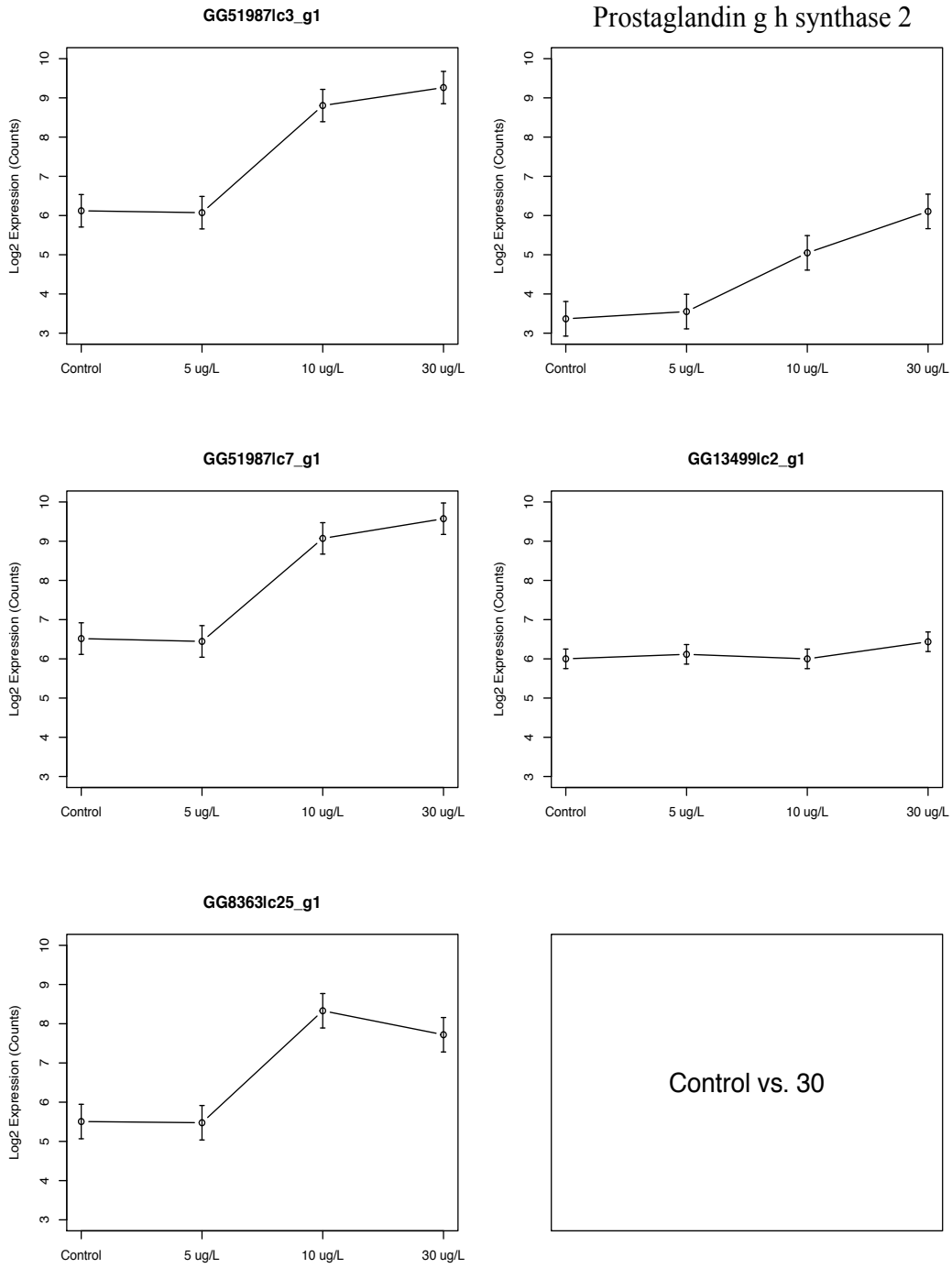


Figure 3.8. Expression of top five genes showing highest fold change in the Cu-L (30 µg/L). Log2expression is based on read count data. X axis represents dose of copper. Confidence intervals reflect p-values for individual tests.



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Appendices

Appendix A

Appendix A.1. Differentially expressed genes in the Cu-L (5 µg/L) copper concentration. Description based on Blast2Go GO database functional grouping. Transcript codes given by Blast2Go database. Italicized gene transcript names indicate genes showing highest fold change.

Transcript	log2Fold Change Cv5	Description
GG68031|c6_g2	2.193	----
GG28255|c73_g1	1.449	----
GG6888|c1_g1	-0.964	----
GG76991|c3_g1	-1.089	Disks large- associated protein partial
GG72502|c8_g1	-0.858	Tubulin beta partial
GG51016|c0_g1	-0.814	----
GG20708|c0_g1	-1.145	----
GG51526|c1_g1	-1.126	----
GG76991|c4_g1	-1.371	----
GG52969|c19_g1	-0.688	----
GG63089|c19_g1	-1.632	----
GG36768|c0_g2	-1.085	----
GG1267|c0_g1	-1.659	----
GG9387|c2_g2	-1.145	----
GG17790|c0_g2	-0.920	----
GG45354|c1_g6	-1.598	----
GG23352|c0_g1	-0.698	----
GG46638|c1_g1	-1.294	RNA-directed DNA polymerase from mobile element jockey
GG47585|c19_g1	-0.727	----
GG36521|c0_g1	-1.519	----
GG22860|c0_g1	-1.515	Proteolysis; peptidase activity
GG43941|c1_g1	-1.477	----
GG2727|c7_g1	-1.140	----
GG71153|c2_g1	-0.812	Serine threonine-protein phosphatase cpped1
GG62741|c149_g1	-0.860	----
GG38478|c3_g1	-0.545	Septin-7-like isoform x2
GG72487|c0_g1	-0.834	
GG79320|c5_g1	-1.048	Transposable element tcb1 transposase
GG22816|c11_g1	-1.062	Retrovirus-related pol polyprotein from transposon
GG63012|c2_g1	-0.927	----

GG8089|c1_g1	-1.088	----
GG6756|c12_g1	-0.959	----
GG76170|c0_g1	-0.816	----
GG50682|c6_g1	-0.849	Synpatosomal-associated protein 25 isoform
GG29315|c1_g1	1.405	GTP binding
GG41215|c0_g1	-1.380	Receptor-type tyrosine-phosphatase
GG39471|c7_g1	-1.092	----
GG10588|c0_g1	-1.325	----
GG24526|c0_g1	-1.269	----
GG26320|c6_g1	-1.065	Potassium channel subfamily k member partial
GG7416|c6_g1	0.998	Prominin-1 isoform
GG57260|c0_g1	-1.322	----
GG73765|c4_g1	-0.971	----
GG29597|c1_g1	-1.186	----
GG80563|c2_g1	-1.250	Pro-partial
GG66331|c2_g1	1.184	----
GG57260|c1_g1	-1.241	----
GG40179|c1_g1	-0.790	----

Appendix A.2. Differentially expressed genes in the Cu-M (10 µg/L) copper concentration. Description based on Blast2Go GO database functional grouping. Italicized gene transcript names indicate genes showing highest fold change.

Transcript	log2Fold Change Cv10	Description
GG37147|c0_g2	-1.831	----
GG51987|c7_g1	2.476	prostaglandin g h synthase 2
GG51987|c3_g1	2.477	----
GG2556|c3_g1	1.677	----
GG71704|c0_g2	1.904	----
GG75099|c0_g1	2.215	----
GG68817|c7_g1	1.748	rho-related gtp-binding protein
GG8363|c25_g1	2.131	----
GG9928|c0_g1	1.426	----
GG37967|c3_g1	1.431	----
GG19578|c2_g1	1.814	----
GG10354|c0_g1	1.507	----
GG56816|c5_g1	-0.683	up-regulated during skeletal muscle growth protein 5
GG47919|c51_g4	-1.375	----
GG5311|c5_g1	0.880	neutral amino acid transporter a

GG52826|c3_g1	1.291	e3 sumo-protein ligase egr2 isoform x2
GG39670|c1_g1	1.771	----
GG75955|c0_g1	-1.836	----
GG40573|c19_g1	1.248	----
GG67201|c0_g1	-1.043	----
GG77353|c2_g1	1.745	----
GG1525|c2_g1	-0.988	----
GG7444|c2_g1	1.060	----
GG13053|c13_g2	1.341	----
GG26520|c22_g1	1.346	----
GG50407|c15_g1	1.519	----
GG73544|c2_g1	-1.684	----
GG19432|c16_g1	1.065	----
GG16688|c0_g1	-0.696	ATP synthase subunit mitochondrial
GG79178|c4_g1	1.274	Nf-kappa-b inhibitor zeta
GG79181|c0_g1	1.476	----
GG75957|c0_g1	-1.263	----
GG10564|c9_g1	-0.499	Alpha-enolase isoform x1
GG52826|c1_g1	1.345	----
GG18550|c3_g2	1.186	----
GG16476|c0_g1	1.625	----
GG13837|c3_g1	1.552	----
GG8363|c21_g1	1.614	Tetraspanin-1 like
GG30616|c4_g1	-1.263	----
GG27935|c0_g1	1.632	Fatty acid 2-hydroxylase
GG69135|c1_g1	1.374	cAMP-responsive element modulator isoform x1
GG33063|c6_g1	0.932	----
GG7447|c7_g1	1.089	Glutamine-fructose-6-phosphate aminotransferase
GG481|c1_g1	1.487	----
GG46866|c0_g2	1.580	----
GG52174|c23_g1	0.926	Thrombospondin-1
GG17196|c2_g1	-1.542	Craniofacial development protein
GG76239|c3_g1	1.483	----
GG40382|c0_g1	1.579	----
GG36173|c6_g1	0.886	Proto-oncogene c-partial
GG31231|c0_g1	1.472	Interleukin-8-like
GG25524|c1_g1	1.449	----
GG70273|c4_g1	0.927	----
GG56859|c2_g1	1.052	----
GG56087|c4_g1	1.502	----
GG75306|c5_g1	0.988	----
GG57115|c1_g1	-0.598	ATP synthase f complex subunit mitochondrial
GG55910|c10_g1	1.039	----

GG68288|c0_g1	1.473	Adapter protein ciks isoform x2
GG6751|c3_g1	1.510	----
GG68712|c1_g1	1.447	----
GG13926|c2_g1	1.492	----
GG8109|c4_g1	-0.974	NADH dehydrogenase
GG35206|c7_g1	0.921	----
GG57427|c4_g1	1.473	----
GG14720|c0_g1	-1.173	----
GG46132|c8_g1	-0.355	----
GG23250|c7_g1	-0.438	----
GG54179|c123_g1	-1.167	Hypothetical protein BVRB_023710
GG5477|c2_g1	1.334	Transmembrane protein 51-like
GG5379|c0_g1	0.895	----
GG47919|c109_g1	-1.372	rRNA promotor binding protein
GG64400|c0_g1	1.232	----
GG69118|c1_g1	-0.577	ATP synthase subunit mitochondrial
GG17008|c6_g1	1.003	Claudin-19 isoform x2
GG66771|c0_g7	-1.176	----
GG25589|c1_g2	1.446	----
GG74670|c5_g1	0.996	Zinc finger protein c3h1 type-like 1
GG11014|c1_g1	1.125	Krueppel-like factor 3
GG77500|c3_g1	1.155	----
GG80077|c7_g1	-0.708	Cytochrome c
GG77987|c6_g1	1.077	----
GG77708|c15_g1	1.020	Ornithine decarboxylase
GG40481|c14_g1	0.553	Catenin alpha-2 isoform x2
GG67147|c2_g1	-0.421	NADH dehydrogenase
GG23103|c1_g1	1.416	----
GG24619|c1_g1	-0.793	----
GG52773|c8_g1	1.288	U3 small nucleolar ribonucleoprotein protein imp4
GG23647|c2_g1	1.373	----
GG47936|c5_g1	-1.052	60s ribosomal I35
GG5985|c1_g1	1.103	----
GG61866|c4_g2	-0.778	----
GG6472|c9_g1	1.357	Craniofacial development protein
GG22047|c0_g1	1.358	Tumor necrosis factor receptor superfamily member 17 isoform x1
GG8363|c21_g2	1.324	Tetraspanin-1 like
GG78855|c1_g1	0.934	Nuclear receptor subfamily 4 group a member 2
GG28100|c3_g1	1.377	----
GG28100|c1_g1	1.377	----
GG13556|c1_g1	-0.634	----
GG35401|c0_g2	-0.843	----

GG62741|c176_g1	1.000	----
GG42592|c2_g3	-0.664	----
GG1102|c1_g1	0.720	----
GG55670|c6_g1	-1.034	Ankyrin repeat domain-containing protein 1
GG40365|c8_g1	1.339	----
GG81373|c3_g1	1.336	Inositol-trisphosphate receptor-interacting protein
GG78571|c0_g2	-0.737	----
GG2428|c1_g1	0.786	----
GG26520|c21_g2	1.020	Serine threonine-protein kinase sgk2 isoform x2
GG80337|c3_g1	-1.273	----
GG70273|c5_g1	0.997	Myosin-iiib-like isoform
GG5311|c11_g1	1.136	----
GG22860|c0_g1	-1.272	Proteolysis; peptidase activity
GG12571|c2_g1	1.087	----
GG71553|c1_g1	-1.136	----
GG50606|c2_g1	1.197	Transmembrane 4I6 family
GG47680|c12_g2	-0.991	----
GG16021|c0_g1	-1.082	Pentapeptide repeat-containing protein
GG68818|c0_g1	1.170	----
GG41107|c4_g1	-0.348	Mitochondrial-processing peptidase subunit beta
GG18550|c6_g1	1.109	----
GG41662|c3_g1	1.031	----
GG25524|c2_g1	1.207	----
GG48852|c1_g1	1.033	----
GG81093|c4_g1	-1.247	----
GG78614|c8_g1	0.962	An1-type zinc finger protein 2a
GG7392|c3_g1	1.034	Zinc finger protein 40 isoform x1
GG30596|c0_g1	0.790	----
GG81086|c0_g1	-0.768	40s ribosomal protein s16
GG24583|c11_g1	1.264	----
GG18900|c5_g1	0.602	Neuronal-specific septin-3 –like isoform x2
GG55670|c4_g1	-0.983	----
GG15844|c4_g1	1.199	----
GG70123|c1_g1	1.245	----
GG54179|c55_g4	-0.991	Hypothetical protein BVRB_023710
GG5210|c6_g1	1.075	----
GG74371|c0_g1	0.825	----
GG75800|c3_g1	1.240	Tumor necrosis factor receptor superfamily member
GG66621|c49_g1	0.792	----
GG55017|c2_g1	1.036	Proto-oncogene c-fos-like
GG47748|c8_g1	-0.787	----
GG577|c9_g1	-0.554	l-lactate dehydrogenase a chain

GG80760|c22_g1	1.087	----
GG30606|c1_g4	0.870	----
GG61787|c4_g1	1.186	----
GG67369|c0_g1	-1.146	----
GG77708|c14_g1	1.219	----
GG36001|c1_g1	-0.361	ATP synthase subunit mitochondrial
GG62448|c11_g2	0.877	Phospholipid-transporting ATPase ic
GG27406|c1_g1	-0.841	----
GG52019|c9_g1	1.060	Ephrin type-a receptor 5 isoform x3
GG58942|c6_g1	0.705	Ubiquitin-conjugating enzyme e2 d2
GG6450|c0_g1	1.220	Phospholipase b-like
GG27042|c7_g1	-0.583	39s ribosomal protein mitochondrial
GG77708|c10_g1	1.210	----
GG15017|c8_g1	1.212	----
GG54179|c12_g1	-0.843	----
GG54209|c0_g1	-1.059	----
GG47919|c112_g1	-1.167	Protein
GG4109|c3_g1	1.088	----
GG52037|c3_g1	0.793	----
GG52904|c0_g1	-0.455	Succinyl-ligase
GG79913|c1_g1	1.156	Calpain-8
GG65958|c5_g1	-0.776	ATP synthase subunit mitochondrial
GG67535|c7_g1	-0.758	RNA-directed DNA polymerase
GG15017|c11_g1	1.130	----
GG3971|c4_g1	0.982	Insulin-like growth factor binding protein 3
GG57427|c3_g1	1.191	----
GG17950|c4_g1	-0.745	----
GG18550|c5_g1	1.163	----
GG57840|c4_g1	0.745	Epithelial membrane protein
GG13724|c0_g1	1.159	----
GG49941|c20_g1	-0.353	Complex I intermediate-associated protein mitochondrial
GG7078|c2_g1	0.965	----
GG72162|c0_g3	-1.058	----
GG80440|c6_g1	0.376	Mitogen-activated protein kinase 6
GG14864|c12_g2	-0.519	39s ribosomal protein mitochondrial
GG78328|c4_g1	-0.379	Succinyl-ligase
GG13053|c13_g1	1.040	----

Appendix A.3. Differentially expressed genes in the Cu-H (30 µg/L) copper concentration. Description based on Blast2Go GO database functional grouping. Italicized gene transcript names indicate genes showing highest fold change.

Transcript	log2Fold Change Cv30	Description
GG51987|c3_g1	3.528	----
GG51987|c7_g1	3.163	Prostaglandin g h synthase 2
GG23647|c2_g1	3.241	----
GG52826|c3_g1	2.069	E3 sumo-protein ligase egr2 isoform x2
GG10354|c0_g1	1.751	----
GG18550|c3_g2	1.532	----
GG52174|c23_g1	1.346	Thrombospondin-1
GG52826|c1_g1	1.998	----
GG8363|c25_g1	2.771	----
GG33798|c0_g1	-2.725	----
GG69135|c1_g1	2.053	cAMP-responsive element modulator isoform x18
GG55017|c2_g1	1.959	Proto-oncogene c-fos-like
GG6751|c3_g1	2.452	----
GG40573|c19_g1	1.654	----
GG16476|c0_g1	2.149	----
GG75099|c0_g1	2.352	----
GG74670|c5_g1	1.429	Zinc finger protein c3h1 type-like 1
GG57427|c4_g1	2.413	----
GG77708|c14_g1	2.417	----
GG36048|c13_g1	1.443	Mitochondrial uncoupling protein 2
GG39670|c1_g1	2.298	----
GG62272|c0_g1	1.749	----
GG13053|c13_g2	1.510	----
GG75306|c5_g1	1.578	----
GG5985|c1_g1	1.493	----
GG8363|c21_g1	2.269	Tetraspanin-1 like
GG77708|c15_g1	1.658	Ornithine decarboxylase
GG51477|c0_g1	2.255	CD44 partial
GG57427|c3_g1	2.196	----
GG57427|c5_g2	2.161	----
GG13837|c3_g1	1.847	----
GG75306|c4_g1	1.233	Protein cyr61
GG55017|c1_g1	2.100	Proto-oncogene c-fos-like
GG57427|c6_g1	2.030	----
GG54022|c17_g1	1.032	Tissue factor pathway inhibitor partial

GG80440|c6_g1	0.636	Mitogen-activated protein kinase 6
GG78614|c8_g1	1.422	An1-type zinc finger protein 2a
GG8812|c1_g1	1.146	Leucine-rich repeat neuronal protein 4
GG9465|c17_g1	1.972	Histone h3 type 2
GG69945|c0_g1	1.672	----
GG11014|c1_g1	1.251	Krueppel-like factor 3
GG7444|c2_g1	1.293	----
GG55020|c1_g1	1.854	Proto-oncogene c-fos-partial
GG72474|c4_g1	-1.202	Filamin-a isoform x2
GG7392|c3_g1	1.221	Zinc finger protein 40 isoform x1
GG59647|c6_g1	-1.066	----
GG80302|c4_g1	1.720	----
GG15079|c12_g1	1.389	----
GG77708|c10_g1	1.917	----
GG26520|c21_g2	1.680	Serine threonine-protein kinase sgk2 isoform x2
GG71704|c0_g2	1.763	----
GG31231|c0_g1	1.871	Interleukin-8-like
GG7447|c7_g1	1.173	Glutamine-fructose-6-phosphate aminotransferase
GG4109|c3_g1	1.455	----
GG18435|c10_g1	-1.738	----
GG62764|c3_g2	-1.868	----
GG57840|c7_g1	-0.789	----
GG6751|c0_g1	1.859	----
GG75955|c0_g1	-1.844	----
GG15079|c5_g1	1.111	----
GG50634|c33_g1	1.721	Hyaluronan synthase 1
GG77500|c3_g1	1.609	----
GG26520|c22_g1	1.748	----
GG69087|c1_g1	-1.755	----
GG27834|c1_g1	-1.509	----
GG52826|c3_g2	1.629	----
GG50366|c3_g2	1.627	----
GG56799|c3_g1	1.038	Chemokine receptor cxcr4
GG58393|c4_g1	1.017	Lecithin retinol acyltransferase-like
GG19578|c2_g1	1.577	----
GG30596|c0_g1	1.108	----
GG52669|c4_g1	1.618	Dual specificity protein phosphatase 1
GG40365|c8_g1	1.692	----
GG45241|c8_g1	1.150	----
GG15017|c11_g1	1.710	----
GG51818|c1_g1	1.621	l-serine dehydratase l-threonine deaminase
GG19206|c4_g1	0.910	Transcription factor ap-1-like

GG81373|c3_g1	1.661	Inositol-triphosphate receptor-interacting protein
GG29982|c71_g1	1.650	Histone partial
GG50366|c4_g1	1.553	----
GG79932|c2_g1	1.221	----
GG5311|c5_g1	0.903	Neutral amino acid transporter a
GG44401|c0_g2	1.217	Carbohydrate deacetylase
GG56087|c4_g1	1.576	----
GG68817|c7_g1	1.535	Rho-related GTP-binding protein
GG25589|c1_g2	1.393	----
GG46866|c0_g2	1.617	----
GG51987|c3_g1	-1.625	----
GG25055|c2_g1	0.745	----
GG66334|c2_g1	1.130	Zinc finger partial
GG22860|c0_g1	-1.613	Proteolysis; peptidase activity
GG9928|c0_g1	1.278	----
GG59820|c0_g1	1.099	----
GG15538|c1_g1	-1.523	Low quality protein: protein phosphatase regulatory 1 subunit
GG39421|c5_g1	-1.354	----
GG12571|c2_g1	1.216	----
GG50249|c0_g1	1.357	----
GG5533|c6_g1	1.450	----
GG66064|c0_g1	1.521	----
GG58009|c28_g1	-1.508	Olfactory receptor 51i1-like
GG77500|c1_g1	1.402	----
GG2093|c2_g1	0.793	----
GG54022|c27_g1	1.417	Tissue factor pathway inhibitor partial
GG5533|c1_g1	0.669	----
GG12945|c2_g1	1.208	----
GG5985|c0_g1	1.505	Transcription factor ap-partial
GG78855|c1_g1	1.029	Nuclear receptor subfamily 4 group a member 2
GG42594|c8_g1	0.819	----
GG46905|c4_g1	1.486	----
GG13052|c0_g3	-1.431	----
GG44881|c7_g1	-1.162	E3 ubiquitin-protein ligase trim39-like
GG36911|c7_g1	1.038	----
GG55018|c3_g1	0.995	Cyclic amp-dependent transcription factor
GG68182|c21_g1	-1.414	----
GG30222|c23_g1	1.073	----
GG29982|c75_g2	1.268	Histone h3 type 2
GG50959|c0_g1	-1.463	Fatty acid synthase
GG65292|c0_g1	1.109	Dual specificity protein phosphatase 10
GG481|c1_g1	1.356	----

GG66334|c1_g1	1.190	----
GG80193|c1_g1	1.302	----
GG13724|c0_g1	1.330	----
GG54353|c0_g1	0.855	----
GG3971|c4_g1	1.032	Insulin-like growth factor binding protein
GG6129|c15_g1	-1.130	----
GG37967|c3_g1	1.200	Rho- related GTP binding protein
GG44534|c10_g1	0.561	----
GG35218|c17_g2	-0.992	----
GG36116|c0_g1	0.932	----
GG2556|c3_g1	1.202	----
GG13926|c2_g1	1.357	----
GG69575|c0_g1	-0.846	----
GG51455|c5_g1	0.599	Monocyte to macrophage differentiation factor 2
GG68209|c9_g1	0.711	PTB domain-containing engulfment adapter protein
GG21709|c1_g1	1.021	----
GG76988|c14_g2	-1.338	----
GG61473|c5_g1	0.938	Cysteine dioxygenase type 1
GG37147|c0_g2	-1.247	----
GG8483|c1_g1	-1.143	Collagen alpha-2 chain isoform x2
GG18435|c12_g1	-1.338	----
GG52019|c9_g1	0.866	Ephrin type-a receptor 5 isoform x3
GG28066|c9_g1	0.741	Arginase-mitochondrial
GG31818|c3_g1	-0.893	----
GG12891|c1_g1	-1.301	Plexin-a1 isoform x2
GG200|c0_g1	0.978	----
GG52586|c4_g1	0.751	----
GG54022|c25_g1	1.327	Tissue factor pathway inhibitor partial
GG49224|c5_g1	0.902	Excitatory amino acid transporter 2
GG5210|c5_g1	1.124	RNA-directed DNA polymerase from mobile element
GG17008|c6_g1	1.002	Claudin-19 isoform x2
GG35027|c1_g1	1.051	Kelch-like protein 38
GG13066|c13_g1	0.986	----
GG68818|c0_g1	1.203	----
GG39015|c4_g1	-0.807	Protein transport protein sec31a isoform
GG71580|c1_g1	1.045	Tribbles homolog 2 isoform x2
GG4290|c0_g1	0.768	Transcription factor sox-4
GG78553|c13_g1	-0.917	----
GG31966|c3_g2	0.453	----
GG18339|c9_g1	-1.226	----
GG22047|c0_g1	1.210	Tumor necrosis factor receptor superfamily

		member 17 isoform x1
GG8363|c21_g2	1.259	Tetraspanin-1 like
GG34253|c4_g1	1.018	Alpha-crystallin b chain
GG55624|c0_g1	-1.224	Forkhead box protein q1
GG19912|c10_g1	0.618	Coenzyme q-binding protein coq10 homolog mitochondrial
GG79155|c0_g1	1.259	Pr domain zinc finger protein 1 isoform
GG5786|c0_g1	1.268	A disintegrin and metalloproteinase
GG5477|c2_g1	1.053	Transmembrane protein 51-like
GG27935|c0_g1	1.270	Fatty acid 2-hydroxylase
GG18073|c271_g1	0.722	Multiple epidermal growth factor-like domains protein 6
GG46319|c5_g2	0.509	Dehydrogenase reductase sdr family member 7
GG16413|c11_g1	0.975	----
GG67200|c4_g1	0.794	----
GG68712|c1_g1	1.228	----
GG66331|c2_g1	1.167	----
GG74788|c0_g1	-1.200	Protein
GG55832|c0_g1	-1.178	----
GG45241|c2_g1	1.046	----
GG40187|c2_g1	0.484	----
GG66343|c7_g1	0.965	----
GG4208|c4_g1	0.607	----
GG7078|c2_g1	0.919	----
GG79274|c15_g1	0.760	----
GG12434|c0_g1	0.771	----
GG33205|c4_g1	0.539	----
GG36911|c7_g2	1.019	----
GG30616|c4_g1	-1.104	----
GG20094|c1_g1	0.815	----
GG63299|c9_g1	1.174	Glyceraldehyde-3-phosphate dyhydrogenase
GG9661|c0_g1	0.611	----
GG55453|c13_g1	0.557	Tyrosine- protein phosphatase non-receptor type 14
GG23268|c15_g1	-1.191	----
GG62967|c1_g1	-0.707	NAD mitochondrial-partial
GG51909|c0_g1	1.222	----
GG66161|c2_g2	0.816	----
GG67955|c0_g1	-0.260	Cytochrome heme mitochondrial
GG13366|c1_g1	0.675	----
GG12385|c4_g1	0.586	Cysteine and glycine-rich protein 2
GG52718|c8_g2	-1.106	Yeats domain-containing protein 2 isoform
GG31743|c2_g1	0.597	

GG59886|c12_g1	0.470	Probable ATP-dependent RNA helicase
GG75991|c2_g1	-0.933	----
GG62976|c1_g1	-1.018	----
GG48847|c0_g1	-1.123	----
GG16328|c1_g1	-0.342	ATP synthase subunit mitochondrial isoform x1
GG50407|c15_g1	1.154	----
GG555|c16_g1	-1.172	----
GG64174|c20_g1	1.093	P2Y purinoceptor 8
GG28464|c14_g1	0.810	Arrestin domain-containing protein 3
GG31743|c3_g1	0.814	----
GG15396|c7_g1	0.652	----
GG21362|c4_g1	0.502	----
GG25753|c13_g1	1.174	----
GG77353|c2_g1	1.179	----
GG37696|c14_g1	0.637	----
GG11011|c2_g1	-0.690	ATP-dependent 6- muscle type
GG49492|c0_g1	1.016	----
GG2821|c16_g2	-0.929	----
GG44401|c1_g1	0.810	Carbohydrate deacetylase
GG47919|c51_g4	-1.099	----
GG67867|c1_g1	0.524	Cell adhesion molecule partial
GG57022|c1_g1	-0.586	Kelch-like protein 40
GG58711|c1_g1	-0.791	Rho-related GTP-binding protein isoform
GG21476|c0_g1	-1.018	Four and a half lim domains protein 3
GG2771|c0_g1	-0.415	Spliceosome RNA helicase ddx39b isoform x2
GG13053|c13_g1	1.065	----
GG29867|c2_g1	-1.142	----
GG28875|c1_g1	-0.957	----
GG7148|c2_g1	-1.150	----
GG58711|c7_g1	-0.542	----
GG79587|c3_g1	-0.474	ADP ATP translocase 1
GG54209|c0_g1	-1.127	----
GG22391|c0_g1	-0.902	Collagen alpha-1 chain isoform
GG20973|c6_g1	0.560	----
GG13066|c14_g1	0.674	Glutamine synthetase
GG68892|c4_g1	0.796	Electrogenic sodium bicarbonate cotransporter 1 isoform
GG32006|c2_g1	0.875	----
GG65293|c0_g1	0.990	----
GG67287|c2_g1	0.571	----
GG13499|c2_g1	2.105	----
GG75809|c2_g1	0.898	----
GG58942|c6_g1	0.630	Ubiquitin-conjugating enzyme e2 d2

GG43158|c2_g1	-0.889	----
GG54619|c2_g1	0.871	----
GG77500|c2_g1	1.067	----
GG20063|c5_g2	-0.740	Magnesium transporter nipa1
GG64289|c10_g1	0.946	Sco-spondin
GG72242|c39_g1	-0.467	Mitogen-activated protein kinase
GG16514|c4_g2	-0.665	----
GG66162|c0_g1	0.764	----
GG65853|c35_g1	-1.068	Ankyrin unc44
GG77325|c30_g1	1.020	Myosin heavy chain b193a04
GG7753|c4_g2	-0.795	----
GG24619|c1_g1	-0.663	----
GG16762|c0_g1	1.062	----
GG2496|c1_g1	0.736	Krueppel-like factor 15
GG35040|c3_g1	1.023	----
GG47936|c5_g1	-0.845	60s ribosomal protein I35
GG7926|c1_g1	0.579	----
GG25524|c1_g1	0.987	----
GG50606|c2_g1	1.002	Transmembrane 4I6 family
GG70553|c4_g1	-0.603	Tropomodulin-1
GG75957|c0_g1	-0.952	----
GG43350|c2_g1	0.607	Elongation of very long chain fatty acids protein 5
GG45065|c1_g1	0.711	Anosmin-1
GG30873|c2_g1	-1.062	Fatty acid synthase
GG650|c1_g1	0.974	----
GG76121|c0_g2	-1.010	----
GG80957|c0_g1	0.906	----
GG33002|c3_g1	-1.059	----
GG54179|c123_g1	-0.998	Hypothetical protein BVRB_023710
GG23103|c1_g1	1.094	----
GG41192|c0_g1	-1.048	----
GG40365|c11_g1	1.039	----
GG20490|c8_g1	-0.926	----
GG25777|c1_g1	0.532	Cell adhesion molecule 2 isoform x2
GG14588|c1_g1	-1.034	Obscurin isoform x8
GG12945|c1_g1	1.085	Apoptosis-enhancing nuclease
GG69726|c3_g1	-0.684	Hypothetical protein, transmembrane transport
GG81086|c0_g1	-0.668	40s ribosomal protein s16

Appendix B

Sea lamprey olfactory organ candidate genes of the chemosensory receptors: olfactory receptors (OR), vomeronasal receptors (V1R) and trace-amine receptors (TAAR) (Libants et al., 2009). All genes were expected to show downregulated expression with increasing copper exposure.

Functional Group	Gene
Olfactory Receptor	OR 320
Olfactory Receptor	OR 330
Olfactory Receptor	OR 325
Olfactory Receptor	OR 343
Olfactory Receptor	OR 345
Olfactory Receptor	OR 230
Olfactory Receptor	OR 361
Olfactory Receptor	OR 328
Olfactory Receptor	OR 320
Olfactory Receptor	OR 322
Olfactory Receptor	OR 381
Olfactory Receptor	OR 326
Vomeronasal Receptor	V1R 320
Vomeronasal Receptor	V1R 342
Vomeronasal Receptor	V1R 311
Vomeronasal Receptor	V1R 312
Trace-amine Receptor	TAAR 351
Trace-amine Receptor	TAAR 355
Trace-amine Receptor	TAAR 353
Trace-amine Receptor	TAAR 358
Trace-amine Receptor	TAAR 355
Trace-amine Receptor	TAAR 353
Trace-amine Receptor	TAAR 348

Chapter 4: City of Windsor pollution plant treatment assessment of waste and storm water for heavy metals

4. 1 City of Windsor pollution plant treatment assessment of waste and storm water for heavy metals

Funding provided by ERASMUS- CREATE program offered a unique opportunity through an internship placement with the City of Windsor. Primary goals of the NSERC CREATE program are to provide additional training and communication to prepare environmental researchers to lead future investigations on sustainable freshwater resources. To address the issues of heavy metal contamination in an urban setting, the City of Windsor water treatment plant data was analyzed to investigate the potential of heavy metals being missed in treatment and subsequently being returned to the Detroit River. It was hypothesized that heavy metal values, specifically copper, would be highest in the influent water quality samples, and during spring data collection.

4.2 Materials and Methods

I was to analyze heavy metal water quality data for the Lou Romano Water Reclamation Plant (LRWRP), Windsor ON between 2013-2015. The plant provides primary and secondary treatment to combined sanitary and storm flows, with a plant capacity of 273,000 m3/d (60 MIGD). Storm water is collected from the core section of the City, as well as sewer systems in recently developed areas of South Windsor. Depending on rainfall events, contribution of both will be variable. Samples compiled of both sanitary and storm water effluent, influent and sludge values were analyzed to determine the abundance of copper and the potential output that is returning directly to the Detroit River.

Two collection sites analyzed were the Lou Romano Water Reclamation Plant, and the Windsor Little River Pollution Control Plant (LRPCP). Little River serves the

eastern portion of the City of Windsor and the surrounding municipality of Tecumseh, with a plant capacity of 73,000 m^3/d (16 MIGD). Heavy metal values were obtained from January 2013- December 2015 for both site locations. With specific interest in copper input and outputs, the effluent, influent, monthly removal efficiencies, as well as sludge data analysis was conducted per month at both site locations.

Influent values are sampled once a month for water coming into the treatment plants. Influent values are sampled at the Outfall at the Detroit River for water that is returning into the Detroit River following treatment. Sludge from primary settling tanks is then dewatered, and turned to dry cake to be used for fertilizer pellets. Final values of copper (µg/L) are reported in Table 1. Based on these values, copper that is present in effluent water (0.4483-0.0262 µg/L) is seemingly removed in the sludge (0.0274-0.2892 µg/L), and does not return to the Detroit River effluent (0.0046- 0.0283 µg/L). Values are below those that are reported to impair fish olfaction (1-3 µg/L) (Sandhal et al., 2007).

4.3 Results and Discussion

In summary, values of copper were highest (0.0262- 0.4483 µg/L) during the influent data sampling collection time point, however did not reach the threshold known to impair fish olfaction from findings in Chapter 2 (5 µg/L), and previous studies (1-3 µg Cu-L) (Sandahl et al., 2007; Kolmakov et al, 2009). In addition, spring sampling time points did not show higher copper values than other seasonal time points. However, treatment at both sites is adequate for removal of heavy metal values of copper during each sampling point, based on removal efficiency percentages between 70- 95% (Table 4.1).

Table 4.1. Copper values for Little River Pollution Control Plant (LRPCP) and Lou Romano Water Reclamation Plant (LRWRP) for 2013- 2015 in influent, effluent and sludge. Copper concentrations reported in µg/L. Removal efficiency reported in percent (%) removed following treatment.

January	Influent µg/L	Effluent µg/L	Removal Efficiency %	Sludge µg/L
LRPCP 2013	0.0890	0.0189	78.68	0.0274
LRPCP 2014	0.0601	0.0154	75.00	0.0455
LRPCP 2015	0.0929	0.0051	94.50	0.0699
LRWRP 2013	0.4366	0.0002	93.82	0.1783
LRWRP 2014	0.2684	0.0231	91.37	0.1473
LRWRP 2015	0.1767	0.0205	88.37	0.1637

February	Influent µg/L	Effluent µg/L	Removal Efficiency %	Sludge µg/L
LRPCP 2013	0.0290	0.0631	78.26	0.0480
LRPCP 2014	0.0262	0.0570	90.00	0.0434
LRPCP 2015	0.0388	0.0538	75.00	0.0458
LRWRP 2013	0.2581	0.2390	90.74	0.1403
LRWRP 2014	0.2824	0.2139	92.42	0.1618
LRWRP 2015	0.2171	0.1884	92.64	0.1919

March	Influent µg/L	Effluent µg/L	Removal Efficiency %	Sludge µg/L
LRPCP 2013	0.0532	0.0064	87.80	0.0413
LRPCP 2014	0.0786	0.0083	89.36	0.0403
LRPCP 2015	0.0851	0.0376	68.42	0.0575
LRWRP 2013	0.1983	0.0022	88.88	0.1594
LRWRP 2014	0.4134	0.0027	93.24	0.1593
LRWRP 2015	0.3184	0.0513	91.22	0.1890

April	Influent µg/L	Effluent µg/L	Removal Efficiency %	Sludge µg/L
LRPCP 2013	0.1250	0.0188	84.93	0.0508
LRPCP 2014	0.0945	0.0064	86.48	0.0489
LRPCP 2015	0.0604	0.0067	88.88	0.0580
LRWRP 2013	0.3849	0.0296	92.30	0.1653
LRWRP 2014	0.1619	0.0231	85.71	0.1911
LRWRP 2015	0.1952	0.0227	88.37	0.2324

May	Influent µg/L	Effluent µg/L	Removal Efficiency %	Sludge µg/L
LRPCP 2013	0.0547	0.0059	89.13	0.0510
LRPCP 2014	0.0847	0.0154	81.81	0.0629
LRPCP 2015	0.0630	0.0151	76.00	0.0491
LRWRP 2013	0.2192	0.0219	90.00	0.2328
LRWRP 2014	0.3632	0.0283	92.18	0.2276
LRWRP 2015	0.2529	0.0242	90.38	0.1733

June	Influent µg/L	Effluent µg/L	Removal Efficiency %	Sludge µg/L
LRPCP 2013	0.0339	0.0070	79.16	0.0080
LRPCP 2014	0.0270	0.0135	84.21	0.0513
LRPCP 2015	0.0183	0.0417	95.45	0.0529
LRWRP 2013	0.2059	0.0264	87.17	0.2576
LRWRP 2014	0.3091	0.0234	92.42	0.2058
LRWRP 2015	0.2296	0.0151	86.84	0.2892

July	Influent µg/L	Effluent µg/L	Removal Efficiency %	Sludge µg/L
LRPCP 2013	0.0569	0.0083	85.29	0.0509
LRPCP 2014	0.0663	0.0115	82.53	0.0549
LRPCP 2015	0.0643	0.0681	90.38	0.0558
LRWRP 2013	0.2255	0.0341	84.84	0.1915
LRWRP 2014	0.1763	0.0219	87.80	0.2157
LRWRP 2015	0.2242	0.0238	89.36	0.1744

August	Influent µg/L	Effluent µg/L	Removal Efficiency %	Sludge µg/L
LRPCP 2013	0.0464	0.0046	89.36	0.0464
LRPCP 2014	0.0581	0.0581	77.35	0.0463
LRPCP 2015	0.0521	0.0521	69.56	0.0542
LRWRP 2013	0.1524	0.0341	86.11	0.1605
LRWRP 2014	0.2731	0.0219	90.90	0.1934
LRWRP 2015	0.4483	0.0238	94.73	0.1910

September	Influent µg/L	Effluent µg/L	Removal Efficiency %	Sludge µg/L
LRPCP 2013	0.0436	0.0111	74.41	0.0411
LRPCP 2014	0.0418	0.0148	64.51	0.0432
LRPCP 2015	0.0371	0.0281	84.84	0.0500
LRWRP 2013	0.2151	0.0202	90.56	0.1415
LRWRP 2014	0.2938	0.0249	91.52	0.1934
LRWRP 2015	0.2443	0.0222	90.90	0.1670

October	Influent µg/L	Effluent µg/L	Removal Efficiency %	Sludge µg/L
LRPCP 2013	0.0728	0.0049	93.24	0.0527
LRPCP 2014	0.0809	0.0120	86.41	0.0529
LRPCP 2015	0.2630	0.0567	78.43	0.0393
LRWRP 2013	0.2807	0.0200	92.85	0.1535
LRWRP 2014	0.2931	0.0212	92.75	0.1962
LRWRP 2015	0.1901	0.0445	76.59	0.1336

November	Influent µg/L	Effluent µg/L	Removal Efficiency %	Sludge µg/L
LRPCP 2013	0.0489	0.0133	72.72	0.0355
LRPCP 2014	0.0485	0.0055	89.58	0.0406
LRPCP 2015	0.0649	0.0193	70.17	0.0395
LRWRP 2013	0.2662	0.0464	82.53	0.1445
LRWRP 2014	0.1527	0.0200	86.84	0.1596
LRWRP 2015	0.2443	0.0211	90.56	0.1899

December	Influent µg/L	Effluent µg/L	Removal Efficiency %	Sludge µg/L
LRPCP 2013	0.0598	0.0059	90.00	0.0209
LRPCP 2014	0.0507	0.0050	90.00	0.0506
LRPCP 2015	0.0629	0.0067	89.36	0.0318
LRWRP 2013	0.2443	0.0226	90.74	0.1277
LRWRP 2014	0.3492	0.0198	94.31	0.1514
LRWRP 2015	0.1369	0.0244	82.14	0.1666

Chapter 5: Closing Remarks

The present study investigated the effect of exposure to environmentally relevant copper concentrations on the olfactory system of the sea lamprey (*Petromyzon marinus)*. To identify environmentally relevant copper concentrations in urban and rural settings, water sampling was conducted throughout the City of Windsor and surrounding area, as well as lamprey spawning streams in Michigan. The investigation of storm water and sewage treatment at the Lou Romano Water Reclamation Plant in Windsor, ON was also conducted to analyze the amount of copper present in wastewater effluent following treatment. The work described in this thesis adds to extensive research relating to the toxic effects of copper on fish olfaction, and identifies gaps in literature in regards to study species and environmentally relevant exposures (5-10 µg/L), as well higher concentrations (30 µg/L) previously shown in waterways, such as those experiencing mining and sewage effluent runoff. This thesis has described the effects of copper on the sea lamprey, an invasive species within the Great Lakes, and a basal vertebrate with early divergence from the main vertebrate line. Water sampling was conducted to identify environmentally relevant values of copper in high traffic areas, as copper contamination through roadway runoff is the main source of contamination into aquatic environments. Based on these levels, effects of copper were investigated using a variety of techniques, addressing functional, mechanistic and genetic processes of toxicity. Copper exposure was found to disrupt olfactory sensory function at concentrations lower than those that cause physical damage. At low copper levels (5 µg/L) there was found to be a reduction in olfactory responses to the lamprey sex pheromone 3KPZS, as well as moderate effects on the structural integrity of the olfactory epithelium and on transcriptome responses. At higher doses (10- 30 µg/L) however, there was significant reductions in the sensory

response to 3KPZS, increased epithelial damage, and increased transcriptome responses, with majority of common genes differently expressed occurring between these two concentrations. This dramatic change in response at higher doses compared to lower may depict a threshold response to copper toxicity in the sea lamprey, where they may be able to adapt to lower levels of copper than those that begin to cause damage to the structural integrity of the cells in the olfactory system.

Effects on the olfactory epithelium led to an understanding of the potential damage to tissues during copper exposure, exposing irregularities in the olfactory sensory neuronal cells, as well as damage to the structure of the olfactory epithelium lamellae occurring at the low levels of 5 μg/L. The ability to label the olfactory epithelium leads to a greater understanding of copper effects on olfactory sensory processes leading to neurotransmission.

Our study has also shown that the cyclostome sea lamprey responds in similar ways as teleost fishes during copper exposures in regards to odor responses (Sandhal et al., 2007; Green 2012), as we report odor response inhibition as well as olfactory epithelium damage at levels as low as 5 μg/L. Odor-evoked local field potential recordings were analyzed through recordings in response to a specific male sex pheromone odour, 3KPZS. A dose-dependent reduction in odor responses was recorded, with significant reduction in response during our highest dose of copper, 30 μg/L. This study furthers the understanding of heavy metal contamination and the effect on the olfactory system in fishes, as well as the potential of recovery thresholds, seen in olfactory bulb recordings following post-copper exposure.

Based on changes in odor response and epithelial damage, underlying molecular mechanisms involved in toxicity were analyzed through transcriptomic analysis on olfactory tissues. Environmentally relevant concentrations (5-10 μg/L), as well as a high dose of copper (30 μg/L) were addressed following a 24-hour exposure, investigating candidate genes and transcriptome-wide modifications during exposures. As presented through the transcriptome-wide analyses, an increasing number of transcripts showed differential expression in a dose dependent manner with increasing copper concentration. In addition, olfactory receptor candidate genes showed a significant downregulated response in the highest dose of copper (30 μg/L). By conducting transcriptomic analysis, a molecular understanding is presented, giving a snapshot of the biological processes affected during copper contamination in the olfactory system of the sea lamprey. This study presents a novel approach to the study of copper toxicity on the olfactory epithelium and olfactory bulb in the sea lamprey, and furthers understanding of the molecular processes involved. While no similar studies have described such an analysis, this provides an initial investigation into copper toxicity in transcriptomic modulation.

It may be advantageous for future research to further investigate a variety of areas in regards to toxicity on the olfactory system of the sea lamprey. One area of focus could analyze the effects of copper toxicity in response to other odor stimuli than pheromones, such as amino acid odors. The sea lamprey has a highly sensitive olfactory response specific to pheromone odors. Because of this, copper toxicity may have a greater effect in olfactory response reduction to odors that do not have such a specialization for response in the olfactory system, and may more detrimental to other

olfactory mediated behaviours, such as alarm response to predation. Additionally, it would be worthwhile to investigate the specific copper concentration in which an adaptive response to copper toxicity is overcome. The current study has shown that low levels of copper exposure (5 μg/L) cause a reduction in odor response, but one that is able to show an adaptive response through the up-regulation of specific processes involved in copper metabolism within the transcriptome. This response is seemingly overcome at higher concentrations, showing significant physical damage to olfactory tissues and changes to the transcriptome. It would be beneficial to increase copper concentrations in smaller increments, to determine what specific threshold the sea lamprey are able to adapt to copper toxicity.

Overall, this thesis has made important contributions to the field of environmental toxicity by investigating the effects of copper on the sea lamprey. To my knowledge, no previous studies have employed these analyses on the sea lamprey, and ours is one of the few that characterizes these effects using multiple techniques to give a holistic analysis of the effects of copper olfactory system tissues. Additionally, the implementation of pheromone abatement strategies, proven highly advantageous, should consider these results upon application to streams. In areas where the lamprey are threatened, this should also be considered for conservation methods, where if copper levels are within the range known to impair olfaction, this could affect population levels. Lastly, guidelines for manufacturing protocols should be applied to anthropogenic sources of copper, such as brake pads in vehicles, and downspout and roofing material, which should consider the effects of heavy metal contamination entering aquatic ecosystems through roadway runoff. In conclusion, it is our belief that the work

presented here has made a distinctive contribution to the fields of toxicology and olfactory biology.

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