PROGRESS TOWARDS A CHEMOSENSORY-BASED BIOTIC LIGAND MODEL

PROGRESS TOWARDS THE DEVELOPMENT OF A CHEMOSENSORY-BASED BIOTIC LIGAND MODEL IN FATHEAD MINNOWS (*PIMEPHALES PROMELAS*) AND WILD YELLOW PERCH (*PERCA FLAVESCENS*)

By

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TITLE: Progress towards the development of a chemosensory-based biotic ligand model in fathead minnows (*Pimephales promelas*) and wild yellow perch (*Perca flavescens*)

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ABSTRACT

Elevated metal concentrations in the aquatic environment may pose a threat to fishes through acute lethal and chronic sub-lethal exposures. To assess the impact of anthropogenic metal contamination on aquatic fauna the Biotic Ligand Model (BLM), a model based on metal-binding to the gills, is now considered to be the best approach for predicting site-specific metal toxicity. The olfactory epithelium (OE) of fishes is exposed directly to the aquatic environment and is vulnerable to waterborne exposures to sub-lethal metal concentrations. These exposures have been shown to impair the olfactory function of fishes. The goal of this thesis was to begin the development of a chemosensory-based BLM (cbBLM) to predict sub-lethal metal toxicity using laboratory-based experiments with fathead minnows (*Pimephales promelas*, FHM) and yellow perch (*Perca flavescens*, YP), the latter chronically exposed to metals in the wild. Our results demonstrate that Cu and Cd binding to the OE of fathead minnows was saturable and that increasing waterborne Ca decreases metal accumulation in the OE. The binding of Cd to the OE of wild YP was saturable but the binding of Cu was not. Acute exposure of the OE to Cu impaired peripheral olfactory function in FHM as demonstrated by a decreased electrophysiological response but this was not observed for exposures to Cd. Moreover, the peripheral and central olfactory system functions of chronically metal-exposed wild YP were also impaired as indicated by an elevated electrophysiological response and a lack of behavioural response to conspecific skin extracts. This is the first study to examine the binding characteristics of Cu and Cd to the OE of fishes and to relate this metal accumulation in the OE to the impairment of peripheral olfactory function. This

information can now be used to begin development of a cbBLM. A cbBLM would use metal-OE binding constants, site-specific water chemistry, and ethological or ecological endpoints. Such models would be useful in devising environmental regulations to predict and therefore prevent chronic, sub-lethal metal toxicity in fishes and improve ecological risk assessment.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PREFACE

The proceeding thesis contains two main chapters (in addition to the general introduction and overall conclusions) that have been prepared as full manuscripts for publication. These chapters describe experiments that I designed, conducted, and data that I have analyzed, interpreted and written.

Chapter 2 is a manuscript prepared for submission that contains metal-OE binding and electrophysiology experiments conducted at Nipissing University and McMaster University between December 2005 - December 2006 and the spring of 2007. I am the primary author of the prepared manuscript and hence had a major role in designing and executing the experiments, analyzing and interpreting the data, and writing the manuscript.

Chapter 3 describes work conducted between May 2005 and October 2005 and during the summer of 2006 by my colleagues and me (as listed in the author line) at the Nipissing University Aquatic Ecotoxicology Lab and McMaster University. This chapter is a modified version of the manuscript that was prepared for submission as I have added additional data regarding a metal-olfactory epithelium (OE) binding experiment that was not in the original version of the manuscript. My contributions to this manuscript include a major role in designing the experiments and collecting the fish from lakes in the Sudbury, ON and North Bay, ON areas needed for the experiments. Furthermore, I conducted the electrophysiology (electro-olfactogram; EOG) and metal-OE binding experiments on these fish as well as analyzed and interpreted the EOG and metal-OE binding data. I contributed to the manuscript by writing the EOG and metal-OE binding

portion of the methods and results sections as well as portions of the introduction and discussion. Finally, as second author on the manuscript I was heavily involved in the final editing in order to prepare the manuscript for submission to a scholarly science journal. During the collection of the metal-OE binding data described in this chapter, two additional experiments were also conducted using the gill and gastrointestinal epithelia of these fish. The results of the gill and gastrointestinal studies have recently been published in the international peer-reviewed journal *Aquatic Toxicology* (see Klinck et al., 2007). The gill and gastrointestinal data are not directly related to the thesis and therefore were not included as a chapter in the thesis.

CHAPTER 1

GENERAL BACKGROUND, SCOPE, AND IMPLICATIONS OF THE THESIS

BACKGROUND

To assess the impact of anthropogenic metal contamination on aquatic fauna and help devise regulations that reduce metal contamination in the aquatic environment, the Biotic Ligand Model (BLM) is now considered to be the best approach for predicting site-specific metal toxicity to freshwater fauna. The BLM is a chemical equilibrium-based computer model that incorporates site-specific water chemistry (i.e., competing ions and complexation factors) and accumulation at the physiological active site to which the free metal ion in question binds (i.e., the biotic ligand) in order to predict a toxicological response (Fig 1-1) (Paquin et al., 2002). These predictions are based on acute lethality tests (96 hr LC50) and laboratory derived metal-ligand binding constants (i.e., log K – the strength of metal binding to the physiological active site, and B_{max} – the maximum number of binding sites available on the biotic ligand). To date, in freshwater fish, acute gill-based BLMs have been developed for Ag, Cd, Cu, and Zn in fathead minnows (*Pimephales promelas*, FHM) and rainbow trout (*Oncorhynchus mykiss*) (reviewed by Niyogi and Wood, 2004).

The gills of fish are an appropriate biotic ligand for acute toxicity modeling due to the fact that they are the primary site of lethal metal toxicant action (Wood, 2001) and predictions of acute toxicity are usually close to actual laboratory-derived acute toxicity data. However, chronic, sub-lethal exposures to waterborne and dietary metals have revealed several factors that complicate BLM predictions. During chronic exposure to

environmentally-realistic low levels of waterborne or dietary metals the gill-metal binding characteristics change causing a shift in the binding affinity of the gill-metal complex and shift in the number of binding sites present on the gill epithelium. This change is due to acclimation to the surrounding water conditions or change in circulating plasma metal concentrations from dietary metal uptake (reviewed by Niyogi and Wood, 2003). The changes in gill-metal log K and B_{max} values occur in order to regulate internal concentrations of nutritive metals such as Cu, Fe, and Zn which are necessary at very low concentrations for normal cellular functioning but are lethal at high concentrations. However, the uptake of non-nutritive metals such as Cd, to the gill is also regulated during chronic waterborne or dietary exposures; hence, metals do not accumulate in the gills to concentrations that are toxic (Niyogi and Wood, 2003). Since the chronic-exposure history of the fish has a direct impact on gill-metal binding properties (log K and B_{max}) the acute gill-based BLM cannot properly predict acute lethality in chronically metal-exposed fish. Moreover, metal concentrations in the environment are rarely high enough to cause acute toxicity. Therefore, the understanding and development of chronic BLMs has been of recent interest.

Chronic BLMs are being developed for the gill and gastrointestinal epithelium of fishes because both are major sites of metal uptake via waterborne and dietary exposures, respectively. Another epithelium that is continuously exposed to the aquatic environment and vulnerable to the toxic effects of waterborne metals is the olfactory epithelium (Klaprat et al., 1992). Briefly, the olfactory epithelium covers the surface of the olfactory rosettes which are part of the peripheral olfactory system in fishes. Olfactory sensory

neurons (OSN) exist within the olfactory epithelium and project their dendritic termina into the mucus layer where fine cilia sit in the olfactory cavity (Zielinski and Hara, 2007). Odour molecules bind to G-protein-coupled receptor proteins located on the surface of the cilia and cause an influx of Ca^{2+} and Na^{+} via membrane channels in order to change the membrane potential. This change in membrane potential depolarizes the OSN and causes the generation of an action potential that travels along the OSN to central olfactory pathways (Firestein, 2001; Zielinski and Hara, 2007).

A large number of odour molecules are known to bind to the surface receptor proteins in the olfactory cavity, including L-amino acids, bile acids, steroids, prostaglandins, and nucleotides (reviewed by Hara, 1992; Zielinski and Hara, 2007). Depending on the odour detected, these compounds indicate the presence of food, convey the reproductive status of a mate, allow for the identification of conspecifics, or signal the presence of a predator. The detection of these odours ultimately affects behavioural decisions relating to feeding, mating, and predator avoidance among other things.

The effects of metal toxicity on olfactory function in fishes have been studied now for almost 30 years. Understanding of the mechanistic underpinnings and ecological impacts has been growing steadily. Exposures to waterborne metals such as aluminum (Al), silver (Ag), cadmium (Cd), copper (Cu), mercury (Hg), manganese (Mn), nickel (Ni) and zinc (Zn) have been shown to disrupt olfactory function in fishes and have the potential to impair activities crucial for survival (Hara et al., 1976; Tallkvist et al., 1998, 2002; Hansen et al., 1999a,b; Beyers and Farmer, 2001; Baldwin et al. 2003; Persson et al., 2003; Scott et al., 2003; McPherson et al., 2004; Sandahl et al. 2004; Carreau and

Pyle, 2005; Bettini et al, 2006; Sandahl et al., 2006, 2007). Of these metals, two of the more commonly studied are Cu and Cd. Copper and Cd are studied due to their prevalence in a variety of compounds of anthropogenic origin and their release into the environment from non-point source (urban runoff, brake dust, use in fish culturing, piping and wiring) and point source (industrial and mining) activities (Sandahl et al., 2004; Pyle et al., 2005; Sandahl et al., 2007). Generally, waterborne exposures, ranging from 30 minutes to several weeks, with Cu concentrations ranging from 5 – 60 $\mu g\ L^{-1}$ have the potential to reduce olfactory sensitivity, cause physical damage to, or reduce the number of, olfactory receptor cells in the olfactory epithelium, and impair the ability of fishes to behaviourally respond to conspecific skin extracts (Julliard et al., 1996; Hansen et al., 1999; Beyers and Farmer, 2001; Baldwin et al., 2003; Sandahl et al., 2007). Moreover, one week exposures to Cd concentrations as low as 2 μg Cd L^{-1} have been shown sufficient enough to impair the olfactory abilities of rainbow trout, as seen by an inability to behaviourally respond to the presence of conspecific skin extracts (Scott et al., 2003).

The development of a chemosensory-based BLM (cbBLM) for fish would be of benefit to the ecological risk assessment community because it would provide another tool by which predictions could be made regarding the impacts of aquatic metal contamination on fish populations. A cbBLM would have the advantage of being conceptually simple (like other BLMs) but would have the added benefit of using the OE as the biotic ligand. The OE of fishes serves a single purpose, to bind molecules from the surrounding aquatic environment, which makes it very amenable to biotic ligand modeling. Moreover, the accumulation of metals in the OE occurs via waterborne

exposures and is not influenced by other metal uptake routes such as the diet; therefore reducing confounding factors that may influence the determination of metal binding constants. Furthermore, environmentally realistic concentrations of metals have been shown to disrupt olfactory function in the peripheral olfactory system of both lab and wild fish populations (Mirza et al., chapter 3; Sandahl et al., 2007). This disruption of olfactory function ultimately leads to the inhibition of olfactory mediated ecologically important behaviours (e.g. inability to avoid predation). Therefore, the development of a cbBLM may provide a tool that has the ability to combine site-specific water chemistry, metal-OE binding constants, and ethological or ecological endpoints to predict chronic, sub-lethal metal toxicity in fishes.

SCOPE AND IMPLICATIONS

The goal of this thesis was to begin the development of a cbBLM to predict sub-lethal metal toxicity using laboratory-based experiments in a standard reference fish species, the FHM. These techniques were then applied to a wild fish species, yellow perch (*Perca flavescens*, YP) which had been chronically exposed to metals in contaminated lakes of the Sudbury area, in order to determine if laboratory-based results can be extrapolated to field situations. The two main areas of research focused on: (1) determining Cu and Cd binding dynamics in the OE of acutely exposed FHM and chronically exposed wild YP, and (2) examining the effects of sub-lethal metal exposure on olfaction-based endpoints in both species.

Chapter 2 is a paper that has been prepared for submission. This paper investigates the acute binding dynamics of Cu and Cd to the OE of FHM under varying waterborne

Ca^{2+} concentrations. In FHM, increasing concentrations of waterborne Ca^{2+} decreased the binding capacity of Cu in the OE, but binding affinity of Cu for the epithelium did not change, indicating a non-competitive inhibition of Cu binding by waterborne Ca^{2+}. However, when waterborne Ca^{2+} concentrations were increased, the binding capacity and affinity of the OE for Cd both decreased in FHM, indicating mixed competitive and non-competitive inhibition of Cd uptake by waterborne Ca^{2+}. This change in metal binding characteristics demonstrates that Ca^{2+} has a protective role against waterborne Cu and Cd toxicity in the OE of FHM and indicates that the determination of binding constants is possible for the OE. Therefore, it may be feasible to develop a cbBLM in FHM. Using a technique known as the electro-olfactogram (EOG) (Ottoson, 1956; reviewed by Scott and Scott-Johnson, 2002) we were able to measure the olfactory sensitivity of FHM to a variety of odours before, during, and after exposure to either Cu or Cd. Exposure to > 10 $\mu g\ L^{-1}$ Cu was sufficient to cause a significant reduction in olfactory sensitivity of FHM to the amino acid L-arginine (a standard food cue). However, exposure of up to 10 $\mu g\ L^{-1}$ Cd did not cause a reduction in olfactory sensitivity to any of the odours tested. These results enable us to make a link, at least for Cu, between metal-OE binding dynamics and impairment of the olfactory system of FHM.

Chapter 3 investigates whether YP living in metal-contaminated lakes have an impaired olfactory system compared to YP living in reference lakes. Histological, electrophysiological (EOG), behavioural, and metal-OE binding techniques were employed to investigate this question. We found that YP from metal-contaminated lakes did not have a lower OSN density than those from a reference lake, indicating that

chronic waterborne metal exposure does not cause a down-regulation of OSN production or large-scale OSN death either. Somewhat surprisingly, the EOG response of YP from metal-contaminated lakes was greater than that of YP from a reference lake. This result is contrary to what we would have expected, as acute waterborne Cu exposures in the lab have been shown to decrease the EOG response in salmonids (Baldwin et al., 2003; Sandahl et al., 2004, 2007) and now in FHM (chapter 2). However, this effect may indicate a compensatory response and/or that the signal transduction pathway of YP is disrupted differently after acute vs. chronic metal exposures. This disruption of olfactory system function is evidenced by the inability of these same YP to respond behaviourally to a conspecific alarm substance. Copper binding dynamics in the OE were not saturable in YP from either reference or metal-contaminated lakes, therefore making the determination of log K and B_{max} not possible. However, the metal binding dynamics of Cd to the OE of YP from both the reference and metal-contaminated lakes were saturable. Yellow perch from the reference lake had a higher log K and lower B_{max} for Cd compared to YP from the metal contaminated-lake. Overall, this study indicates that there is a disruption somewhere beyond the peripheral portion of the olfactory system and that chronic metal exposures in fish are more complicated than in fish acutely exposed in the laboratory.

The results of this thesis provide the first set of metal-OE binding constants in FHM and wild YP and provide evidence that the production of a cbBLM is possible. Moreover, this study demonstrates that metal-OE binding dynamics are different in lab vs. wild fish species and that acute and chronic waterborne metal exposures produce

different metal-OE binding dynamics. Furthermore, this thesis has provided more evidence that environmentally realistic exposures to waterborne metals can impair olfactory function in fishes at a variety of levels (i.e., at the peripheral olfactory organ and at higher brain centers).

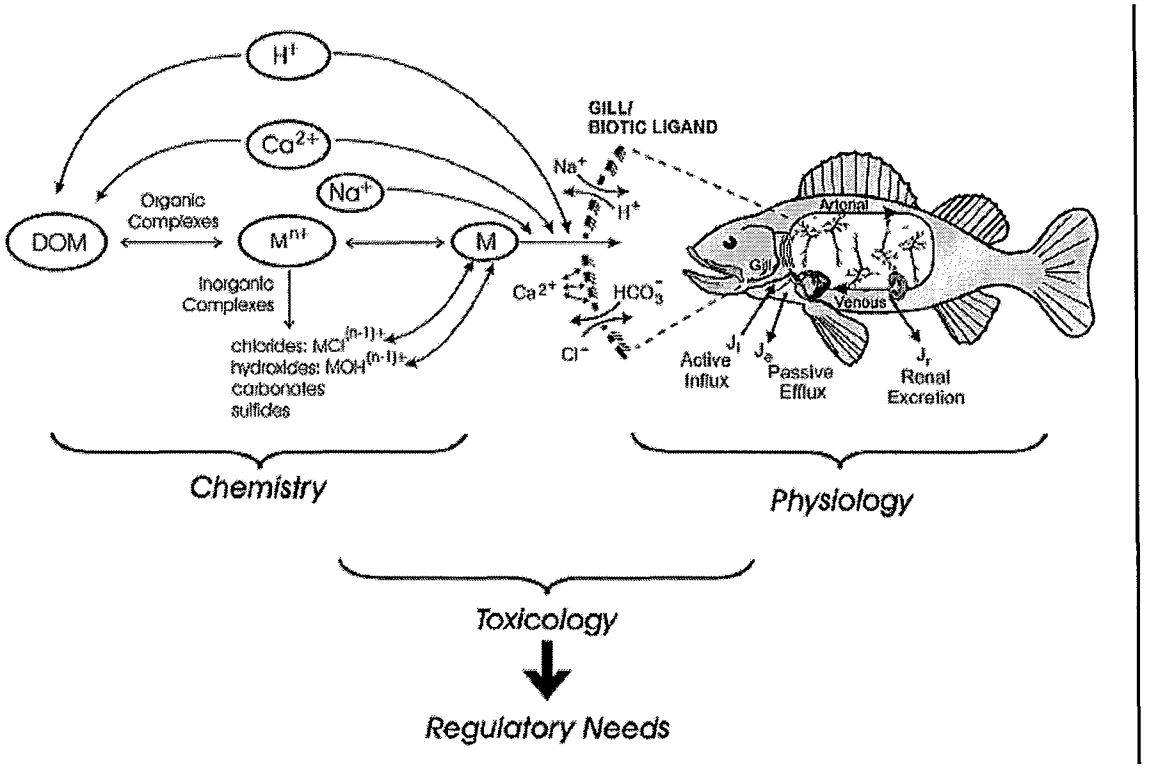

Figure 1-1 A conceptual illustration of the acute biotic ligand model (BLM) in fish (taken from Paquin et al., 2002).

CHAPTER 2

BINDING CHARACTERISTICS OF COPPER AND CADMIUM AND THE EFFECTS OF SUBLETHAL METAL EXPOSURE ON ELECTRO-OLFACTOGRAM RESPONSES IN THE OLFACTORY EPITHELIUM OF FATHEAD MINNOWS (*PIMEPHALES PROMELAS*)

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ABSTRACT

Elevated metals of anthropogenic origin in the aquatic environment may pose a serious threat to fishes through acute lethal and chronic sub-lethal exposures. Due to the direct contact of the branchial epithelium with the external aquatic environment, the effects of copper (Cu) and cadmium (Cd) on the gills have been extensively studied to produce acute gill biotic ligand models (BLM). These BLMs are used to predict acute, lethal metal toxicity in fishes. However, a BLM that predicts the effects of chronic sub-lethal metal toxicity is needed. Olfaction is particularly important in aquatic environments and chemical cues play a crucial role in many basic biological functions such as locating food, finding mates, and avoiding predators. Like the gills, the olfactory epithelium (OE) of fishes is exposed directly to the aquatic environment making it vulnerable to toxicants. Our goals were to investigate the accumulation of waterborne Cu and Cd in the OE of fathead minnows (FHM), examine the influence of waterborne calcium (Ca^{2+}) on the accumulation of both metals, to produce OE-metal binding constants, and to determine the effect of short-term waterborne Cu and Cd exposures on peripheral olfactory sensitivity. Using short-term (3 h) *in vivo* waterborne exposures to ^{64}Cu and ^{109}Cd in synthetic soft water (SSW), we found that both metals accumulate rapidly in the OE of FHM. The binding affinity (log $K_{Cu\text{-}OE}$) and binding capacity (B_{max}) of ^{64}Cu in the OE were 6.6 and 11.7 nmol Cu g^{-1} (of olfactory tissue) respectively in SSW. ^{109}Cd binding to the OE in ASW produced log $K_{Cd\text{-}OE}$ and B_{max} values of 7.5 and 10.1 nmol Cd g^{-1}. As waterborne Ca^{2+} was increased, the B_{max} of Cu and Cd decreased by ~ 50% and 90%, respectively, while the log $K_{Cu\text{-}OE}$ remained the same and log $K_{Cd\text{-}OE}$ decreased slightly.

Using electro-olfactograms (EOG), short-term (1 h) exposures to 10 and 15 μg Cu L^{-1} (~ 160 and 240 nM) were found to significantly reduce olfactory responses to 10^{-5} M L-arginine by 72 and 79%, respectively after 10 min. However, exposure to 2.5 or 10 μg Cd L^{-1} (~ 22 and 89 nM) did not affect olfactory sensitivity to 10^{-5} M L-arginine. The production of a chemosensory-based BLM using metal accumulation in the OE, site-specific water chemistry, and ecological endpoints (such as the inability to detect odours involved in predator avoidance) may provide a useful tool for predicting chronic sub-lethal metal toxicity in fishes and improve ecological risk assessment.

Keywords:

Copper, cadmium, olfactory epithelium, fathead minnow (*Pimephales promelas*), binding kinetics, electro-olfactogram.

INTRODUCTION

It has long been known that elevated metal concentrations in aquatic environments may pose a serious threat to fishes through acute lethal and chronic sub-lethal exposures. Two of the more widely studied metals that can cause such a threat are copper (Cu) and cadmium (Cd). In general, these metals are studied due to their prevalence in a variety of compounds of anthropogenic origin and their release into the environment from non-point source (urban runoff) and point source (industrial and mining) activities (Sandahl et al., 2004; Pyle et al., 2005; Sandahl et al., 2007).

Due to its direct exposure to the aquatic environment and its importance in ionoregulatory functions, the gill epithelium of fishes has been extensively studied with respect to the effects of Cu and Cd toxicity on fish physiology and survival (Wood, 2001). The accumulation of Cu on the gills of freshwater fish causes toxicity by inhibiting Na^+/K^+-ATPase and weakening cell junctions, which ultimately leads to ionoregulatory dysfunction due to disrupted sodium (Na^+) and chloride (Cl^-) homeostasis (Lauren and McDonald, 1985, 1986; Taylor et al., 2003). Similarly, Cd is also highly toxic to freshwater fish but the mechanism of toxicity is fundamentally different (Wood, 2001). Cadmium enters gill chloride cells via high affinity Ca^{2+}-binding sites and inhibits Ca^{2+}-ATPase at the basolateral membrane, effectively disrupting calcium (Ca^{2+}) homeostasis and causing mortality due to hypocalcaemia (Verbost et al., 1987, 1988, 1989). Furthermore, increasing water hardness and alkalinity have been shown to have a protective effect against both Cu and Cd toxicity (Playle et al., 1992; Playle et al., 1993a, 1993b; Wood, 2001; Taylor et al., 2003).

The OE of fishes is another epithelium that is continuously exposed to the surrounding water conditions, and also plays a vital role in the survival and reproductive success of fish populations. Olfaction is particularly important in aquatic environments because chemical cues are better than other sensory modalities especially at night or when the water is turbid (Wisenden, 2000). Moreover, water is an excellent medium for dispersing persistent chemical cues that can be detected by fishes at low concentrations using olfaction. These chemical cues play a crucial role in many basic biological functions which include, but are not limited to: locating food (Hara, 1986), assessing mating potential (Yambe et al., 1999), recognizing kin (Brown and Brown, 1996; Olsen et al., 1998), and avoiding predators (Smith, 1992; Chivers and Smith, 1998; Wisenden 2000).

The OE of fishes does not possess a protective membrane, making it extremely vulnerable to toxicants (Klaprat et al., 1992). Elevated levels of Cd and Cu have been shown to accumulate within different areas of the peripheral olfactory system of fishes (Julliard et al., 1995; Scott et al., 2003) and both metals are known to impair the ability of fishes to behaviourally respond to important chemical cues (listed above) (Beyers and Farmer, 2001; Scott et al., 2003; McPherson et al. 2004; Carreau and Pyle, 2005; Pyle and Mirza, 2007; Sandahl et al., 2007). Moreover, prolonged, low concentration exposures of Cu have been shown to cause peripheral degeneration of the olfactory epithelium and reduce the number of ciliated and microvillar olfactory sensory neurons (OSN) in fishes (Julliard et al., 1996; Hansen et al., 1999; Bettini et al., 2006).

An electrophysiological technique known as the electro-olfactogram (EOG) has provided insight into the effects of metals on peripheral olfactory function. The EOG is a well-established method for measuring responses to odourants from the peripheral olfactory organ of fishes (Scott and Scott-Johnson, 2002). The neurophysiological response measured by the EOG is a large, negative voltage transient recorded from the extracellular surface of the olfactory epithelium and represents the summation of generator potentials of the OSNs (Hara, 1994). Baldwin et al. (2003) used EOGs to demonstrate that 30 – 60 minute exposures to environmentally-relevant concentrations of Cu (1-20 $\mu g\ L^{-1}$) impair olfactory responses to 10^{-5} M L-serine in juvenile coho salmon. Moreover, Cu concentrations > 1 $\mu g\ L^{-1}$ impaired EOG responses within 10 minutes of exposure. Thresholds for sub-lethal Cu toxicity in juvenile coho salmon were determined to be ~ 3 μg Cu L^{-1} based on a 25% reduction in EOG response to 10^{-5} M L-serine, 10^{-6} M TCA, and an amino acid mixture (Baldwin et al., 2003). Interestingly, increasing hardness (using $CaCl_2$) up to 240 mg Ca L^{-1} did not have a protective effect against a 10 μg Cu L^{-1} exposure (Baldwin et al., 2003). Sandahl et al. (2004) demonstrated that a 7 day waterborne exposure to Cu concentrations ranging from 5 – 20 $\mu g\ L^{-1}$ significantly reduced the EOG and the electro-encephalogram (EEG) responses to 10^{-4} M L-serine and 10^{-5} M TCA. This result shows that Cu not only impairs peripheral (EOG) but also more central (EEG) olfactory responses, and that Cu inhibits, non-selectively, different classes of OSNs (Sandahl et al., 2004). Moreover, in a subsequent study Sandahl et al. (2007) provided evidence that a 2 μg Cu L^{-1} exposure (3 hrs in duration) was sufficient to impair EOG and behavioural responses to a conspecific skin extract (i.e., alarm substance) in

juvenile coho salmon. Furthermore, they showed that decreases in EOG response to skin extracts, due to increasing waterborne Cu concentrations, were highly correlated with decreases in behavioural responses to skin extracts (Sandahl et al., 2007). To my knowledge the effects of Cd on peripheral olfactory function have not been investigated using EOGs.

In order to predict gill-metal toxicity in fishes and ultimately devise environmental regulations to reduce the impact of elevated metal concentrations in the aquatic environment, the accumulation of metals in the gills of fishes has been investigated using a Biotic Ligand Model (BLM) construct (McGeer et al., 2000; DiToro et al, 2001; Santore et al., 2001). The BLM uses the laboratory-derived gill-metal binding constants (log K – an index of the strength of binding between the metal and the biotic ligand) and B_{max} - the maximum number of binding sites on the biotic ligand) as well as site-specific water chemistry in order to predict acute lethal metal toxicity in fishes (i.e., LA50 – lethal accumulation of metals on the biotic ligand that causes mortality in 50% of the test organisms) (Paquin et al., 2002; Niyogi and Wood, 2004). In fathead minnows (*Pimephales promelas*, FHM), the Cu and Cd gill-metal binding affinity (log K) and binding capacity (B_{max}) constants have been determined , as well as how a variety of different water chemistry parameters (e.g., Ca^{2+}, Na^{+}, Mg^{2+}, H^{+}, DOC) influence gill-metal accumulation (Playle et al., 1992; Playle et al., 1993a, 1993b). This research has led to the production of Cu and Cd BLMs for FHM (Santore et al., 2001). However, in many situations, metal exposure concentrations are too low to cause mortality but may cause disruption of physiological processes in other tissues, such as the olfactory epithelium,

ultimately causing ecological death (Scott and Sloman, 2004). In such cases, the use of the BLM construct could be helpful in designing a chemosensory-based BLM that uses metal-OE binding constants, site-specific water chemistry, and ethological or ecological endpoints such as the inability to detect chemical cues that facilitate predator avoidance. Such models would be useful in devising environmental regulations to predict and therefore prevent chronic, sublethal metal toxicity in fishes.

The aim of the current study was therefore to determine if environmentally-relevant concentrations of waterborne Cu and Cd cause metal accumulation in the OE, examine how increasing waterborne calcium influences the accumulation of both metals in the OE, and to determine if exposures to waterborne Cu and (or) Cd impair peripheral olfactory responses to a suite of odourants in FHM.

METHODS

Metal-ligand binding experiments

Experimental animals

Adult fathead minnows (3-5 g) were obtained from Thamesville fish farm (Thamesville, ON) and from Aquatic Biosystems (Fort Collins, CO). Fathead minnows were acclimated to synthetic softwater (SSW; mean ± SEM; Ca^{2+} = 122.7 ± 4.4 μM, Na^{+} = 106.6 ± 6.6 μM, Mg^{2+} = 35.7 ± 1.3 μM, dissolved organic carbon ~ 1 mg L^{-1}, temp = 12 °C) in holding tanks (up to 100 fish/tank) for at least two weeks prior to experimental testing. Holding tanks consisted of 200 L flow-through polyethylene tanks supplied with water at a rate of 1 L min^{-1}. Each day, debris was siphoned from the tanks and fish were fed until satiation with frozen brine shrimp (Hikari® Bio-Pure frozen brine shrimp,

Hayward, CA, USA) in the morning and commercial flake food (Nutrafin® Goldfish Food, Hagen Ltd., QC) in the evening. In all of the following experiments, the pH of the experimental water was determined (PHM 82 standard pH meter with electrode, Radiometer Copenhagen) prior to the beginning of all experiments, and was always in the range 6.72 – 6.93.

The time course of ^{64}Cu and ^{109}Cd binding to the olfactory epithelium

Seven 3 L exposure containers were placed onto a wet table (to keep temperature constant) and filled with 2 L of SSW (baseline condition) (mean ± SEM; Ca^{2+} = 41.7 ± 2.2 μM, Na^{+} = 51.6 ± 0.9 μM, Mg^{2+} = 11.1 ± 0.06 μM, dissolved organic carbon ~ 0.3 mg L^{-1}, temp = 14 °C), continuously aerated, and spiked with the appropriate level of radioactively labeled metal. Radioactive ^{64}Cu (as $Cu(NO_3)_2$) was created by irradiation in the McMaster Nuclear Reactor. ^{64}Cu (specific activity = 2 μCi μg^{-1}) or Cd (as Cd $(NO_3)_2$) plus ^{109}Cd (10 μCi L^{-1}) (as $CdCl_2$, specific activity = 3.65 μCi μg^{-1}; I.I.C.H., Kansas, USA) were added to achieve measured concentrations (mean ± SEM) of 311.6 ± 8.5 nM Cu or 116.5 ± 3.1 nM Cd, respectively in each container (n = 5-8). Water chemistry of low ionic composition was chosen in order to achieve maximum metal uptake with minimal interference of competitive ions or complexation. Each exposure container represented a time period within a 24 h range (1, 2, 3, 4, 8, 16, and 24 h). Five fathead minnows were added to each container for the duration of their respective exposure period. At the end of the exposure period, fish were removed from the container and placed into a lethal solution of MS-222 (500 mg L^{-1}). Fish were then rinsed with fresh SSW to remove excess mucus. The olfactory rosettes (a pair) were then removed, rinsed

in deionized water, blotted dry, and placed into a pre-weighed 0.5 mL micro-centrifuge tube. Water samples (10 mL) were taken in duplicate at the beginning and end of each exposure period. The water samples were filtered through a 0.45 μm filter and subsequently acidified to 1% with trace metal grade HNO_3 (Fisher Scientific, Oakville, ON).

The effect of waterborne Ca on ^{64}Cu binding to the OE

Five exposure containers (3 L) were placed onto a wet table and filled with 2 L of SSW with continuous aeration. Initial specific activity of ^{64}Cu was approximately 2 μCi/μg Cu (half life = 12.9 h). The ^{64}Cu solution was then added to each exposure container to produce nominal Cu concentrations of 79, 157, 236, 315, 787 nM respectively (measured concentrations mean ± SEM; 100.5 ± 8.2 nM, 176.7 ± 10.8 nM, 252.9 ± 9.2 nM, 331.7 ± 15.2 nM, 780.9 ± 36.2 nM). Seven fathead minnows were then added to each exposure for a period of 3 hours, a time selected based on the results of the previous 24 hour exposure. Water samples were taken at the start and end of the exposure period and processed as outlined above. At the end, the fish were removed from the exposure, euthanized in a lethal MS-222 solution, and rinsed with fresh synthetic soft water to remove excess mucus. The pair of olfactory rosettes were then removed and processed as described earlier.

To determine the effect of Ca^{2+} on Cu binding in the OE of fathead minnows, the above experiment was repeated but the Ca^{2+} concentration of the baseline SSW was manipulated to produce nominal water Ca^{2+} concentrations of 100, 500, and 1000 μM (measured concentration mean ± SEM; 104.1 ± 0.9 μM, 474.7 ± 2.1 μM, 971.5 ± 5.8

μM), by the addition of Ca (as Ca $(NO_3)_2$) (Fisher Scientific, Oakville, ON) to the water in each exposure container

The effect of waterborne Ca on ^{109}Cd binding to the OE

Five exposure containers (3 L) were placed onto a wet table and each container was filled with 2 L of synthetic soft water (baseline water chemistry – as mentioned above) and continuously aerated. Each container was then spiked with Cd [as $Cd(NO_3)_2 \cdot 4H_2O$. Fisher Scientific, Canada] and ^{109}Cd (10 μCi L^{-1}) (as $CdCl_2$, specific activity = 3.65 μCi μg^{-1}; I.I.C.H. Kansas, USA) to produce nominal Cd concentrations of 45, 90, 150, 200 nM (measured concentration mean ± SEM; 55.2 ± 11.4 nM, 105.1 ± 9.9 nM, 166.7 ± 16.1 nM, 221.0 ± 13.5 nM). Six fathead minnows were then placed into each exposure container for 3 hours, a time again selected based on the results of the earlier 24 hour experiment. Water samples were taken as above and the pair of olfactory rosettes removed and processed at the end of 3h as in the previous experiments.

To determine the effect of Ca^{2+} on Cu binding in the OE of fathead minnows, the above experiment was repeated but the Ca^{2+} concentration of the baseline synthetic soft water was manipulated to produce nominal water Ca concentrations of 100, and 1000 μM (measured mean ± SEM; 109.7 ± 4.7 μM and 931.2 ± 1.3 μM). The water Ca^{2+} concentrations were changed by the addition of Ca (as Ca $(NO_3)_2$) to the water in each exposure container.

Electro-olfactogram experiments

Experimental animals

Fathead minnows were obtained from the in-house breeding culture at Nipissing University. Minnows were fed twice daily with frozen brine shrimp (Hikari® Bio-Pure frozen brine shrimp, Hayward, CA, USA) in the morning and commercial flake food in the afternoon (Nutrafin® Goldfish Food, Hagen Ltd., QC). Fish were kept at 19 ± 1°C on a 16 h:8 h light:dark cycle. Fish were held in a 300 L aquarium containing dechlorinated North Bay municipal tap water (Mean ± SEM; Ca^{2+} = 167.5 ± 2.0 μM, Na^{+} = 560.9 ± 12.5 μM, Mg^{2+} = 63.3 ± 2.3 μM) that was filtered and aerated using a Fluval 404 system (Hagen Ltd., QC).

Stimulus preparation

Stock solutions of 10^{-2} M L-arginine, L-serine, L-alanine, and L-aspartic acid as well as 10^{-3} M taurocholic acid (TCA) were made fresh once a week using artificial softwater and stored at 4 °C. Each day new working solutions of 10^{-4} M L-arginine, 10^{-5} M TCA, and a 10^{-4} M amino acid mixture was made fresh by diluting from the stock solutions. The amino acid mixture working solution was made by adding a known volume of 10^{-2} M L-arginine, L-serine, L-alanine, and L-aspartic acid to a known volume of synthetic softwater (SSW) (mean ± SEM; Ca^{2+} = 45.8 ± 0.57 μM, Na^{+} = 166.6 ± 1.73 μM, Mg^{2+} = 14.9 ± 0.27 μM), thereby producing a solution containing 10^{-4} M of each of the amino acids. All working solutions were made in SSW. Blank solutions consisted of only SSW, the same water being used to perfuse the nares.

The delivery of 10^{-4} M L-arginine during the metal exposure phase required that a 10^{-4} M L-arginine working solution was also made. This solution was made from the same 10^{-2} M L-arginine stock (as mentioned above) but using the metal (Cu or Cd) contaminated SSW instead of non-metal contaminated softwater. The metal-contaminated water used to make this working solution was the same water as used for naris perfusion during the metal exposure phase of an experimental trial. Metal blank solutions consisted of only metal contaminated SSW.

Electro-olfactogram recording

Fathead minnows were prepared for EOG experimentation using methodology adapted from Sveinsson and Hara (2000). Fathead minnows were anaesthetized in a solution of MS222 (185 mg L^{-1}; ethyl 3-aminobenzoate methanesulfonate salt, Sigma, Oakville, ON, Canada) and subsequently immobilized by an epaxial intramuscular injection of Flaxedil (gallamine triethiodide, 3 mg kg^{-1} body mass, Sigma, Oakville, ON, Canada) (Sveinsson and Hara, 2000). Anaesthetized and immobilized fish were wrapped in wet tissue (to prevent desiccation), leaving the head and tail regions exposed. Fish were secured and electrically grounded in a Plexiglas perfusion chamber where the gills were perfused with a constant supply of oxygenated, dechlorinated water containing 50 mg L^{-1} MS222 via a tube inserted into the mouth (Sveinsson and Hara, 2000). The right olfactory rosette was exposed by removing the nasal septum separating the anterior and posterior nares and the exposed olfactory chamber was perfused with SSW.

Electro-olfactogram responses were differentially recorded (difference between the recording and reference electrodes) using saline (0.9%)-gelatine (4%) filled glass

capillary tubes (tip diameter, 60-80 μm) bridged to an Ag-AgCl electrode (Type MEH8, WP Instruments, Sarasota, FL, U.S.A) filled with 3 M KCl. The recording electrode was placed in the olfactory chamber, close to but not touching the olfactory epithelium, near the most caudal lamellae along the midline raphe to obtain the largest response to a standard (10^{-4} M L-arginine; Sigma, Oakville, ON, Canada) and a minimal response to an SSW blank. A reference electrode was placed on the surface of the skin posterior to the perfused naris (Sveinsson and Hara, 2000). Electro-olfactogram responses were amplified using a DC headstage and DC preamplifier (AD Instruments, Colorado Springs, CO, U.S.A.) and recorded using a computer-assisted data acquisition system (model ML 750, AD Instruments, Colorado Springs, CO, U.S.A.) and Chart® version 5 software.

Electro-olfactogram experimental protocol

Before testing, the fish olfactory epithelium was allowed to acclimate to SSW in the plexiglas perfusion chamber for 1 hour. The experimental period for each fish tested consisted of three phases: (1) pre-metal exposure, (2) metal exposure, and (3) post-metal exposure. During the pre-metal exposure phase 10^{-4} M L-arginine, 10^{-5} M TCA, and 10^{-4} M amino acid mixture cues were delivered once every 10 minutes for the first 30 minutes. Within a given 10 minute period, experimental stimuli were delivered one at a time using a gravity-flow stimulus delivery system for three seconds, after which the flow of SSW was immediately restored. Two minutes were left between each stimulus delivery to minimize olfactory receptor adaptation to odourants (as determined by previous experimentation). After the pre-metal exposure period was complete, the source water flowing through the olfactory chamber of the fish was switched from clean SSW to metal-

contaminated SSW containing nominal concentrations of 5, 10, or 15 μg L^{-1} Cu (~ 80 – 240 nM) ((measured mean (μg L^{-1}) ± SEM; 4.8 ± 0.14, 8.6 ± 0.25, 10.4 ± 0.35)) or nominal concentrations of 2.5 or 10 μg L^{-1} Cd (~ 22 and 90 nM) ((measured mean (μg L^{-1}) ± SEM; 2.6 ± 0.06, 9.1 ± 0.08)). To track the time to effect of the metal exposure, the fish's EOG response to 10^{-4} M L-arginine was tested once every 10 minutes for a total of 60 minutes (representing the metal exposure period). Once the metal exposure period was complete the flow of water through the olfactory chamber of the fish was switched back to clean SSW (post-metal exposure period). The EOG responses to the same set of olfactory cues as used in the pre-metal exposure period were again tested once every 10 minutes for a period of 30 minutes; which represented the post-metal exposure period. At the time of testing, gill perfusion water, nares perfusion water, and all experimental stimuli were at room temperature (18-22°C).

Water and Tissue analysis

Immediately after the completion of a metal-ligand binding experiment, the water and tissue samples were counted for radioactivity using a 1480 Wallac Wizard 3" automatic gamma counter (Perkin Elmer, Toronto, Canada). The weights of the tissue samples were determined for use in subsequent calculations. The dissolved Cu and Cd concentrations of the water samples from all metal-ligand binding and electro-olfactogram experiments were determined using graphite furnace atomic absorption spectroscopy (GFAAS) (Varian GTA 110, Varian Scientific, Mulgrave, Australia). A certified reference material (TM-15) from the National Research Council of Canada was used to ensure accuracy. The concentrations of Ca^{2+}, Na^{+}, and Mg^{2+} in water samples

were measured using flame atomic absorption spectroscopy (FAAS) (Varian SpectrAA-220 FS, Varian Scientific, Australia) against standard curves produced using reference standards from Fisher Scientific, USA.

Calculations and Statistical Analysis

To determine the amount of newly bound Cu and Cd to the OE the following equation was employed:

$$a(bc^{-1})^{-1}$$

where: a = radioactivity counts in the OE (cpm g^{-1} wet tissue weight); b = radioactivity counts in the water (cpm L^{-1}); c = dissolved metal concentration in the water (μg L^{-1}). The resulting values were then converted from μg g^{-1} to nM g^{-1}.

Non-linear regressions were employed for the analysis of metal binding to the OE of FHM. B_{max} (binding capacity) and log K (binding affinity) were then determined from these non-linear regressions. B_{max} was the value on the y-axis where the maximum amount of metal binding occurred. Log K was determined as the log (x^{-1}) where x represents the total dissolved metal concentration in the water that provides metal binding of half the B_{max} in molar units. Non-linear regressions were performed using Sigmaplot 8.0 for Windows.

To determine the magnitude of EOG response to an odour stimulus, the difference between baseline and peak depolarization was determined for each odour delivery. Relative responses to a particular odourant (e.g., 10^{-5} M L-arginine) were calculated by dividing each response value for a given fish by the respective pre-metal exposure response average for that odourant. When comparing pre-metal and post-metal exposure

responses, means were determined by averaging the 3 responses for a given exposure period (i.e., pre-metal exposure or post-metal exposure) for each fish and then determining a mean of means.

All data are presented as means ± SEM (n) where n = number of fish. In order to determine the time at which maximum accumulation of Cu or Cd in the OE of FHM occurred over a 24 h exposure period, a one-way analysis of variance (ANOVA) was used followed by a modified Student's post hoc. The effects of increasing waterborne [Cu] and [Ca^{2+}] or [Cd] and [Ca^{2+}] on metal-OE accumulation were examined by two-way ANOVA. Subsequent ANOVA's with a Tukey-Kramer HSD post hoc tests were used to determine if increasing waterborne [Ca^{2+}] reduced metal-OE accumulation at each of the metal concentrations tested. Values were considered statistically significant if $p \leq 0.05$. To determine the effect of Cu or Cd exposures on EOG response to 10^{-5} M L-arginine throughout the entire testing period, repeated measures two-way ANOVA were utilized. The difference in EOG responses between pre-metal and post-metal exposures to 10^{-5} M TCA and a 10^{-4} M amino acid mixture was analyzed using individual paired t-tests for each metal concentration. A Bonferroni adjustment to α (0.05) was used to control for family-wise error rates when conducting paired t-tests. Values were considered significant when $p \leq 0.016$ after the Bonferroni adjustment. All statistical analyses were performed using JMP 5.0 for Windows.

RESULTS

Metal-ligand binding experiments

To characterize Cu binding to the OE of FHM the time course of Cu-OE binding had to first be determined. Newly accumulated Cu was measured in the OE of FHM over a 24 hour time period. Copper accumulation in the OE did not significantly increase after 3 hours ($F_{4,20} = 0.43$; $p = 0.79$ (Fig.2-1). Next, the binding affinity (log K) and binding capacity (B_{max}) of the OE for Cu in baseline synthetic softwater (SSW) was determined using a 3 hour radiolabelled Cu^{64} binding assay. The uptake of Cu in the OE was sigmoidal in shape over the [Cu] range of 100 – 800 nM. Log $K_{Cu\text{-}OE}$ and B_{max} were calculated to be 6.6 and 11.7 nmol g^{-1}, respectively (Fig. 2-2) (Table 2-1). Furthermore, to determine if increasing waterborne Ca had a protective effect against Cu binding to the OE, the [Ca^{2+}] of the SSW was increased from 50 μM (baseline) to 100, 500, or 1000 μM. Two-way ANOVA revealed that increasing waterborne [Cu] significantly increased Cu-OE binding ($p < 0.0001$) and that increasing waterborne [Ca^{2+}] reduced Cu-OE binding ($p < 0.0001$). Moreover, the interaction of waterborne Cu and Ca significantly affected Cu-OE binding because increasing waterborne [Ca^{2+}] reduced Cu-OE binding between 2 to 6 times relative to Cu-OE binding in baseline synthetic softwater at each of the levels of waterborne [Cu] tested (whole model $F_{19,119} = 22.28$; $p < 0.0001$) (Fig. 2-2). Finally, as waterborne [Ca^{2+}] increased, the B_{max} of Cu decreased by 50% and the log $K_{Cu\text{-}OE}$ remained the same (Table 2-1).

As with the copper characterization experiments, the time course of cadmium (Cd) binding to the OE of FHMs was first determined. Newly accumulated Cd was measured in the OE of FHM over a 24 hour time period. Maximum Cd accumulation occurred after 3 hours and did not significantly increase over the remaining 21 hours ($F_{4,34} = 0.62$; $p =$

0.65) (Fig.2-1). The binding affinity (log K) and binding capacity (B_{max}) of Cd to the OE of FHM in baseline synthetic softwater were determined using a 3 hour radiolabelled Cd^{109} binding assay. The uptake of Cd in the OE exhibited Michaelis-Menten kinetics over the concentration range of 45 – 250 nM. Log $K_{Cd\text{-}OE}$ and B_{max} were calculated to be 7.5 and 10.0 nmol g^{-1}, respectively (Fig. 2-3) (Table 2-1). Furthermore, to determine if increasing waterborne Ca^{2+} had a protective effect against Cd binding to the OE, the $[Ca^{2+}]$ of the softwater was increased from 50 μM to 100 or 1000 μM. Each of these experiments was conducted using the same [Cd] range as the previous experiment. Two-way ANOVA revealed that increasing waterborne [Cd] significantly increased Cd-OE binding ($p < 0.0001$) and that increasing waterborne $[Ca^{2+}]$ reduced Cd-OE binding ($p < 0.0001$). Moreover, the interaction between waterborne Cd and Ca^{2+} significantly affected Cd-OE binding because increasing waterborne $[Ca^{2+}]$ reduced Cd-OE binding between 9 to 11 times relative to Cd-OE binding in baseline synthetic softwater at each of the waterborne [Cd] tested (whole model $F_{11,58} = 26.42$; $p < 0.0001$) (Fig. 2-3). Overall, as waterborne $[Ca^{2+}]$ increased, the B_{max} of Cd decreased ~ 90 % and the log $K_{Cd\text{-}OE}$ decreased by approximately one half of a log unit (Table 2-1).

Electro-olfactogram experiments

After 10 minutes, exposure to 10 and 15 μg Cu L^{-1} (~ 160 and 240 nM) reduced EOG response to 10^{-4} M L-arginine in FHM by approximately 72 % and 79 %, respectively ($F_{11, 121} = 3.47$, $p < 0.0001$) (Fig. 2-4). This inhibition of the EOG response continued throughout the Cu exposure period. During the post-Cu exposure period, the EOG response to 10^{-4} M L-arginine returned to pre-Cu exposure levels within 10 minutes

after a 10 μg Cu L^{-1} exposure and 20 minutes after a 15 μg Cu L^{-1} exposure. Interestingly, even after a 1 hour exposure to 5 μg Cu L^{-1} (80 nM), EOG responses to 10^{-4} M L-arginine were not affected (Fig. 2-4). Moreover, when comparing pre-metal and post-metal exposure responses within a given concentration, a 1 hr exposure to 5, 10, or 15 μg Cu L^{-1} did not inhibit EOG responses to 10^{-5} M TCA (paired t-test 5μg L^{-1}: $t = 0.66$, d.f. = 4, $p = 0.55$; 10μg L^{-1}: $t = 1.19$, d.f. = 4, $p = 0.30$; 15 μg L^{-1}: $t = 1.86$, d.f. = 4, $p = 0.14$) (Fig. 2-5) and 10^{-4} M amino acid mixture (paired t-test 5 μg L^{-1}: $t = 2.33$, d.f. = 4, $p = 0.08$; 10 μg L^{-1}: $t = 1.91$, d.f. = 4, $p = 0.13$; 15 μg L^{-1}: $t = 1.06$, d.f. = 4, $p = 0.35$) (Fig. 2-6).

Exposure for 1 hour to 2.5 and 10 μg Cd L^{-1} (22 and 90 nM) did not reduce EOG responses in FHM to 10^{-4} M L-arginine ($F_{11, 66} = 0.86$, $p = 0.58$) (Fig. 2-7). Moreover, when comparing pre-metal and post-metal exposure EOG responses to 10^{-5} M TCA (paired t-test 2.5 μg L^{-1}: $t = 1.13$, d.f. = 3, $p = 0.34$; 10 μg L^{-1}: $t = 1.20$, d.f. = 2, $p = 0.35$) and 10^{-4} M amino acid mixture (paired t-test 2.5 μg L^{-1}: $t = 2.06$, d.f. = 3, $p = 0.13$; 10 μg L^{-1}: $t = 1.63$, d.f. = 2, $p = 0.24$) stimuli, it was found that 2.5 and 10 μg Cd L^{-1} did not reduce EOG responses (Fig. 2-8 and 2-9, respectively).

DISCUSSION

The accumulation of Cu and Cd in the OE of FHM was determined under varying water Ca^{2+} concentrations in order to characterize metal binding to the OE and ultimately to begin the development of a chemosensory-based biotic ligand model. In order to relate the Cu and Cd OE binding characteristics to an inhibition of olfactory perception, responses to a variety of odourants were measured in FHM before, during, and after

exposure to a similar range of Cu and Cd concentrations used in the metal-ligand binding experiments.

The accumulation of both metals in the OE was rapid and saturable when tested in SSW and Cu accumulated to a slightly greater concentration to that of Cd. This accumulation reflected a slightly greater binding site density (B_{max}) for Cu compared to Cd, even though the affinity of the biotic ligand for Cd was almost 10 times greater than for Cu as indicated by the difference in log K values (Table 2-1). Previous studies have shown that Cu accumulates to a much greater concentration in the gills of FHM due to a larger number of binding sites present even though Cd has a greater affinity for the biotic ligand (Playle et al., 1993). Interestingly, for both Cu and Cd, the log K values are ~ 1 log unit lower in the OE than the gill of FHM but the B_{max} values are greater in the OE, especially for Cd (~ 5 times greater) (Table 2-2). This difference in B_{max} values is likely due to qualitative differences in the two different epithelia. The gill is an ionoregulatory organ and there is a constant bidirectional flux of ions across the epithelial membranes in order to maintain blood plasma ion homeostasis; however, the main function of an OE in fishes is to bind odour molecules and take up Ca^{2+} and Na^{+} in order to generate action potentials. This difference in biological function and structure may explain the greater binding site density observed in the OE relative to that in the gill epithelium.

It is believed that Cu binding in the OE occurs on cellular surface proteins, membrane structures, or internal organelles and at high enough concentrations can cause cell death (Brown et al., 1982; Julliard et al., 1996; Hansen et al., 1999). Moreover, Cu may disrupt EOG responses by blocking ligand-gated and (or) voltage-gated ion channels

(Sandahl et al., 2006). The results of the current electro-olfactogram experiment indicate that short-term (1 hr) waterborne exposures to environmentally-relevant concentrations of Cu can severely inhibit peripheral olfactory function in FHM. This result is in general agreement with findings of previous EOG studies using juvenile coho salmon; specifically that Cu reduces the EOG responses to a variety of odourants. Interestingly, studies with juvenile coho salmon demonstrated that 2-3 μg Cu L^{-1} is sufficient to significantly reduce EOG response (Baldwin et al., 2003; Sandahl et al., 2007) but in the present study, EOG responses of FHM were not significantly reduced until exposure to > 5 μg Cu L^{-1}.

As waterborne [Ca^{2+}] increased from ~ 50 to 1000 μM the Cu B_{max} decreased but log K remained the same as it was in baseline SSW, indicating that Ca^{2+} non-competitively inhibited the binding of Cu to the OE. Similarly, despite some conflicting results, it is generally accepted that the binding and consequent toxic effects of Cu in fish gills are reduced with increasing waterborne [Ca^{2+}] (Playle et al., 1992; Playle et al., 1993a; Erickson et al., 1996) and increasing waterborne [Na^{+}] (Erickson et al., 1996; Santore et al., 2001; Grosell and Wood, 2002). Although Cu uptake in the gill of freshwater fishes is through apical sodium channels (Grosell and Wood, 2002), not Ca^{2+} channels, ambient [Ca^{2+}] has a greater effect than ambient [Na^{+}] on reducing Cu toxicity due to Ca^{2+} controlling the permeability of the membrane thereby reducing Na^{+} and Cl^{-} loss (Hunn, 1985). Interestingly, Baldwin et al. (2003) found that increasing water hardness (using $CaCl_2$) up to 240 mg Ca L^{-1} did not prevent degradation of the EOG signal in response to a variety of odourants during a 10 μg Cu L^{-1} exposure.

In comparison, increases of waterborne [Ca^{2+}] reduced the accumulation of Cd binding to the OE of FHM as seen by the Bmax decreasing ~ 9 times and log K also decreasing, thus indicating that Ca^{2+} was inhibiting Cd binding by both competitive and non-competitive mechanisms. Furthermore, the fact that Ca competitively inhibited Cd binding indicates that Ca and Cd possibly share common receptor sites. In the gills of FHM (as well as other freshwater fishes) Cd is known to bind to high-affinity Ca^{2+} channels on the apical surface of the gill; however, when waterborne [Ca^{2+}] is increased the amount of Cd that binds to the gill decreases because Ca^{2+} and Cd are known to be antagonists (Playle et al., 1993a; 1993b; Niyogi and Wood, 2004). Recently, the presence of Ca^{2+} sensitive receptors (Ca-SR) and possible Na^{+} uptake channels in the OE of goldfish (*Carassius auratus*) have been reported that independently take up Ca^{2+} and Na^{+} in order to maintain an ionic gradient in the mucus layer of the OE (Hubbard et al., 2002; Hubbard and Canario, 2007). The maintenance of this ionic gradient in the OE of fishes is necessary to facilitate signal transduction for odour perception (Hubbard et al., 2002). Since Ca^{2+} and Cd are known antagonists, they could have been competing for binding sites on the Ca^{2+}-SR so that as waterborne [Ca^{2+}] increased, a competitive inhibition of Cd binding was seen. However, more research is necessary to determine the mechanism of non-competitive inhibition by Ca^{2+} on Cd binding in the OE of FHM.

The most interesting finding of this study was that although Cd bound to the OE of FHM, waterborne concentrations of Cd upwards of 10 $\mu g\ L^{-1}$ did not inhibit EOG responses to any of the three cues tested and did not reduce responses to 10^{-4} M L-arginine even during the Cd exposure period. Cadmium has been shown to accumulate in

the olfactory system of fishes and be transported along OSNs by axonal transport to the olfactory bulb, but does not cross the synapses in the bulb and therefore, does not get transported to the brain (Tjalve and Henriksson, 1999; Scott et al., 2003). However, there is evidence that Cd is poorly permeant across the blood-brain barrier of rainbow trout from exposure to elevated concentrations of Cd in the diet (Szebedinszky et al., 2001). Brown et al. (1982) found that lethal concentrations of Cd decreased EEG responses but that a 2 week exposure to Cd (146 $\mu g\ L^{-1}$) did not inhibit EEG responses in rainbow trout (*Oncorhynchus mykiss*). Finally, Scott et al. (2003) demonstrated that a 7 day waterborne exposure to 2 μg Cd L^{-1} was sufficient to significantly reduce behavioural responses to a conspecific skin extract in rainbow trout. Although Cd has been extensively studied with respect to its accumulation and transport in the olfactory system, the electrophysiological consequences of Cd seem to have received less attention. To my knowledge, this is the first study to investigate if Cd affects peripheral olfactory function in FHM and quite possibly in fish in general. Taken together, the results of this and previous studies suggest that for Cd to affect peripheral olfactory function in fishes may require more extended exposure periods than those used in the current study.

The accumulation of Cu in the OE of FHM was measured over the waterborne Cu concentration range of ~ 100 – 800 nM to determine log $K_{Cu\text{-}OE}$ and B_{max} values. The range of Cu used was representative of the concentrations of Cu that can be found in metal-contaminated aquatic environments (Pyle et al., 2005); therefore, the binding constants determined can be related to Cu concentrations found in the environment. However, in aquatic environments with elevated levels of Cd the concentrations typically

range from ~ 0.5 to 7 nM (Couture and Rajotte, 2003; Giguere et al., 2005). These low concentrations of Cd can make the determination of Cd accumulation in tissues difficult to measure. The current study used elevated levels of Cd (~ 50 – 200 nM) that were not environmentally relevant to ensure that Cd accumulation in the OE of FHM was saturable making the determination of log K and B_{max} values possible.

The inhibition of peripheral olfactory function in FHM due to exposure to sub-lethal concentrations of Cu has implications for the survival and reproductive success of FHM in metal-contaminated aquatic habitats due to the role olfaction plays in mediating these vital biological functions. When examining the binding dynamics of Cu in the OE and the inhibition of EOG responses across the same range of Cu concentrations, it is clear that there is a dose-response relationship between increasing accumulation of Cu and the reduction of EOG response. To the best of the authors' knowledge, the current study is the first to examine the binding kinetics and accumulation of Cu and Cd in fish olfactory epithelia under varying waterborne Cu, Cd and Ca^{2+} concentrations. This approach allowed for the determination of binding constants log K and B_{max} for Cu and Cd and the evaluation of how these constants change over a range of ambient Ca^{2+} concentrations. By understanding the relationship between metal binding dynamics in the OE and how that relates to inhibition of peripheral olfactory function, it may be possible to develop a chemosensory-based biotic ligand model (cbBLM).

A cbBLM may be used to relate metal accumulation in the olfactory rosettes and site-specific water chemistry to predict endpoints such as, a 50 % inhibition of olfactory response leading to impaired behavioural responses to ecological odours (e.g., chemical

alarm cues). This model would ultimately be used to predict ecologically-relevant, sub-lethal metal toxicity in freshwater fishes and improve ecological risk assessment. Future studies should investigate how other water chemistry variables such as Na^+ and DOC influence Cu and Cd binding kinetics and also determine the binding constants of Ca^{2+}, Mg^{2+}, K^+, and Na^+ to the OE—all of which could contribute to the development of a cbBLM.

The work presented here is only the beginning of what needs to be accomplished in order to create a cbBLM for Cu in FHM. Future studies should determine how increasing concentrations of ions such as Ca^{2+}, Na^+, Mg^{2+}, H^+, and DOC influence the toxic effects of Cu in the peripheral olfactory system (i.e., EOG response) and relate the findings to similar work suggested for OE-metal binding dynamics. Moreover, since Cd did not impair EOG response in FHM, future studies should investigate whether environmentally-relevant concentrations of waterborne Cd inhibit central processes of the olfactory system (i.e., EEG responses) in FHM.

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Table 2-1. Log K and B_{max} values for Cu and Cd binding to the olfactory epithelium of fathead minnows after 3 hour exposures to waterborne Cu or Cd as a function of increasing waterborne $[Ca^{2+}]$.

Metal	[Ca] (μM)	B_{max} (nmol g^{-1})	Log K
Cu	50	11.7	6.6
	100	14.4	6.4
	500	10.2	6.5
	1000	5.8	6.5
Cd	50	10.1	7.5
	100	8.6	7.2
	1000	1.1	7.1

Table 2-2. A comparison of log K and B_{max} values for the gill epithelium and olfactory rosettes of fathead minnows after short term waterborne exposures to Cu or Cd in artificial softwater with low concentrations of competitive ions and other complexing agents (e.g., DOC). Asterisks indicate values from Playle et al., 1993 a,b.

	Gill		OE	
	Cu	**Cd**	**Cu**	**Cd**
Log K	7.4*	8.6*	6.6	7.5
B_{max} (nmol g^{-1})	10*	2*	11.7	10.1

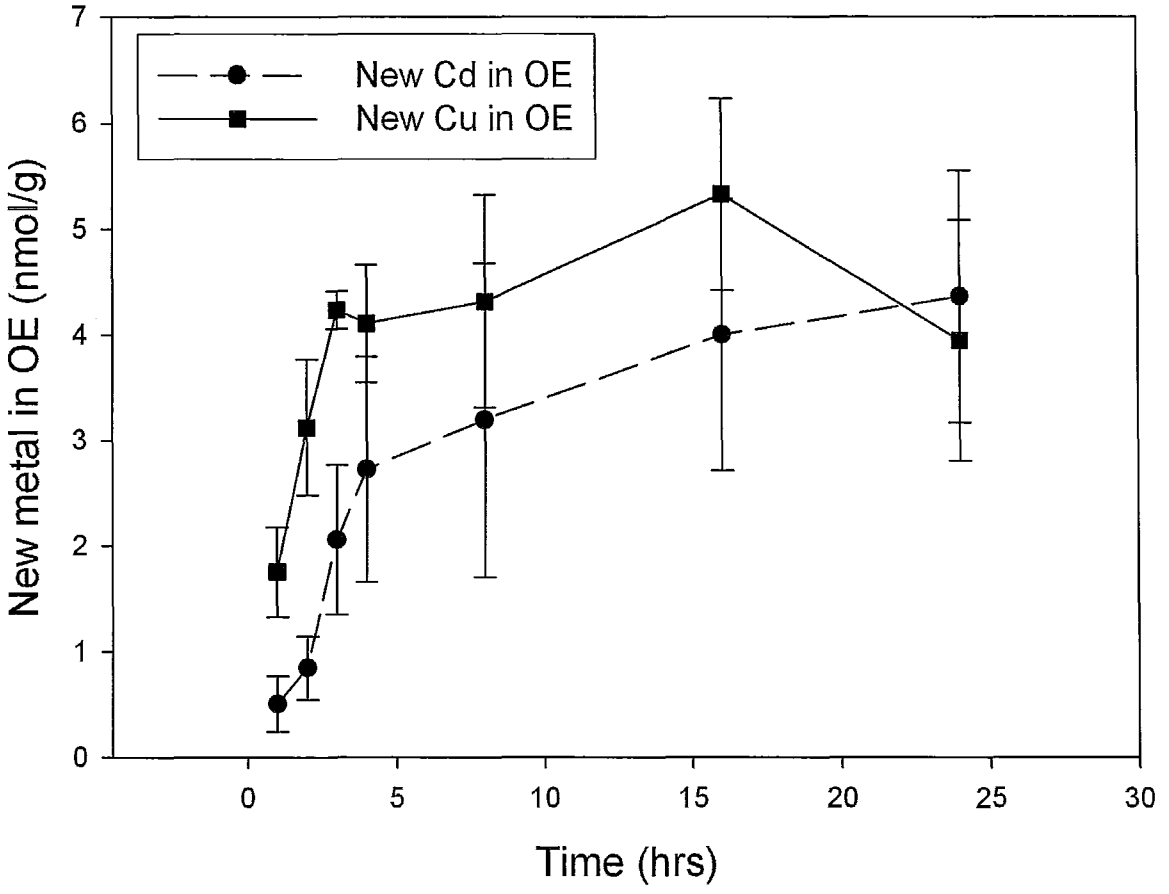

Figure 2-1. New copper or cadmium binding to the olfactory rosettes of fathead minnows over a 24 hour period during exposure to (mean ± SEM) 311.6 ± 8.5 nM waterborne Cu or 116.5 ± 3.1 nM waterborne Cd. Points represent means ± S.E.M (n = 5-8).

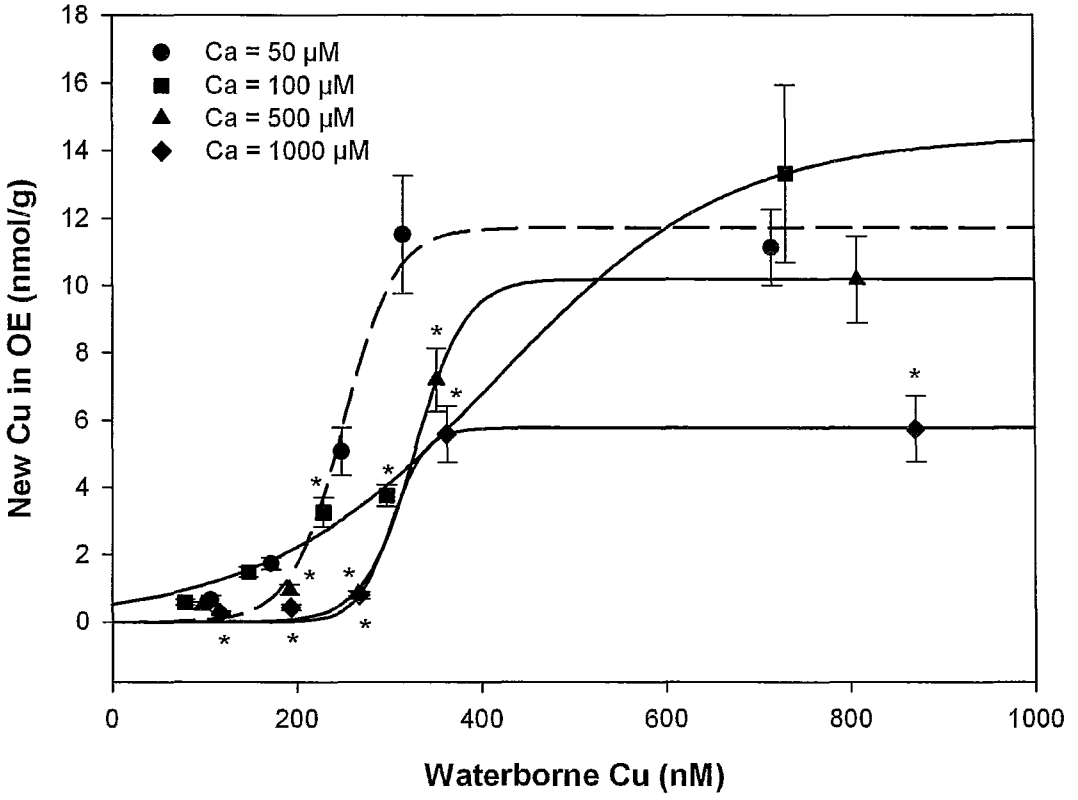

Figure 2-2. New copper binding to the olfactory rosettes of fathead minnows across a range of waterborne Cu concentrations in different waterborne Ca concentrations after a short-term 3 hour copper exposure. Points represent means ± S.E.M (n = 6-7). Asterisks represent significant difference of Cu binding from Cu binding in baseline softwater (hatched line) within the respective Cu concentration.

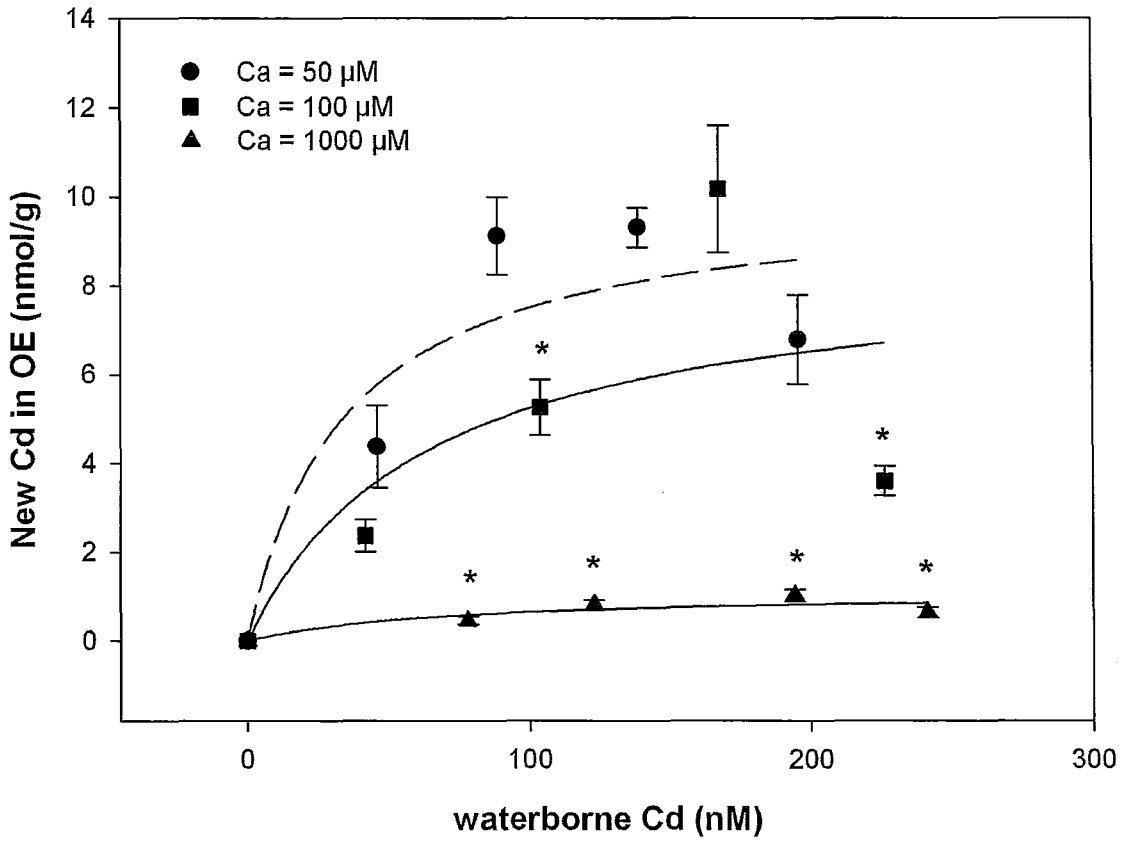

Figure 2-3. New cadmium binding to the olfactory rosettes of fathead minnows across a range of waterborne Cd concentrations in varying waterborne Ca concentrations after a short-term 3 hour cadmium exposure. Points represent means ± S.E.M (n = 6). Asterisks represent significant differences in Cd binding from Cd binding in baseline softwater (hatched line) within the respective Cd concentration.

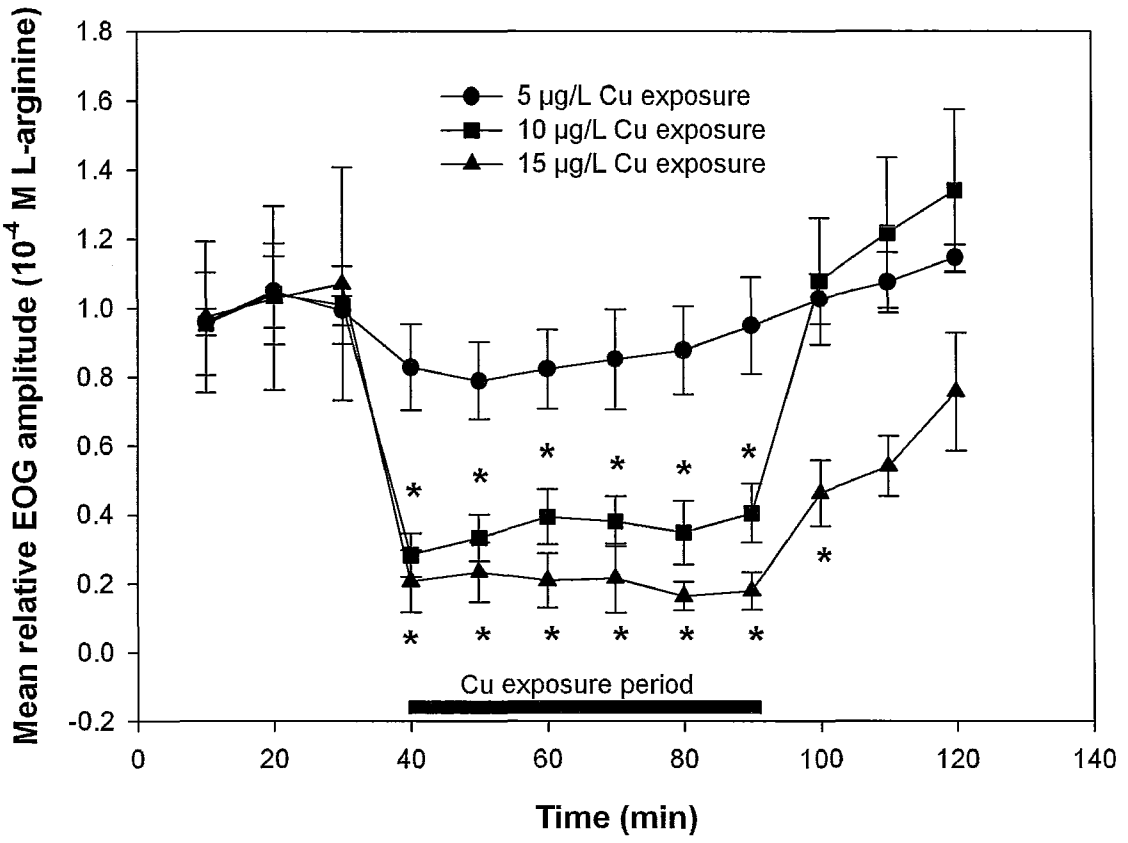

Figure 2-4. Mean ± SEM of the relative EOG response of fathead minnows to 10^{-4} M L-arginine for the pre-copper, copper exposure, and post-copper exposure periods at concentrations of 5, 10, and 15 µg Cu L^{-1} (~ 80 – 240 nM). Relative responses were determined by dividing each response value for a given fish by the respective pre-metal exposure response average. Plotted means were determined by averaging the response to 10^{-4} M L-arginine of all 5 fish for a given time period. Repeated measures two-way ANOVA was conducted for each Cu concentration and asterisks indicate significant differences from the respective pre-metal exposure responses ($p \leq 0.05$).

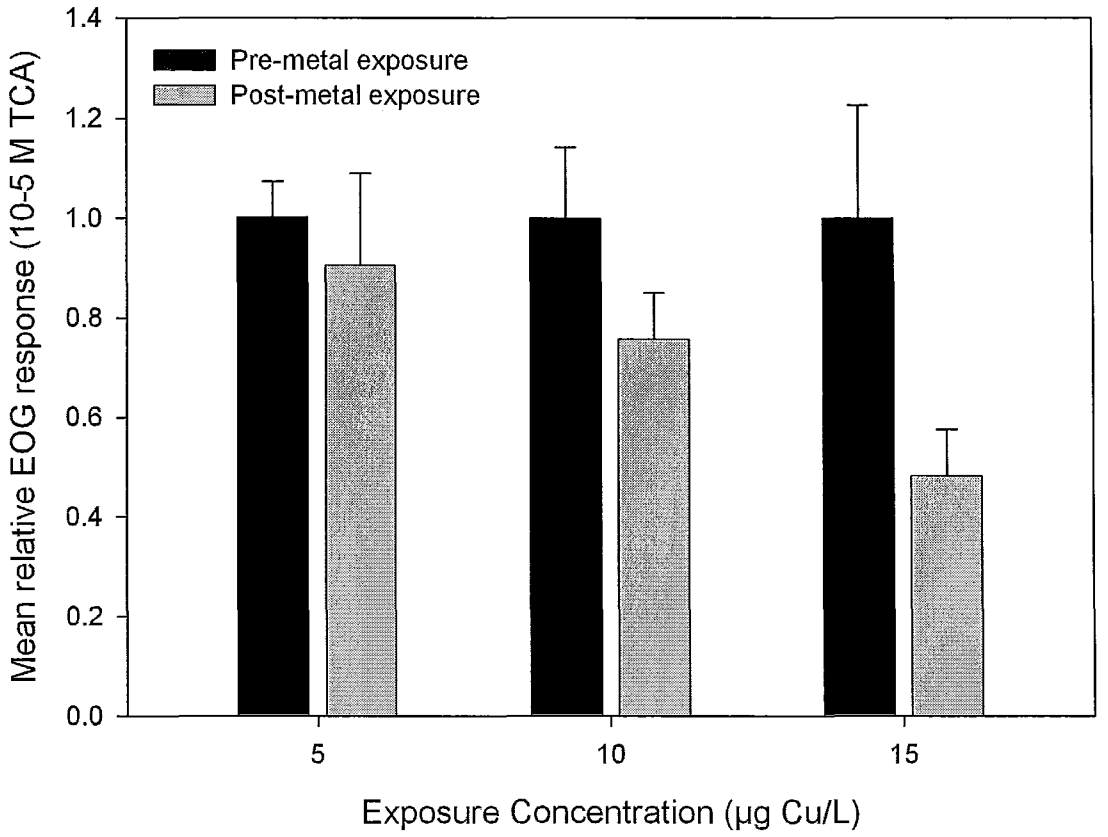

Figure 2-5. Relative EOG response (mean ± SEM, n = 5) of fathead minnows to 10^{-5} M taurocholic acid (TCA) prior to and following exposure to 5, 10, or 15 µg Cu L^{-1} (~ 80 – 240 nM) for 1 hour. Relative responses were determined by dividing each response value for a given fish by the respective pre-metal exposure response average. Means were determined by averaging the 3 responses for a given exposure period (i.e., pre-metal exposure or post-metal exposure) for each fish and than determining a mean of means for all 5 fish.

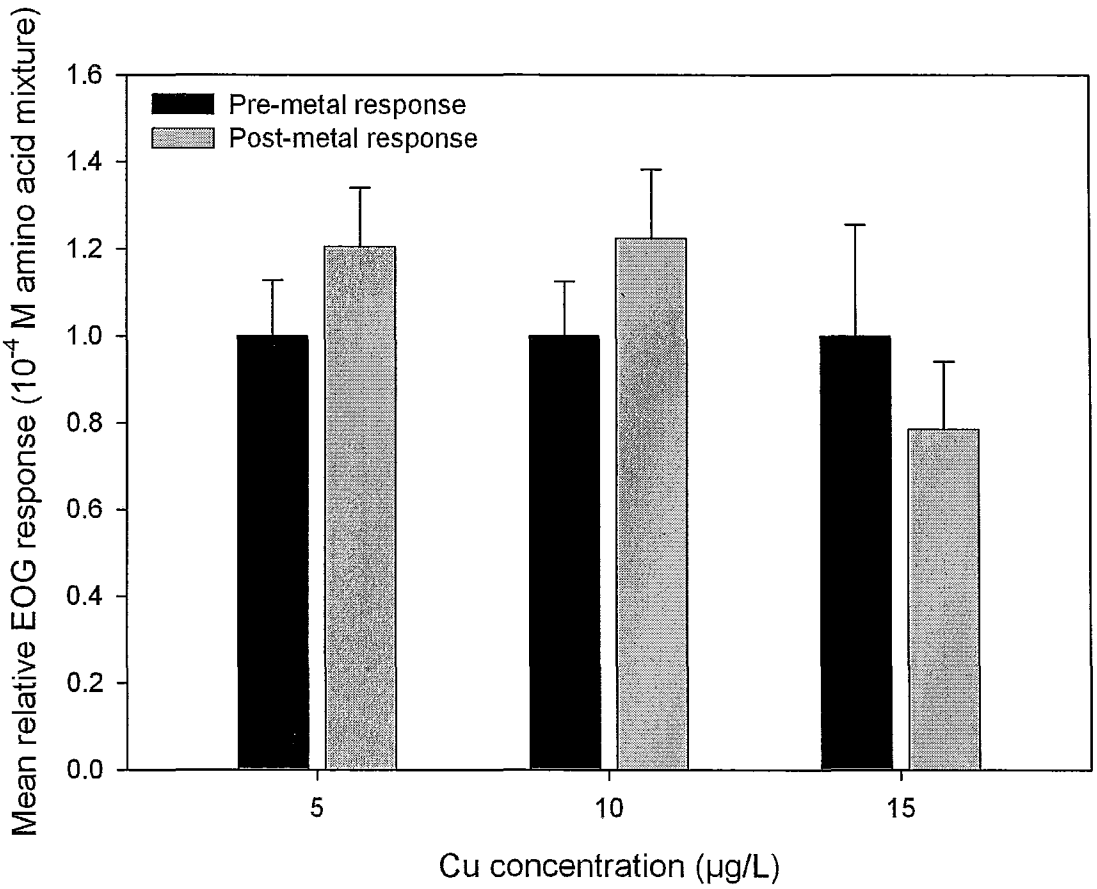

Figure 2-6. Relative EOG response (mean ± SEM, n = 5) of fathead minnows to 10^{-4} M amino acid mixture (10^{-4} M L-arginine, L-alanine, L-serine, L-aspartic acid) prior to and following exposure to 5, 10, or 15 µg Cu L^{-1} (~ 80 – 240 nM) for 1 hour. Relative responses were determined by dividing each response value for a given fish by the respective pre-metal exposure response average. Means were determined by averaging the 3 responses for a given exposure period (i.e., pre-metal exposure or post-metal exposure) for each fish and then determining a mean of means for all 5 fish.

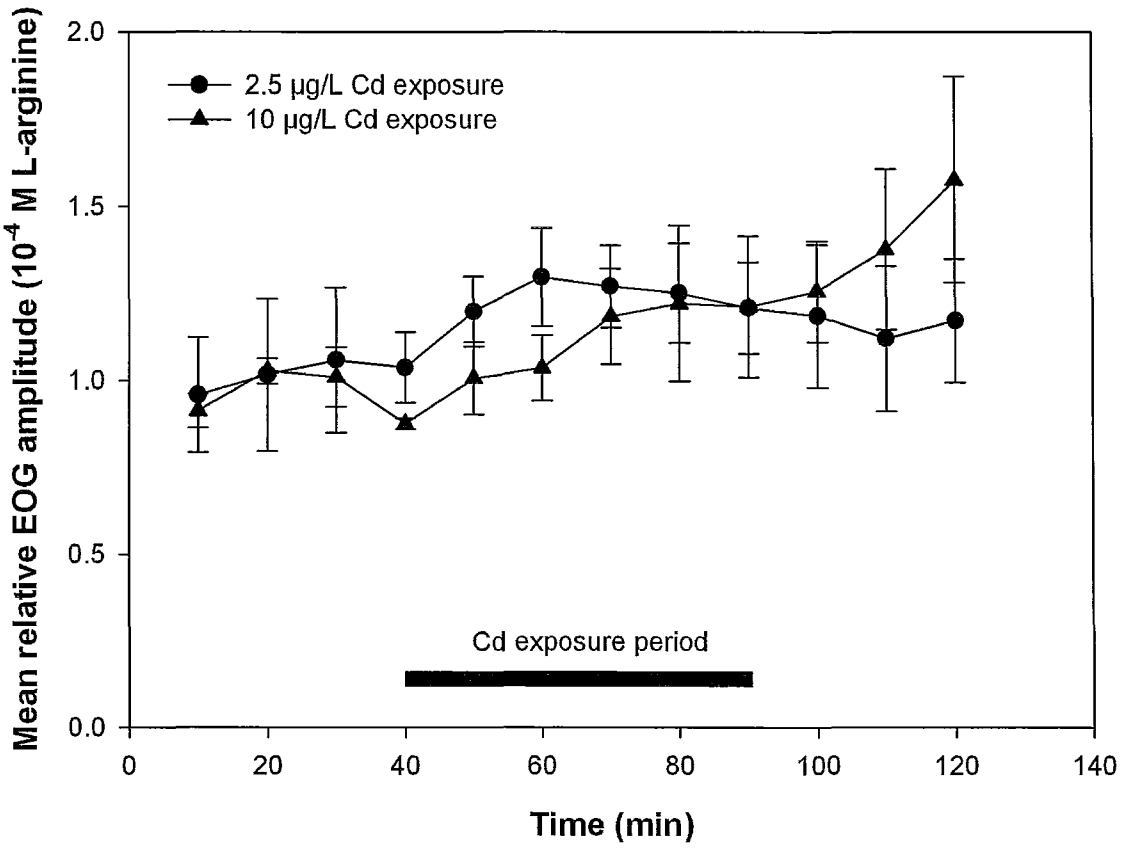

Figure 2-7. Mean ± SEM of the relative EOG response of fathead minnows to 10^{-4} M L-arginine for the pre-cadmium, cadmium exposure, and post-cadmium exposure periods at concentrations of 2.5 (n = 6) and 10 (n = 3) µg Cd L^{-1} (~ 22 and 90 nM). Relative responses were determined by dividing each response value for a given fish by the respective pre-metal exposure response average. Plotted means were determined by averaging the response to 10^{-4} M L-arginine of all fish in a given time period.

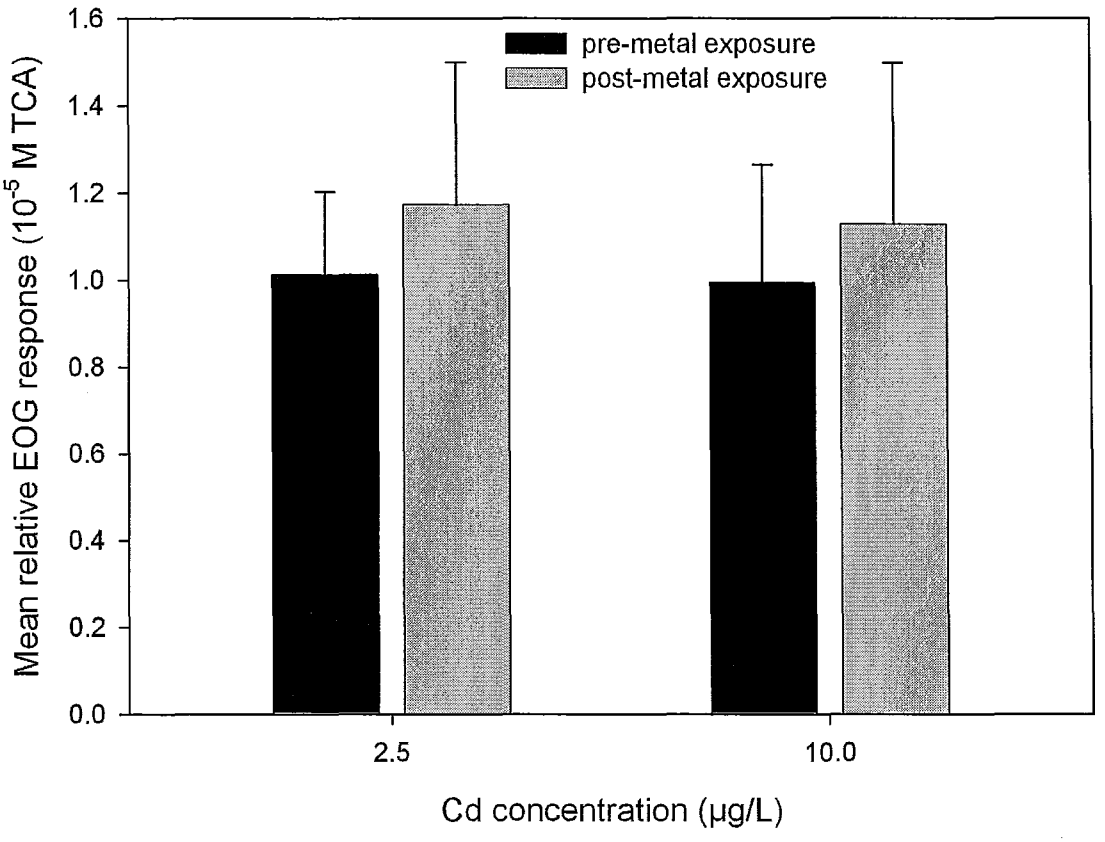

Figure 2-8. EOG response (mean ± SEM) of fathead minnows to 10^{-5} M taurocholic acid (TCA) prior to and following exposure to 2.5 (n = 4) or 10 (n = 3) µg Cd L^{-1} (~ 22 and 90 nM) for 1 hour. Relative responses were determined by dividing each response value for a given fish by the respective pre-metal exposure response average. Means were determined by averaging the 3 responses for a given exposure period (i.e., pre-metal exposure or post-metal exposure) for each fish and then determining a mean of means.

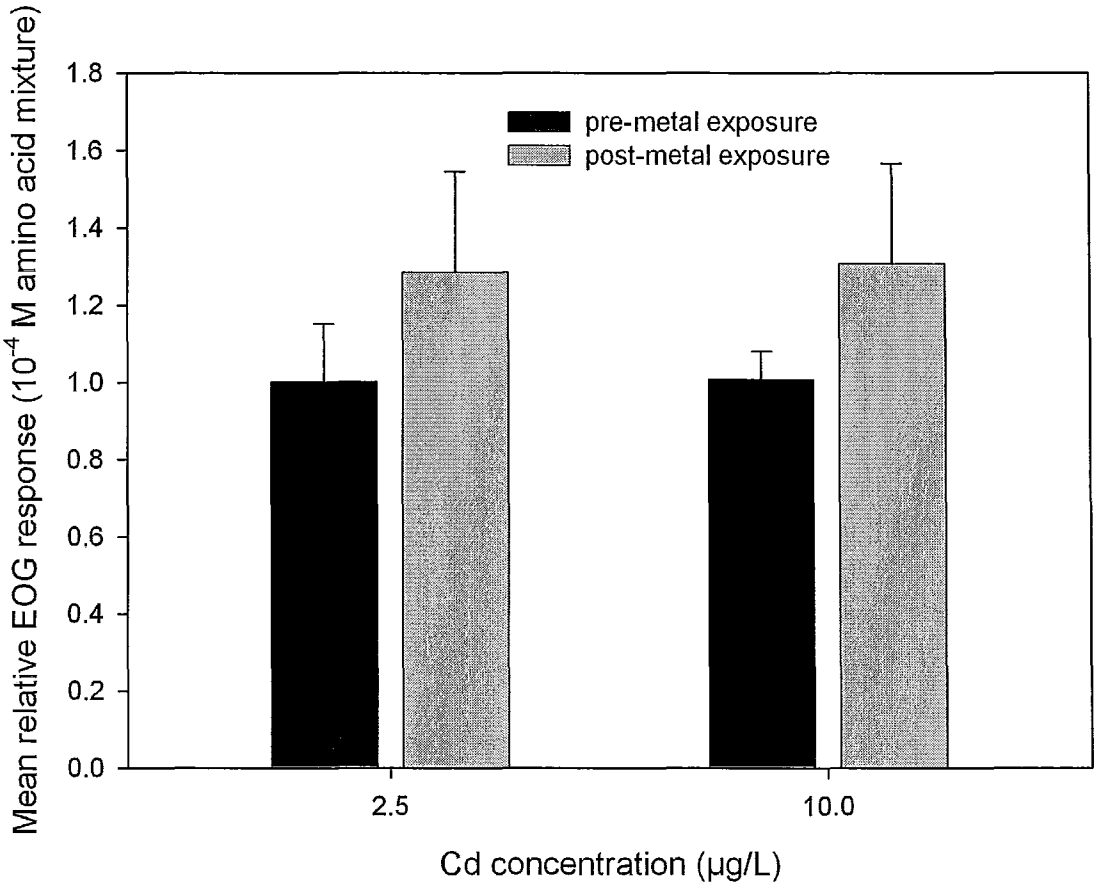

Figure 2-9. EOG response (mean ± SEM) of fathead minnows to 10^{-4} M amino acid mixture (10^{-4} L-arginine, L-alanine, L-serine, L-aspartic acid) prior to and following exposure to 2.5 (n = 4) or 10 (n = 3) µg Cd L^{-1} (~ 22 and 90 nM) for 1 hour. Relative responses were determined by dividing each response value for a given fish by the respective pre-metal exposure response average. Means were determined by averaging the 3 responses for a given exposure period (i.e., pre-metal exposure or post-metal exposure) for each fish and then determining a mean of means.

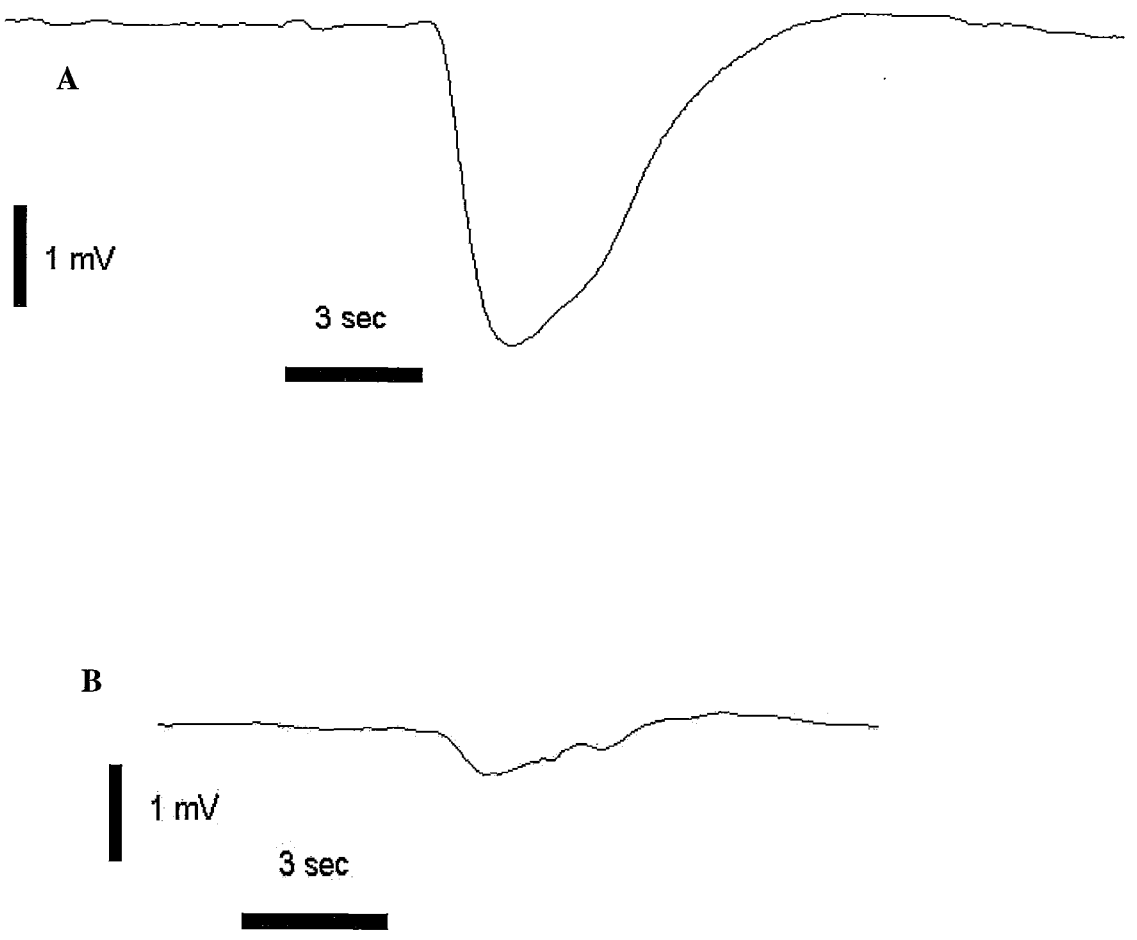

Figure 2-10. Example of a typical electro-olfactogram response of fathead minnows to 10^{-4} M L-arginine during A) pre-metal exposure period and B) 10 $\mu g\ L^{-1}$ metal exposure period. Vertical bar indicates millivolts scale and horizontal bar denotes stimulus delivery period and duration.

CHAPTER 3

DO YOU SMELL WHAT I SMELL? OLFACTORY IMPAIRMENT IN WILD YELLOW PERCH (*PERCA FLAVESCENS*) FROM METAL-CONTAMINATED WATERS IN NORTHERN ONTARIO, CANADA

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ABSTRACT

Metal contamination can pose serious problems for aquatic organisms. Many aquatic organisms can suffer changes in physiology and behaviour due to exposure to sublethal concentrations of waterborne metals. A wide diversity of aquatic organisms utilize chemical information to find food, find mates, assess habitat quality, recognize kin and assess predation risk. Recently it has been found that waterborne metals disrupt olfactory function in aquatic invertebrates and fishes which can potentially result in large-scale ecological consequences. However, these studies were conducted mainly with laboratory reared populations. We sampled yellow perch from three lakes along a polymetallic contamination gradient between North Bay and Sudbury, Ontario, Canada and conducted a series of physiological and behavioural studies to test their olfactory abilities. In our first experiment, we tested the integrated extracellular field potential response of yellow perch to stimulation of olfactory epithelium using an electro-olfactogram (EOG). We exposed perch to native yellow perch skin extract (alarm cue), rainbow trout skin extract (injured fish odour control), L-alanine (amino acid standard) and a water blank. Yellow perch from contaminated lakes responded with significantly greater intensity EOG responses to yellow perch skin extract and rainbow trout skin extract than yellow perch from the reference lake. In our second experiment, we exposed yellow perch from each lake to yellow perch skin extract, rainbow trout skin extract or dechlorinated tap water and observed their anti-predator behaviour. Yellow perch from the reference lake exhibited decreased activity (fright response) to yellow perch skin extract, but there was no significant change in activity for yellow perch from the

contaminated lakes. In our third experiment we examined neuron density in the olfactory rosette and olfactory bulb. We found no significant difference in neuron counts at both the olfactory rosette and the olfactory bulb among yellow perch from all three lakes. Finally, using short-term (3 h) *in vivo* waterborne exposures to ^{64}Cu and ^{109}Cd we found that Cd accumulated rapidly in the olfactory epithelium (OE) of yellow perch. The binding affinity (log $K_{Cu\text{-}OE}$) and binding capacity (B_{max}) of Cd in the OE of yellow perch from a reference and metal contaminated lake were 6.96 and 2.96 nmol Cd g^{-1} and 6.86 and 6.65 nmol Cd g^{-1} (of olfactory tissue) respectively in SSW. However, Cu accumulation in the OE was not saturable over the concentration range tested, preventing the determination of binding constants. Taken together, these results suggest a metal-induced decoupling between signal reception and perception, an alteration of metal binding dynamics in the OE, that signal propagation is impaired along the olfactory pathway, and that these phenomena are not due to a change in olfactory neuron density.

Key words: olfaction, predation, metals, yellow perch, electro-olfactogram, behaviour

INTRODUCTION

Metal contamination, at elevated levels, can pose serious problems for many aquatic organisms. The influx of metals into an aquatic system can dramatically alter water chemistry causing short and long-term changes in the physiology and behaviour of fishes (see reviews: Klaprat, et al., 1992; Scott and Sloman, 2004). Historically, metal toxicity was often evaluated on the basis of acute lethality toxicity testing. However, most natural metal-contaminated environments are substantially below contaminant levels required to induce acute toxicity, calling into question the ecological relevance of acute toxicity testing. Acute toxicity tests do not account for effects on ecological activities, such as foraging, reproduction or assessing predation risk. Although exposure to subacute metal concentrations may not be directly lethal to fishes, it may impede a fish's ability to function in an ecological context if their normal physiology and (or) behaviour is altered (Scott and Sloman, 2004).

Fishes use chemosensory information to mediate several important ecological activities such as foraging, assessing predation risk, finding mates, reproduction, kin recognition, locating and recognizing conspecifics, assessing habitat quality and homing/migration (Smith, 1992; Wisenden, 2000; Sandahl et al., 2006; Whitlock, 2006). The olfactory epithelium is constantly in direct contact with the aquatic environment and therefore to any contaminants that are present (Klaprat et al., 1992; Baldwin et al., 2003; Sandahl et al., 2004; Bettini et al., 2006). Moreover, waterborne metals such as aluminum (Al), silver (Ag), cadmium (Cd), copper (Cu), mercury (Hg), manganese (Mn), nickel (Ni) and zinc (Zn) have been shown to disrupt olfactory function in fishes (Hara et al.,

1976; Tallkvist et al., 1998, 2002; Hansen et al., 1999a,b; Beyers and Farmer, 2001; Baldwin et al. 2003; Persson et al., 2003; Scott et al., 2003; McPherson et al., 2004; Sandahl et al. 2004; Carreau and Pyle, 2005; Bettini et al, 2006; Sandahl et al., 2006). Direct exposure to contaminants may result in damage or death of olfactory receptor neurons. Previous studies have shown that exposure to trace amounts of metals over 4 h led to ORN cell death (Hansen et al., 1999b; Baldwin et al., 2003; Bettini et al., 2006) and although ORNs are capable of regenerating it may be weeks before recovery (Zielinski and Hara, 1992). Since ORNs are continually exposed to waterborne metals, neuron density at the olfactory epithelium may be affected which could influence propagation of the olfactory neuro-electrical signal to the olfactory bulb. Impairment of chemosensory function can lead to disruption of activities crucial for survival, potentially resulting in large-scale effects that have gone relatively unexplored by the ecological risk assessment community.

The olfactory system is activated when odourant molecules bind reversibly to the receptor of the olfactory receptor neuron (ORN). Once the receptor is activated it initiates one or more signal transduction cascades that cause an influx of Ca^{2+} into the ORN resulting in depolarization and propagation of a signal down the axon to the olfactory bulb (reviewed in Restrepo et al., 1990; Delay and Restrepo, 2004; Cens et al., 2006; Craven and Zagotta, 2006). Olfactory neurons synapse with the terminal ends of specialized structures in the olfactory bulb called glomeruli which provide the first processing of information. Information is coded and then sent to the olfactory cortex for further processing (Hara, 1992; Firestein, 2001; Yoshihara et al., 2001). Olfactory

systems, particularly signal transduction cascades, show a high degree of phylogenetic conservation among both invertebrates and vertebrates. Consequently, once the mechanism for metal-impaired olfaction is identified in one taxon, it may be possible to extrapolate the mechanism to a wider range of taxonomically diverse animals.

Industrial activities have been taking place in the Sudbury region (northern Ontario, Canada) for more than a century, resulting in some of the most heavily metal-contaminated environments in the world (Keller and Gun, 1995). The metals of interest in this region are primarily Cd, Cu, Ni and Zn (Brodeur et al., 1997; Rajotte and Couture, 2002; Couture and Rajotte, 2003). The 'zone of influence' from industrial activities extends approximately 17,000 km^2 around the epicenter of mining and processing activities and includes approximately 7000 lakes (Keller et al., 1992). Although remediation efforts have been ongoing for decades, several metal intolerant species have been extirpated. One of the few metal-tolerant fishes found in lakes inside the zone of influence are yellow perch, *Perca flavescens*. Yellow perch are widely distributed throughout North America (Scott and Crossman, 1973), and occupy important trophic positions in aquatic ecosystems depending on their age and resource availability (i.e., planktivore, benthivore, and piscivore) (Sherwood et al., 2002). The majority of toxicity studies are conducted on laboratory-reared fishes (e.g., rainbow trout *Oncorhynchus mykiss*, fathead minnows *Pimephales promelas*), however; these species are not typically found in Sudbury lakes. Therefore it is important to study fishes specifically from metal-contaminated systems to provide the highest degree of ecological relevance.

A wide diversity of fishes, including yellow perch (Mirza et al., 2003), mediate their risk of predation using chemosensory information (reviewed in: Smith, 1992; Chivers and Smith, 1998; Kats and Dill, 1998; Wisenden, 2000; Chivers and Mirza, 2001). Prey that have been injured or captured release a chemical alarm cue that 'warns' nearby individuals of potential danger and increases their probability of survival (reviewed in Chivers and Smith, 1998; Mirza and Chivers, 2001). Detection of alarm cues sets off a cascade of events from activation of the receptor and signal transduction pathway leading to processing of that information in higher brain centers (Hara, 1992; Firestein, 2001; Yoshihara et al., 2001) resulting in appropriate antipredator behaviour, such as reduced movement, reduced foraging, tighter group cohesion or area avoidance (Lima and Dill, 1990). Therefore, it is important to understand how chemosensory information is used by yellow perch to mediate ecological processes that are influenced by environmental contamination in order that environmental risk assessments provide the highest degree of ecological relevance.

A chemosensory-based biotic ligand model (cbBLM) would be a tool by which to predict chronic sub-lethal metal toxicity in wild fishes and would incorporate ecologically relevant endpoints such as the inability to chemically detect the presence of a predator. However, before a cbBLM can be created for chronically exposed fishes, the binding dynamics of fishes from these populations need to be evaluated and binding constants (i.e., the binding affinity and binding capacity) of metals to the biotic ligand must be determined. The determination of these binding constants will allow for the field validation of a laboratory model that is being produced and ensure that results in the lab

can be extrapolated to wild fish populations chronically exposed to metals in contaminated lakes.

In this study, we examined metal-induced chemosensory impairment in yellow perch from contaminated and non-contaminated lakes along a metal-contamination gradient around Sudbury, Ontario, Canada. We collected juvenile yellow perch from three different lakes: Hannah Lake (high contamination), Ramsey Lake (intermediate contamination) and James Lake (low contamination). First, we measured the integrated extracellular field potential response of yellow perch to stimulation of olfactory epithelium (OE) using electro-olfactogram (EOG) to yellow perch alarm cues. Second, using yellow perch from the same three lakes, we tested their antipredator behaviour in response to yellow perch alarm cues. Third, we examined neuronal cell density at the OE and olfactory bulb to evaluate whether changes in the number of neurons at either site could have contributed to the observed differences among fish from the three lakes. Finally, we determined the binding dynamics of Cu and Cd to the OE of wild yellow perch and compared the results to those determined in laboratory-raised FHM (Chapter 2) in order to determine if laboratory derived binding constants can be extrapolated to chronically metal-exposed wild fish populations.

METHODS

Collection and maintenance of animals

For EOG and behavioural experiments as well as neuron density counting juvenile yellow perch were collected by angling from Hannah (high metal-contaminated) and Ramsey Lakes (intermediate contamination) and James Lake (reference) in early August

and early October 2005 and transported in aerated native lake water to Nipissing University (Fig. 3-1). Yellow perch were held in 60 L plastic tanks at 20 °C under a 16:8 L:D photoperiod for 48 h before being used in experiments. Fish were not fed during this period because past experience has shown that yellow perch from these northern lakes will not readily feed in the laboratory (personal observation). Waterborne concentrations of metals of interest are listed in Table 3-1 and basic water quality parameters of the study lakes are listed in Table 3-2.

For metal-ligand binding experiments yellow perch (*Perca flavescens*) (10-26 g) were collected over a two day period during the summer of 2006 from two lakes in north-eastern Ontario. James Lake (46° 17′21″N, 78°59′26″W), an uncontaminated reference lake, is located on the Nipissing University –Alcan Environmental Research Preserve near North Bay, Ontario. Hannah Lake (46°26′35″N, 81°02′24″W) is located within the city limits of Sudbury, Ontario and represents a metal-contaminated lake. Fish were captured by angling and placed into 20 L containers (20-25 fish) filled with native lake water (16-20 °C) and aerated using battery powered air pumps. Fish were then transported to McMaster University in Hamilton, Ontario; mortality due to transport was ~ 5%. Upon arrival fish were place into 200 L flow-through tanks containing synthetic softwater (SSW) which was produced by mixing reverse osmosis and dechlorinated Hamilton municipal tap water. Final water composition was Ca = 125.7 μM, Na = 153.6 μM, Mg = 37.4 μM, hardness ~ 16 mg L^{-1} as $CaCO_3$, dissolved organic carbon ~ 1 mg L^{-1}, and pH 6.8. Temperature was 12 °C, an a 12h:12h light:dark photoperiod was used. Fish were

held under these conditions without food for approximately 24 hours prior to experimentation.

EOG experiments

Stimulus preparation

For testing electrophysiological responses, a 10^{-5} M L-alanine standard test stimulus was prepared fresh daily by diluting a fresh 10^{-2} M L-alanine stock solution with dechlorinated municipal water. We also prepared skin extract stimuli (chemical alarm cue) from juvenile rainbow trout (mean ± SEM standard length, 8.05 ± 0.33 cm, n = 11), James Lake yellow perch (9.17 ± 0.32 cm, n = 7), Ramsey Lake yellow perch (9.15 ± 0.56 cm, n = 10), and Hannah Lake yellow perch (11.90 ± 0.75 cm, n = 6). Fish were sacrificed by severing the spinal cord in accordance with guidelines of the Canadian Council on Animal Care. Skin was removed from both sides of each fish and placed into 100 mL of ice-chilled dechlorinated tap water. For yellow perch, 81.03 cm^2 (Hannah Lake), 82.64 cm^2 (James Lake) and 80.91 cm^2 (Ramsey Lake) of skin were used to produce yellow perch skin extract stimuli. For rainbow trout 134.43 cm^2 of skin was used; this skin extract served as a control for stimuli from an injured fish. Skin was homogenized, then filtered through polywool filter floss to remove any large particles and then diluted in dechlorinated tap water to a final volume of 1600 mL for yellow perch from each lake and 2685 mL for rainbow trout giving us final concentrations of 0.051 cm^2 skin mL^{-1} (Hannah Lake), 0.052 cm^2 skin mL^{-1} (James Lake), 0.051 cm^2 skin mL^{-1} (Ramsey Lake) and 0.050 cm^2 skin mL^{-1} rainbow trout. Skin extracts (yellow perch and rainbow trout) were then frozen at -20 °C in 50 mL aliquots until used.

Electro-olfactogram recording

Yellow perch were prepared for EOG experimentation using methodology adapted from Sveinsson and Hara (2000). Yellow perch were anaesthetized in a solution of MS222 (150 mg L^{-1}; ethyl 3-aminobenzoate methanesulfonate salt, Sigma, Oakville, ON, Canada) and subsequently immobilized by an epaxial intramuscular injection of Flaxedil (gallamine triethiodide, 3 mg kg^{-1} body mass, Sigma, Oakville, ON, Canada) (Sveinsson and Hara, 2000). Anaesthetized and immobilized fish were wrapped in wet tissue (to prevent desiccation), leaving the head and tail regions exposed. Fish were secured and electrically grounded in a Plexiglas perfusion chamber where the gills were perfused with a constant supply of oxygenated, dechlorinated water containing 50 mg L^{-1} MS222 via a tube inserted into the mouth (Sveinsson and Hara, 2000). The right olfactory rosette was exposed by removing the nasal septum separating the anterior and posterior nares and the exposed olfactory chamber was perfused with dechlorinated water.

Electro-olfactogram responses were differentially recorded (difference between the recording and reference electrodes) using saline (0.9%)-gelatine (4%) filled capillary tubes (tip diameter, 60-80 μm) bridged to an Ag/AgCl electrode (Type MEH8, WP Instruments, Sarasota, FL, U.S.A) filled with 3 M KCl. The recording electrode was placed in the olfactory chamber, close to but not touching the olfactory epithelium, near the posterior most lamellae to obtain the largest response to a standard (10^{-5} M L-alanine; Sigma, Oakville, ON, Canada) and a minimal response to a dechlorinated water blank. A reference electrode was placed on the surface of the skin posterior to the perfused naris (Sveinsson and Hara, 2000). Electro-olfactogram responses were amplified using a DC

headstage and DC preamplifier (AD Instruments, Colorado Springs, CO, U.S.A.) and recorded using a computer-assisted data acquisition system (model ML 750, AD Instruments, Colorado Springs, CO, U.S.A.) and Chart® version 5 software.

EOG experimental protocol

Experimental stimuli were delivered one at a time using a gravity-flow stimulus delivery system for five seconds, after which the flow of dechlorinated water was immediately restored. Two minutes were left between each stimulus delivery to minimize olfactory receptor adaptation to odourants. Before testing, fish were allowed to acclimate in the Plexiglas perfusion chamber for 15-20 minutes. Using a randomized block design, four stimuli were delivered in random order: 1) dechlorinated water (blank), 2) 10^{-5} M L-alanine (standard cue), 3) yellow perch skin extract (conspecific alarm cue), and rainbow trout skin extract (control for odour of an injured fish). Each stimulus was delivered three times consecutively. At the time of testing, gill perfusion water and all experimental stimuli were at room temperature (18-22^{O}C). Ten fish per lake were used in these experiments.

A subset of five yellow perch from each lake also received two additional types of yellow perch skin extract in order to determine if yellow perch from each lake respond differently to alarm cues produced by non-native yellow perch; i.e., yellow perch skin extract donors came from the other two lakes.

Behavioural experiments

Stimulus preperation

For behavioural trials, 14.12 cm^2 (Hannah Lake), 14.23 cm^2 (James Lake) and 14.09 cm^2 (Ramsey Lake) of skin was removed from three yellow perch from each lake (mean ± SEM James Lake: 11.0 ± 0.85 cm; Ramsey Lake: 7.97 ± 0.23 cm; Hannah Lake: 8.63 ± 0.27 cm). Skin was homogenized and filtered (as described above), then diluted in distilled water to make a final volume of 275 mL for each population, producing concentrations of 0.051 cm^2 skin mL^{-1} (Hannah Lake), 0.052 cm^2 skin mL^{-1} (James Lake) and 0.051 cm^2 skin mL^{-1} (Ramsey Lake). Stimuli were frozen in 25 mL aliquots at – 20 °C until used in the experiment. Rainbow trout stimulus was from the same batch used in the EOG experiments.

We produced a brine shrimp stimulus by placing 1 g of frozen brine shrimp (*Artemia salina*) into 250 mL of dechlorinated water and then filtering through polywool filter floss to remove large particles (final concentration 0.004 g brine shrimp mL^{-1} water). The supernatant was used to stimulate swimming activity in yellow perch during the pre-stimulus period. Yellow perch typically remain stationary when isolated thus an additional stimulus is required to promote swimming/searching activity (Mirza et al., 2003). Brine shrimp stimulus was made fresh before every set of trials.

Behavioural assays

Juvenile yellow perch from each lake were exposed to either: yellow perch skin extract, rainbow trout skin extract or dechlorinated tap water (n = 15 per treatment for James and Hannah Lakes; Ramsey lake n = 12 for yellow perch skin extract, n = 13 for rainbow trout skin extract, n = 12 for dechlorinated tap water). We tested 45 yellow perch from each of James and Hannah Lakes and 37 yellow perch from Ramsey Lake (mean ±

SEM; James Lake: 11.0 ± 0.15 cm SL, 17.3 ± 0.48 g; Ramsey Lake: 8.37 ± 0.27 cm SL, 9.03 ± 0.82 g; Hannah Lake: 9.03 ± 0.24 cm SL, 10.5 ± 0.79 g). Trials were conducted in 37-l aquaria (50 x 25 x 30 cm). All yellow perch were tested individually and exposed to conspecific alarm cues from their respective populations to mimic natural exposure conditions. Each test aquarium had a single air stone located in the middle of the back wall of the short side of the tank. A plastic tube was situated next to the air stone for introduction of a chemical stimulus into the tank. Tanks were wrapped with black plastic on three sides to visually occlude test fish from those in adjacent tanks to avoid any behavioural influences from other fish. All yellow perch were tested in clean North Bay dechlorinated tap water (median pH: 7.0, range: 6.7 – 7.6; alkalinity: 23.4 ± 2.5 mg L^{-1} as $CaCO_3$; hardness: 35.6 ± 3.9 mg L^{-1} as $CaCO_3$, n = 13).

Trials were conducted after a 24 h period of acclimation to the test tank. Each trial was 17 min in length and consisted of an 8-min pre- and 8-min post-stimulus period, with a 1-min stimulus introduction period between the pre- and post-stimulus periods. At the beginning of each trial, 60 mL of water was removed from the tank through the stimulus injection tube with a syringe and discarded. This procedure removed any stagnant water in the injection tube. A second and third 60 mL syringe of water was removed and retained. We then injected 5 mL of brine shrimp stimulus and slowly flushed it through the injection tube with one of the two retained 60 mL syringes at a rate of 1 mL s^{-1}. After the pre-stimulus period, 5 mL of the test stimulus was injected into the tank and again flushed slowly through the stimulus delivery line with the 60-mL of water at the same rate. Dye trials indicated that it took approximately 30 s for the test stimulus to distribute

to all parts of the tank. After trials fish were euthanized as described above and used for histological analysis (see below).

Neuron density counts

To ensure that the neuron density analysis was conducted blind to group designation, all animals were coded prior to tissue harvesting. Six adult fish from each lake (used in behavioural tests) were included in the histological analysis.

Tissue preparation and sectioning

Animals were sacrificed by severing the spinal column. Subsequently, both olfactory rosettes and the olfactory bulb were extracted from each fish and drop fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). Tissue blocks remained in fixative for an additional 72 h, before being placed in three ten minute washes of phosphate buffer followed by 1% osmium tetroxide for 1 h. The tissue blocks were then dehydrated in a graded series of ethanol solutions and embedded in Spurr's embedding medium (Ladd Research Industries, Burlington, Vermont).

Excess embedding medium was removed and thick sections (1 μm) were taken from the olfactory rosettes and olfactory bulbs. Thick sections were stained with toluidine blue (2%) in order to quantify the number of neurons in the tissue volume. Thick sections were photographed using a digital camera affixed to a Leica light microscope (400X original magnification).

Neuron counting

Estimates of the number of neurons were determined using an unbiased dissector technique (Black et al., 1990). This stereological technique produces an estimate of the

neuronal density in a given volume of tissue. This method is necessary to account for changes in neuronal density due to hypertrophy or tissue processing effects, and to ensure that neurons are only counted once regardless of their size and configuration.

Neurons were distinguished from glial or support cells by the presence of a central nucleolus within a pale nucleus (Fig 3-2). Neurons were counted by comparing pairs of adjacent thick sections in the series. Neurons were counted if they were observed in one of the sections within the area limited by an unbiased sampling frame (400 μm^2), but not observed in the other section in the dissector pair. This sampling technique ensured that neurons were only counted once regardless of the number of images in which they appeared. The symbol Q was used to denote the total number of neurons counted in each series. The total volume for neuronal counting (V_{neur}) was derived as: $V_{neur} = A*H$, where A was the area of the counting frame and H was the total thickness of the series. Neuron density (N_{neur}) was calculated as: $N_{neur} = Q/V_{neur}$. These data were subsequently converted to neurons per mm^3 which served as the final units of analysis.

For the rosette tissue, randomly selected sampling regions were chosen within bands of sensory neurons, as these bands contain the primary sensory neurons involved in olfaction. In the olfactory bulbs, the randomly chosen sampling frames were selected from the peripheral region of the bulb.

Metal-ligand binding experiments

Olfactory epithelium ^{109}Cd kinetic binding assays

To determine the binding characteristics of cadmium to the olfactory epithelium (OE) of yellow perch, a radiolabelled ^{109}Cd *in vivo* binding assay was used (adapting the

methods of Niyogi et al. 2004; Niyogi and Wood, 2004). Fish from Hannah L. were randomly assigned to four 18 L Rubbermaid® containers for a total of 6-7 fish per group. Each container represented a different Cd concentration, forming a range of exposures. This arrangement was duplicated for James L. fish, for a total of eight tanks. The containers were placed on a flow-through wet table to keep temperature constant at 12 °C. Before the addition of fish, each container was filled with 6 L of SSW (same water as lab holding water) and subsequently spiked with Cd (as $Cd(NO_3)_2 \cdot 4\ H_2O$; Fisher Scientific, Canada) and 3 $\mu Ci\ L^{-1}$ ^{109}Cd (as $CdCl_2$, specific activity = 3.65 $\mu Ci\ \mu g^{-1}$, (I.I.C.H., Kansas, USA) to achieve measured dissolved Cd concentrations of 31.4, 56.1, 105.1, 313.7 nM (for James L.) and 30.0, 54.2, 100.0, and 339.5 nM (for Hannah L.).

Duplicate water samples (10 mL) were taken at the beginning and end of the assay for each exposure replicate. Water samples were filtered through a 0.45μm syringe tip filter (PALL Acrodisc™ 25 mm syringe filter) and acidified to 1% using concentrated trace-metal grade HNO_3 (Fisher Scientific, Canada). After a three hour exposure period, the yellow perch were removed from their containers and euthanized by an overdose of MS-222 (600 mg L^{-1}). The entire gastrointestinal tract (GIT) and whole gill basket was removed by dissection for a subsequent ^{109}Cd *in vitro* gastro-intestinal binding assay and for determination of yellow perch ^{109}Cd gill binding kinetics (see Klinck et al., 2007 for details). Immediately after the removal of the GIT and whole gill basket, the pair of olfactory rosettes were excised, rinsed with deionized water, blotted dry, and placed into a pre-weighed 0.5 mL micro-centrifuge tube.

Olfactory epithelium ^{64}Cu kinetic binding assays

To determine the binding characteristics of Cu to the olfactory epithelium of yellow perch, a radiolabelled ^{64}Cu *in vivo* binding assay was employed using the methods described above, however, with one fewer concentration in the Hannah L. series (total of seven tanks). ^{64}Cu was prepared by irradiating dried $Cu(NO_3)_2$ (300 μg) at McMaster University Nuclear Reactor to produce a radioactivity level of 0.6 mCi (half life 12.9 h). Assay water (6 L) was then spiked with the ^{64}Cu solution to produce measured total copper concentrations of 262, 352.4, 489.3, and 968.4 nM (for James L.) and 308.9, 597.8, and 1025.8 nM (for Hannah L.), in 7 different tanks.

Water and Tissue analysis

Immediately after the completion of an experiment the water and tissue samples were counted for radioactivity using a 1480 Wallac Wizard 3" automatic gamma counter (Perkin Elmer, Toronto, Canada). The weight of the tissue samples was then determined for use in subsequent calculations. The dissolved Cu and Cd concentrations of the water samples were then determined using GFAAS (Varian GTA 110, Varian Scientific, Mulgrave, Australia). A certified reference material (TM-15) from the National Research Council of Canada was used to ensure accuracy. The concentrations of Ca^{2+}, Na^{+}, and Mg^{2+} in water samples were measured using FAAS (Varian SpectrAA-220 FS, Varian Scientific, Australia) against standard curves produced using reference standards from Fisher Scientific, USA.

Calculations and statistical analyses

All data are presented as means ± SEM. In the EOG trials, we conducted two MANOVAs to determine if there was a significant interaction of lake and stimulus on the

integrated extracellular field potential at the olfactory epithelium of yellow perch. The first MANOVA determined the interaction of yellow perch from each lake and three stimuli (10^{-5} M L-alanine, native yellow perch skin extract, rainbow trout skin extract). Subsequent one way ANOVAs were conducted with Dunnett's post hoc analysis to determine if significant differences existed when comparing the extracellular field potentials at the OE of yellow perch from the two contaminated lakes to that of James Lake yellow perch. The second MANOVA was to determine the interaction of yellow perch from each lake and three skin-extract stimuli (James, Ramsey, and Hannah yellow perch skin extract) on the integrated extracellular field potential at the olfactory epithelium of yellow perch. Subsequent one way ANOVAs were conducted with Tukey-Kramer post hoc analysis to determine if significant differences existed when comparing the extracellular field potential among yellow perch exposed to each individual stimulus only.

In the behavioural trials, during both the pre- and post-stimulus periods we recorded time spent moving, dashing (a burst of rapid, disorientated swimming) and freezing (remaining motionless in the water column or on the substrate for a minimum of 30 s). Changes between the pre-stimulus and post-stimulus periods were calculated (post-stimulus minus pre-stimulus), and the difference in changes among treatments was analyzed using a 2-way ANOVA testing for main effects of lake, stimulus and lake x stimulus interactions followed by Tukey HSD post-hoc analysis. Dashing and freezing were analyzed using Chi-square tests for independence followed by post hoc pairwise comparisons.

We compared neuron counts at the olfactory epithelium and olfactory bulb from yellow perch among the three lakes with a one-way ANOVA.

To determine the amount of newly bound Cu and Cd to the OE the following equation was employed:

$$a(bc^{-1})^{-1}$$

where: a = radioactive counts in the OE (cpm g^{-1} wet tissue weight); b = radioactivity counts in the water (cpm L^{-1}); c = dissolved metal concentration in the water (μg L^{-1}). The resulting values were then converted from μg g^{-1} to nM g^{-1}. Non-linear regressions were employed for the analysis of Cd binding to the OE of YP. B_{max} (binding capacity) and log K (binding affinity) were then determined from these non-linear regressions. B_{max} was the value on the y-axis where the maximum amount of Cd binding occurred. Log K was determined as the log (x^{-1}) where x represents the total dissolved metal concentration in the water that provides metal binding of half the B_{max} in molar units. The binding of Cu to the OE of FHM was not saturable and therefore log K and B_{max} could not be determined. To determine if new Cd binding in the olfactory rosettes of wild YP from James L. and Hannah L. were statistically different, independent t-tests were utilized at each waterborne Cd concentration. A Bonferroni adjustment to α (0.05) was used to control for family-wise error rates when conducting independent t-tests. Values were considered significant when $p \leq 0.013$ after the Bonferroni adjustment. Statistical analyses were performed using JMP 5.0 for Windows and non-linear regressions were performed using Sigmaplot version 8.0 for Windows.

RESULTS

Yellow perch from clean and contaminated lakes exhibited significantly different extracellular field potentials at the olfactory epithelium when exposed to the three chemical stimuli (L-alanine, native yellow perch alarm cue, rainbow trout skin extract). Moreover, yellow perch from the different lakes exhibited significantly different extracellular field potentials when exposed to native yellow perch alarm cue, rainbow trout skin extract and L-alanine (Table 3-3). When exposed to native yellow perch skin extract, yellow perch from the contaminated lakes (Ramsey and Hannah Lakes) exhibited significantly higher extracellular field potentials (2.7 and 2.3 times greater, respectively) than yellow perch from the reference lake (James Lake) ($p < 0.05$; Fig. 3-3A). Similarly, yellow perch from Ramsey and Hannah lakes had increased EOG responses to rainbow trout skin extract (2.3 and 1.8 times greater, respectively) and L-alanine (3.3 and 1.9 times greater, respectively) compared to responses of James Lake yellow perch ($p < 0.05$; Fig. 3-3B).

When yellow perch from each lake were exposed to conspecific alarm cue of donors from each of the three lakes, there was an overall significant interaction of lake and donor population on the extracellular field potential at the OE. Comparing the EOG response of yellow perch exposed to alarm cues from donors of all three populations within each lake, only yellow perch from James Lake (clean) exhibited a significantly different extracellular field potential to native and non-native alarm cues. There was no significant difference in extracellular field potential in yellow perch from Ramsey Lake and Hannah Lake (contaminated) exposed to alarm cues from all three populations (Fig.

3-3B, Table 3-3). James Lake yellow perch responded with a significantly greater EOG response to alarm cues from both Hannah Lake (1.68 times; $p < 0.05$) and Ramsey Lake (1.73 times; $p < 0.05$) donors compared to native alarm cue donors from James Lake (Fig. 3-3B). Yellow perch from Ramsey and Hannah Lakes did not significantly differ in their EOG response to alarm cues from donors from all three lakes (Fig 3-3B).

Testing the antipredator behaviour of yellow perch we found that perch from different lakes responded differentially to chemical stimuli after we stimulated initial activity during the pre-stimulus period with the brine shrimp cue (Table 3-3). Yellow perch from James Lake (reference lake) exposed to conspecific alarm cue decreased activity significantly more than yellow perch exposed to both rainbow trout skin extract (14.9 times) and control water (18.2 times) (all p's < 0.05; Fig. 3-4) . Similarly, yellow perch from James Lake exposed to yellow perch alarm cues decreased activity significantly more than yellow perch from either of the contaminated lakes (Ramsey Lake: 4.4 times and Hannah Lake: 400 times) exposed to yellow perch alarm cues (both p's < 0.05; Fig. 3-4). Yellow perch from both contaminated lakes did not exhibit any differential response to rainbow trout extract or dechlorinated water, in any of the behavioural tests (Fig. 3-4).

A greater proportion of yellow perch from the reference lake exhibited freezing behaviour when exposed to conspecific alarm cues than yellow perch from the contaminated lakes ($\chi^2 = 17.78$, df = 2, $p < 0.0001$; Table 3-4). James lake yellow perch also exhibited freezing behaviour significantly more when exposed to conspecific alarm

cue than when exposed to rainbow trout skin extract or control water ($\chi^2 = 13.37$, df = 2, p = 0.001; Table 3-4).

Neuron counts did not differ statistically in either the olfactory rosettes (mean neurons per mm^3 ± SE, James Lake = $1.63 \times 10^6 \pm 8.42 \times 10^4$, Ramsey Lake = $1.73 \times 10^6 \pm 1.56 \times 10^4$, Hannah Lake = $1.71 \times 10^6 \pm 1.41 \times 10^4$) or olfactory bulbs (James Lake = 15589 ± 965.88 neurons, Ramsey Lake = 17881 ± 1632.45 neurons, Hannah Lake = 17184.22 ± 1054.01 neurons) in yellow perch among the three lakes ($F_{2,15} = 0.144$, p = 0.867; $F_{2,15} = 0.880$, p = 0.435).

The OE of YP exhibited saturation kinetics for Cd-binding in fish from both lakes within the [Cd] range of 30 - 340 nM. The OE of YP from James lake had log $K_{Cd\text{-}OE}$ = 6.96 and a B_{max} = 2.96 nmol g^{-1}, while the OE of YP from Hannah lake had a log $K_{Cd\text{-}OE}$ = 6.86 and a B_{max} = 6.65 nmol g^{-1} (Fig. 3-5). Moreover, the amount of new Cd that bound to the OE of Hannah L. YP was greater than that of James L. YP at each of the concentrations tested (independent t-tests; 30-31 nM: t = 2.95, d.f. = 11, p = 0.01; 54-56 nM: t = 3.62, d.f. = 11, p = 0.004; 100-105 nM: t = 5.65, d.f. = 11, p = 0.0001; 314-340 nM: t = 3.45, d.f. = 12, p = 0.0048).

The binding kinetics of Cu in the OE of yellow perch from these two lakes could not be determined due to the linear relationship of the binding. Therefore no saturation occurred within the Cu concentration range tested, and B_{max} and log K could not be calculated (Fig. 3-6).

DISCUSSION

Taken together, results from this study are the first to demonstrate chemosensory impairment in wild juvenile yellow perch from metal-contaminated lakes. Juvenile yellow perch from the contaminated lakes exhibited a significantly higher extracellular field potential at the OE than yellow perch from the clean lake. However, when we conducted behavioural assays yellow perch from the contaminated lakes did not respond to conspecific alarm cue with a fright response unlike yellow perch from the clean lake. These results suggest that despite greater signals being generated at the OE those signals are either not reaching the olfactory bulb or are not being processed in the olfactory bulb and (or) higher brain centers. However, neuronal density counts at the olfactory rosette and olfactory bulb did not differ for juvenile yellow perch among the three lakes.

Our study did not focus on the effects of a single metal as documented in past studies testing for metal effects on chemosensation (Hara et al., 1976; Hansen et al., 1999a, b; Beyers and Farmer, 2001; Baldwin et al. 2003; Persson et al., 2003; Scott et al., 2003;; Sandahl et al. 2004; Carreau and Pyle, 2005; Sandahl et al., 2006). Moreover, the majority of the aforementioned studies used acute exposures in typical laboratory species such as rainbow trout and fathead minnows. However, these species are not typically found in metal-contaminated lakes of the Canadian Shield and single metal acute exposures do not reflect natural conditions. We collected yellow perch from the wild that had been chronically exposed to elevated metal concentrations throughout their lives and for numerous generations. This allowed us to examine the effects of chronic exposure on

olfaction in wild fishes exposed to a mixture of metal contaminants providing a higher degree of ecological relevance.

Although numerous studies have examined the effects of metals on olfaction, little information is available regarding the specific cellular targets for different metals. Different metals may be sequestered in different parts of the olfactory system and brain. Metals such as methyl Hg, Mn and Pb are transported to the olfactory bulb and can be passed on to the olfactory cortex and other brain regions (Tallkvist et al., 1998; Persson et al., 2003). Other metals such as inorganic Cd, Hg and Ni enter the neuronal cell body and are transported to the terminal end of the axon, but do not pass into the olfactory bulb (Tallkvist et al., 1998; Persson et al., 2003; Scott et al., 2003). Persson et al. (2003) also found Zn within the glomerular and granular layers of the olfactory bulb, but no evidence that Zn passes onto higher brain centers. Julliard et al. (1995) found that Cu is not sequestered in the olfactory cell, but stored in melanophores of the lamina propria (the region surrounding the axon outside the myelin sheathe). The authors argue that this storage of Cu may be part of the detoxification process because Cu tends to be tightly regulated, but these results do not coincide with what is reported for other divalent metals. Taken together these studies demonstrate that the presence of metals in various parts of the ORN, olfactory bulb and higher brain centers provide metal ions access to the molecular signal transduction machinery where they can potentially interfere with signal transduction within various levels of the olfactory system (Sloman et al., 2005).

In our study, the change in extracellular field potential at the olfactory epithelium was greater in yellow perch from contaminated lakes than the reference lake, which is

contradictory to results from olfactory studies of salmonids exposed to aqueous Cu. Hatchery-reared salmonids exposed to environmentally-relevant concentrations of Cu exhibited a significantly lower extracellular field potential at the olfactory epithelium compared to non-exposed fish (Hansen et al., 1999; Baldwin et al., 2003; Sandahl et al., 2004; Sandahl et al., 2006). The differences in results may be simply explained by the fact that we used wild-caught fish instead of hatchery-reared fishes and that our exposure was to a mixture of metals instead of a single metal. Furthermore, fish in our study were exposed to metals for years compared to the range of 30 minutes to 7 days in the other studies. However, both sets of results may be explained by how metals influence the signal transduction pathways. Cyclic nucleotide gated (CNG) channels are selective towards monovalent ions such as Na^+ and K^+, but are nonselective towards divalent ions such as Cd^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} (Audersirk, 1993; Vijerberg et al., 2002; Craven and Zagotta, 2006). At high concentrations, divalent ions can block the channel (Elinder and Arhem, 2004). Without sufficient influx of Ca^{2+} the ORN may not reach the threshold potential required to generate an action potential; however, there would still be a small change in the extracellular field potential which would be reflected in the EOG recording. Metals could also act directly upon components of the signal transduction pathway. Metal ions may act on adenylyl cyclase (Song et al., 1997) thereby inhibiting production of cAMP, which is required to activate CNG channels. Divalent metal ions, such as Pb^{2+} and Cd^{2+}, can compete with and (or) mimic Ca^{2+} within neurons (Vijerberg et al., 2002). Metal ions can bind to and activate calmodulin which would shut down the CNG channels before the threshold required for depolarization is reached (Audersirk et al.,

1998). Similarly, metal ions can also block IP_3 activated currents (Ma and Michel, 1998).

Although these effects could explain a decreased extracellular field potential recorded in the salmonid olfactory studies, they do not explain the increased extracellular field potential at the olfactory epithelium that we recorded in yellow perch from metal-contaminated lakes. An increase in olfactory signal in chronically metal-exposed fish may be explained by metal interference on different components of the signal transduction pathway. In fishes, ORNs are believed to be broadly tuned to a subset of odours and not single odours (Delay and Restrepo, 2004). Binding of odour molecules may activate multiple signal transduction pathways. Some of these pathways are excitatory while others are inhibitory, which may allow for odour discrimination (Dubin and Dionne, 1993; Dionne and Dubin, 1994; Schlid and Restrepo, 1998; Delay and Restrepo, 2004; Cens et al., 2006). Within a single ORN there will be multiple signal transduction pathways activated and the resulting signals have to be modulated somehow. Signal modulation has been shown to occur via the IP_3 pathway. Delay and Dionne (2002) found that when phospholipase C (PLC) was inhibited in the ORNs of newts (*Necturus maculosus*), the cAMP pathway was enhanced leading to an increased neuro-electrical signal being generated. Similarly, Vogl et al. (2000) found that when PLC was inhibited in ORNs of rats (*Rattus rattus*), cAMP levels were elevated and neuro-electrical signal strength was increased. Metal ions such as Hg and Cu have been shown to impair PLC activity (Panfoli et al., 2000). Without PLC to mediate the production of IP_3, there would be no inhibitory signal modulation which would lead to an enhanced olfactory signal as

demonstrated in our study. Additionally, the yellow perch from the contaminated lakes are the product of multiple generations dating back to over 100 years of metal exposure. The increased signal may be an adaptation to combat the influences of metals on impairing the signal transduction pathway. Further work is necessary to elucidate these effects.

Despite generating an increased olfactory signal, yellow perch from metal-contaminated waters did not produce an overt behavioural response to conspecific alarm cues. This result suggests that the olfactory signal is not being propagated to higher brain centers responsible for processing the information and stimulating an appropriate response to the cue. Primary olfactory neurons synapse within the glomerular layer of the olfactory bulb (Hara, 1992; Firestein, 2001; Laberge and Hara, 2001; Yoshihara et al., 2001). At the presynaptic terminal the signal is propagated across the synaptic cleft by the release of neurotransmitters from membrane bound vesicles. Release of neurotransmitters is mediated by Ca^{2+} influx through voltage gated channels and interference with these channels would inhibit neurotransmitter release (Kim et al., 2004; Wachowiak et al., 2005). A moderate change in pre-synaptic terminal Ca^{2+} concentration can strongly modulate the amount of neurotransmitter release (Wachowiak et al., 2005). Metal ions such as Cd^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} have been shown to interfere with voltage-gated Ca^{2+} channels at the pre-synaptic terminal in various neuronal cell types (Audesirk, 1993; Rozsa and Salanki, 1994; Elinder and Arhem, 2004). A similar effect in ORNs could inhibit release of neurotransmitters shutting down olfactory signal transmission. Metals that can pass the primary olfactory neurons, such as Hg^{2+} and Pb^{2+}, may also inhibit Ca^{2+}

channels or components of signal transduction pathways in the olfactory bulb or higher brain centers, thereby disrupting the propagation and/or the processing of the olfactory signal (Chetty et al., 1996).

Impairment of the olfactory system may also be due to other causes. Carreau and Pyle (2005) found that fathead minnows exposed to environmentally relevant levels of Cu (10 μg/L) as embryos and then raised in clean, dechlorinated tap water for 84-96 d post hatch did not respond to conspecific alarm cues. This impairment of the olfactory system may be the result of a congenital defect during the development of the olfactory system due to Cu exposure at the highly sensitive embryonic stage. Our yellow perch would also have been exposed to metals as embryos and may possess a similar developmental defect. However, after multiple generations of living in metal-contaminated waters, yellow perch may have adapted to deal with olfactory impairment.

Initially we had two hypotheses regarding ORN density of yellow perch from metal-contaminated lakes compared to yellow perch from the reference lake. Olfactory receptor neuron density could have been either higher or lower in contaminated lake fish compared to fish from the clean lake which could account for the significantly higher extracellular field potential we recorded in yellow perch from the contaminated lakes. However, there was no significant difference in ORN density at either the olfactory rosette or olfactory bulb in yellow perch from the reference and contaminated lakes. Therefore, the differences in extracellular field potential we recorded between yellow perch from clean and contaminated environments are due to other factors and not ORN density.

Another means by which yellow perch may have adapted to metal contamination is possibly by modifying the alarm cue itself to help increase the signal. Yellow perch from James Lake exhibited a significantly greater olfactory signal to alarm cues generated from skin extracts of Hannah and Ramsey Lake yellow perch compared to alarm cues generated from skin extracts of James Lake yellow perch. Modification of alarm cue may be due to an increase in the quantity of the alarm cue present in the skin. This increase could occur as increased amounts of alarm cue within the perch epidermal club cells believed to sequester the alarm cue (Wisenden et al., 2004) or an increase in the number of perch club cells per unit area. Alternatively there could be a change in the chemical composition of the alarm cue itself. However, the chemical identity of the alarm cue is not known. A change in the quantity or quality of the alarm cue may have been a contributing factor to results observed here, but does not fully explain the increased signal observed in fish from contaminated lakes because we also found a significantly higher EOG response to L-alanine in yellow perch from the contaminated lakes relative to yellow perch from the reference lake.

Aquatic organisms also use chemical information to locate food, find potential mates, assess habitat quality, and recognize kin. Impairment of the olfactory/chemosensory system that interferes with any of these ecological activities may also alter population and community structure. Our study also demonstrates the importance of using behavioural assays in ecotoxicological studies. If we had only conducted the electrophysiological study, then we may have had made a false conclusion that yellow perch from metal-contaminated lakes were not olfactory impaired. Scott and

Sloman (2004) argue that behaviour provides the link between physiological and ecological processes. By conducting studies that integrate physiological and behavioural indicators of toxicity we can gain deeper understanding of ecosystems and apply more appropriate environmental risk assessments to provide the highest degree of ecological relevance.

Yellow perch from metal-contaminated lakes bound more Cd to the OE than did YP from a reference lake (as seen by the more than 2-fold increase in binding capacity, B_{max}) and that the binding affinity (log K) of the OE for Cd decreased slightly in chronically metal-exposed YP. This pattern of decreased binding affinity and increased binding capacity in the OE of YP from the metal-contaminated lakes was also present in the corresponding gill binding kinetics from the same YP (James log K = 7.5 and B_{max} = 0.5 nmol g^{-1}; Hannah log K = 7.2 and B_{max} = 0.7 nmol g^{-1}) (see accompanying study by Klinck et al., 2007). In addition, Niyogi et al. (2004) saw a similar pattern of Cd binding to the gills of YP where saturation of Cd-gill binding was exhibited in YP from a reference lake (Geneva L.) but a lack of saturation (i.e., linear kinetics) was exhibited for Cd-gill binding in YP from two metal-contaminated lakes (Hannah L. and Whitson L.). Moreover, a similar pattern of decreased binding affinity and increased binding capacity of Cd to the gills of rainbow trout chronically exposed to waterborne Cd in the laboratory has been seen in several studies (Hollis et al., 1999, 2000a,b; Szebedinszky et al., 2001; reviewed by Niyogi and Wood, 2003). The observed decrease in affinity of the gills for Cd in YP and RBT is likely a protective mechanism to prevent toxicity and the increased Cd binding capacity is likely due to increased protein pools (to which Cd is bound) in the

gill related to storage and detoxification mechanisms (Niyogi and Wood, 2003). However, the OE is not involved in ionoregulation and detoxification of metals and therefore a different explanation for the difference in binding dynamics in the OE of YP from uncontaminated and contaminated lakes is required.

The binding characteristics of the OE of FHM for Cd were different than those of wild YP. Fathead minnow OE (table 2-1) had an approximately 5 times greater binding affinity for Cd compared to that of YP. Moreover, FHM had approximately 3 times greater binding capacity for Cd than YP from a clean lake and a little less than double that of YP from a metal contaminated lake. In wild YP log K and B_{max} for Cu could not be determined due to a lack of saturation. Although the log K and B_{max} values for Cd (of the OE) of wild YP and lab-reared FHM are not the same, saturation of the OE binding sites in both the reference lake and chronically metal-exposed wild YP indicates that with further research a cbBLM for Cd may be feasible in wild YP.

In conclusion, we found that yellow perch from metal-contaminated lakes are olfactory impaired compared to yellow perch from a clean lake. Although yellow perch from the contaminated lakes exhibited a significantly greater extracellular field potential than yellow perch from the reference lake they did not exhibit a fright response to yellow perch alarm cue. There were no differences in ORN density at either the olfactory rosette or olfactory bulb among the three populations of yellow perch, thus differences in the levels of extracellular field potential recorded are mostly likely due to metal effects on the signal transduction pathway and (or) modification of the alarm cue itself. Similarly, metals are likely interfering with propagation of the olfactory neuro-electrical signal at

the ORN pre-synaptic terminal thus the signal is not propagated to higher brain centers for processing. Finally, Cu and Cd OE binding dynamics are different in wild YP compared to laboratory raised FHM; however the production of a Cd cbBLM for chronically metal-exposed wild YP may be possible. Further studies are needed to determine the underlying mechanisms of metal-induced olfactory impairment and further characterize metal-OE binding in wild YP.

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Table 3-1. Total metal concentrations (μg L^{-1}) including metals of interest, in water samples (n=3) collected from Hannah, Ramsey and James Lakes. *Note.* Water chemistry from Hannah and Ramsey Lakes (Pyle et al., 2005), water chemistry from James Lake (Couture et al., in press) MDL= minimum detection limit; NA= not available; ND= not detected; SEM= standard error of the mean; §= (Ilse and Rasmussen, 2005);* = (Shuhaimi-Othman et al., 2006).

Total concentration in water		Metals														
Lake		Al	As	Ba	Ca	Cd	Cu	Fe	Mg	Mn	Ni	Pb	Rb	Se	Sr	Zn
	MDL	1	1	NA	100	0.1	1	50	NA	1	1	1	NA	1	NA	1
James	Mean	19	0.3	19	16143	0.1	1.5	505	15537	186	1	0.3	1	NA	21	16
	SEM	5	0	2	1837	0	0.1	115	2957	88	0.2	0.1	- 0.04	-	1	13
Ramsey	Mean	ND	1.3	14.0	14003	0.08*	9.7	36	4224	72	52	0.62*	2.0	2.0	48.7	5.7
	SEM	---	0.3	1.0	1030	0.003*	0.3	11	240	4	2.6	0.02*	0.0	0.0	2.2	0.7
Hannah	Mean	ND	2	24	11603	§0.5	25	71	4137	251	181	NA	3	2	65	10
	SEM	-	0	2.4	1150	-	2	4	377	19	45	-	0	0.3	6	3

Table 3-2. Water quality parameters for study lakes (n = 3).

Lake	pH	Hardness (mg L^{-1} as $CaCO_3$)	Alkalinity (mg L^{-1} as $CaCO_3$)
James (Reference)	6.6-7.0	58-63	40-43
Ramsey (Contaminated)	7.9-8.0	52-58	30-35
Hannah (Contaminated)	6.8-7.2	38-45	16-18

Table 3-3. MANOVA and ANOVA results for the interaction and main effects of chemical stimuli and yellow perch skin donors on the extracellular field potential recorded at the olfactory epithelium (electrophysiological experiments) and time spent moving (behavioural experiments) of wild juvenile yellow perch from clean and contaminated lakes.

	F	df	p
Electrophysiological experiments			
MANOVA			
Chemical stimuli x lake	11.98	6, 48	< 0.0001
ANOVA			
Yellow perch alarm cue	18.12	2, 26	<0.0001
Rainbow trout skin extract	23.41	2, 26	<0.0001
L-alanine	43.57	2, 26	<0.0001
MANOVA			
Yellow perch skin donor x lake	1.35	6, 20	0.28
ANOVA			
James Lake	5.57	2, 12	0.019
Ramsey Lake	0.08	2, 12	0.92
Hannah Lake	0.22	2, 12	0.80
Behavioural experiments			
ANOVA			
Chemical stimuli x lake	11.91	4, 118	<0.0001

Table 3-4. Percentage of individuals exhibiting freezing behaviour in wild juvenile yellow perch exposed to either dechlorinated tap water, rainbow trout skin extract or yellow perch skin extract. Yellow perch were collected from three different lakes in Northern Ontario representing a metal-contamination gradient.

Lake	Stimulus	n	Percentage
James	Control water	15	0
(Reference)	Rainbow trout extract	15	7
	Yellow perch extract	15	60
Ramsey	Control water	13	0
(Contaminated)	Rainbow trout extract	12	0
	Yellow perch extract	13	0
Hannah	Control water	15	0
(Contaminated)	Rainbow trout extract	15	0
	Yellow perch extract	15	0

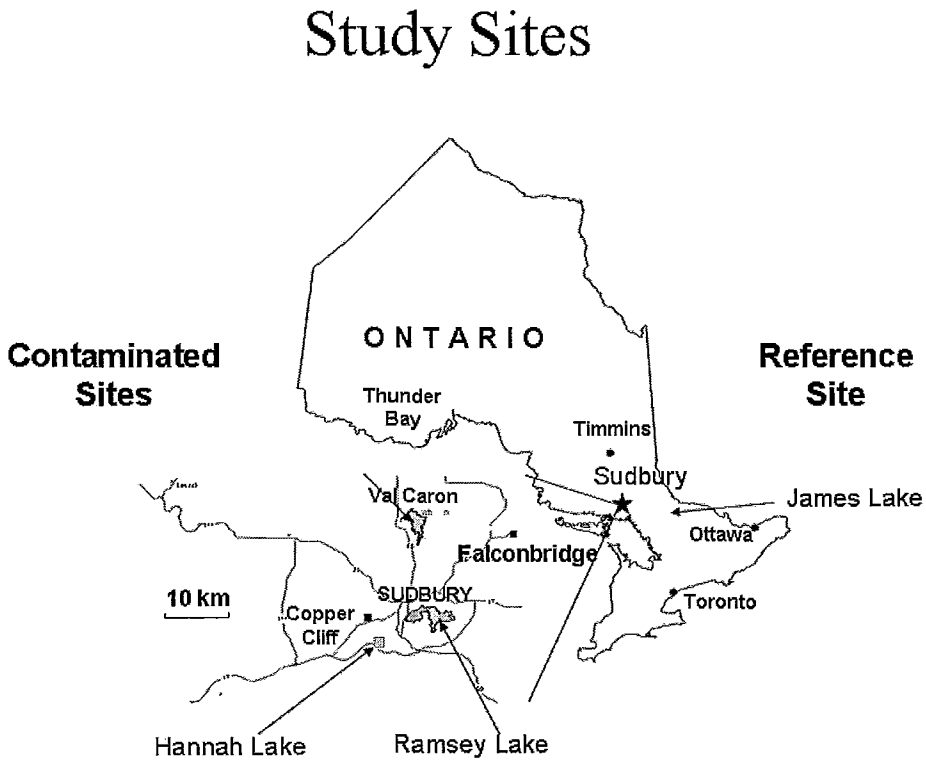

Figure 3-1. Map of study sites, where water samples and yellow perch were collected.

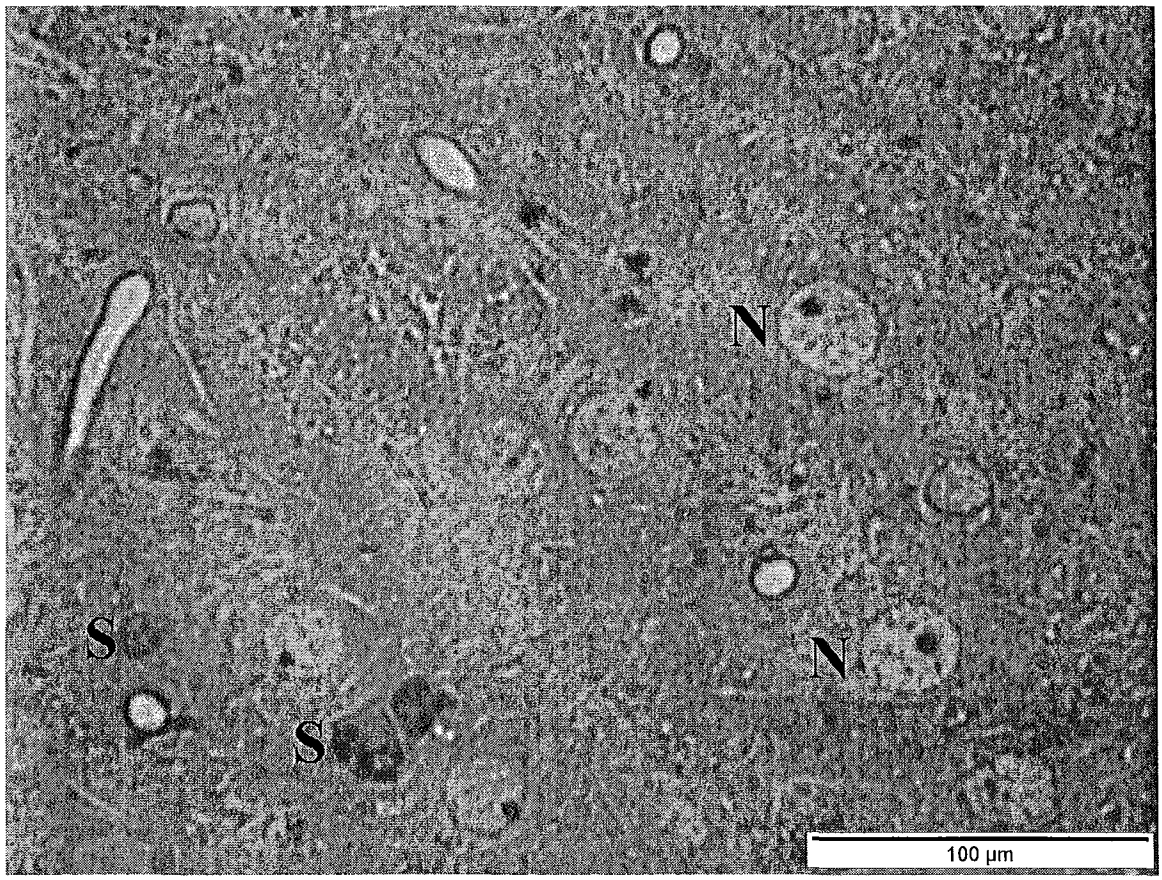

Figure 3-2. Representative section of olfactory bulb one micron thick stained with toluidine blue. N indicates a neuron with a central nucleolus within a pale nucleus. S indicates support cells such as glia with irregular shaped, smaller nuclei with extensive chromatin.

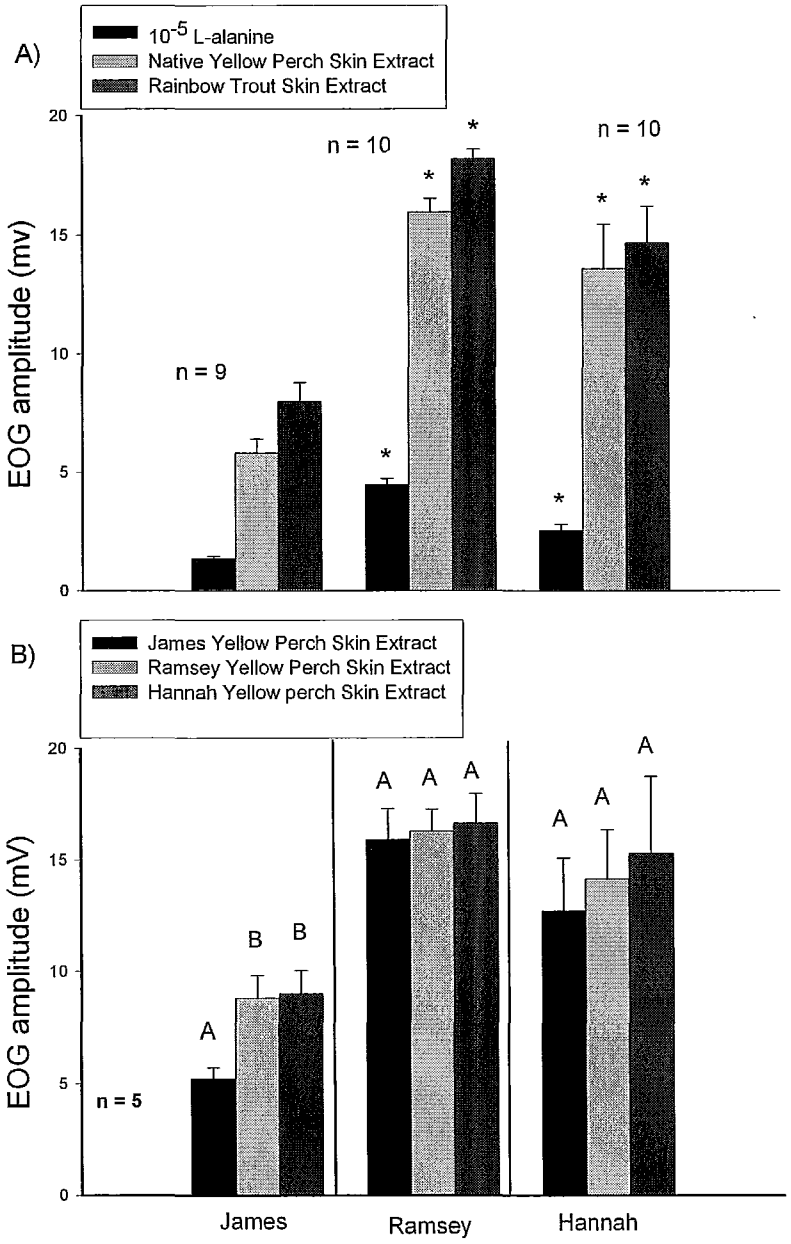

Figure 3-3. Mean extracellular field potential response at the olfactory epithelium ± SEM of juvenile yellow perch from three lakes collected along a metal contamination gradient (James: reference, Ramsey: intermediate contamination and Hannah: high contamination) exposed to: A) yellow perch skin extract from donors from each of the three lakes and B) to native yellow perch skin extract, and two control stimuli, 10^{-5} M L-alanine and rainbow trout skin extract (control for odour of injured fish). In A) asterisks represent a significant difference from the James lake yellow perch response to the respective odour. In B) different letters represent a significant difference within the respective lake ($p < 0.05$) (see text for statistical details).

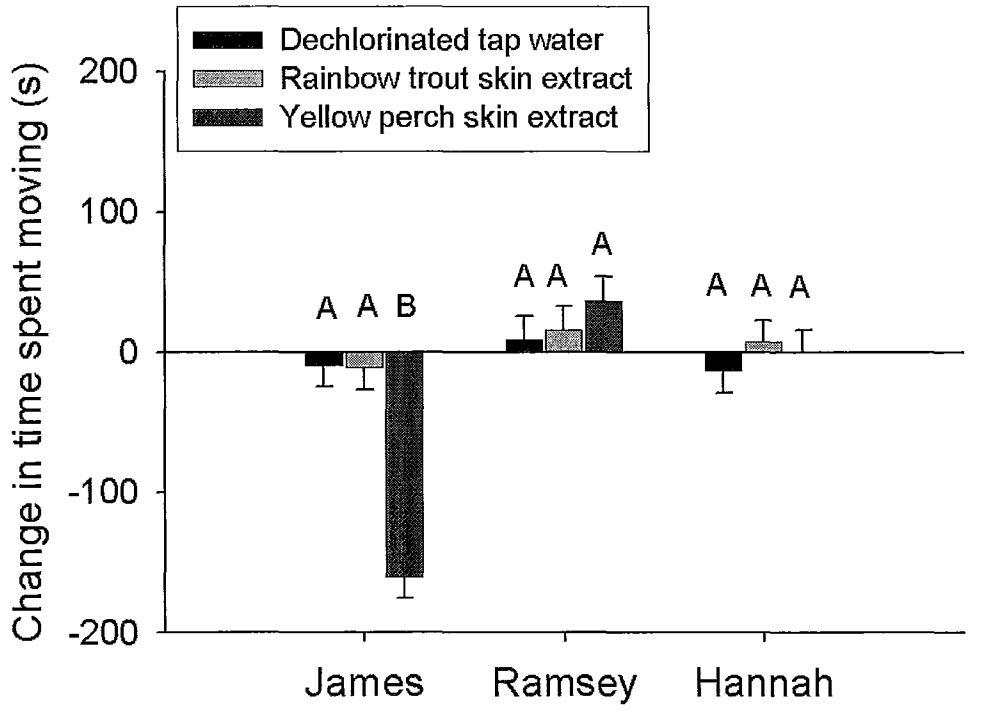

Figure 3-4. Mean ± SEM of change (post-stimulus – pre-stimulus) in time spent swimming (s) of juvenile yellow perch from three lakes along a metal contamination gradient. Perch from each lake were exposed to: 1) native yellow perch skin extract, 2) rainbow trout skin extract or 3) dechlorinated tap water. Different letters indicate a significant difference ($p < 0.05$, n = 12-15 per treatment, see text for statistical details).

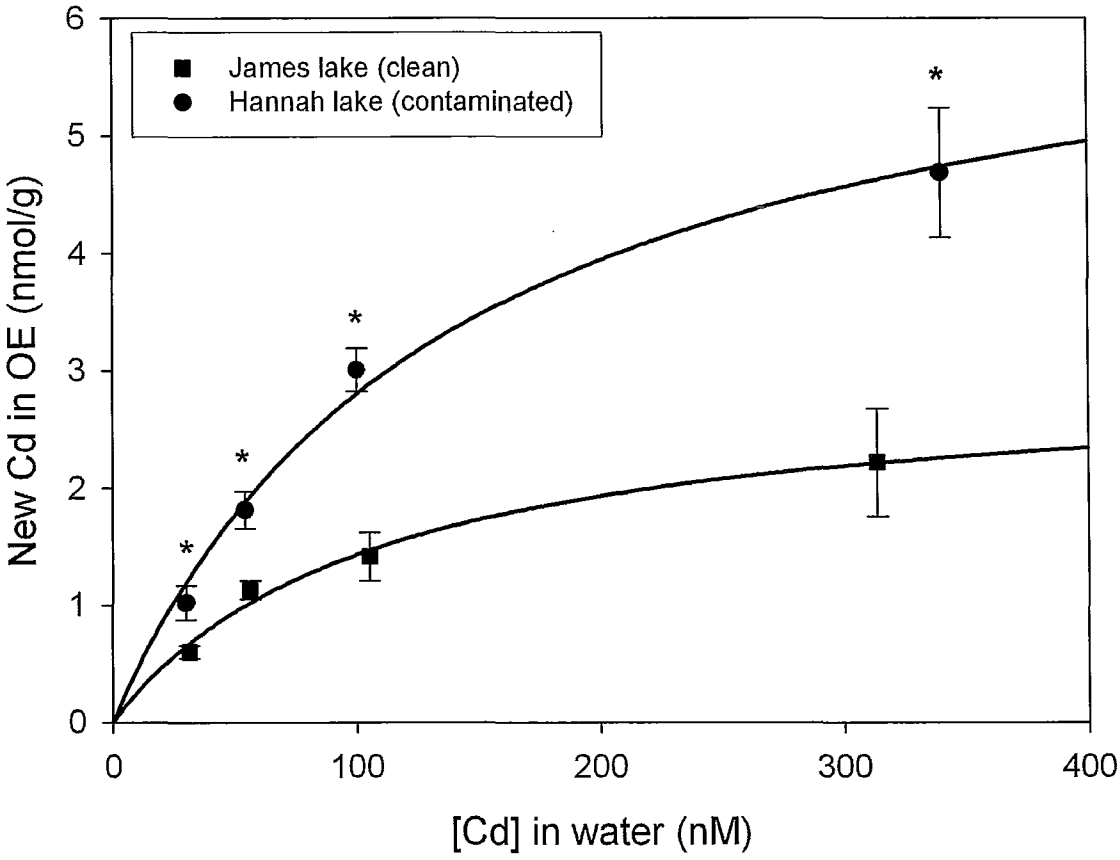

Figure 3-5. New cadmium binding to the olfactory rosettes of wild yellow perch from James L. (reference L.) and Hannah L. (metal contaminated) across a range of waterborne Cd concentrations in synthetic softwater after a short-term 3 hour cadmium exposure. Points represent means ± S.E.M (n = 6-7). Asterisks represent significant difference of Cd binding between the two lakes within the respective Cd concentration ($p \leq 0.05$).

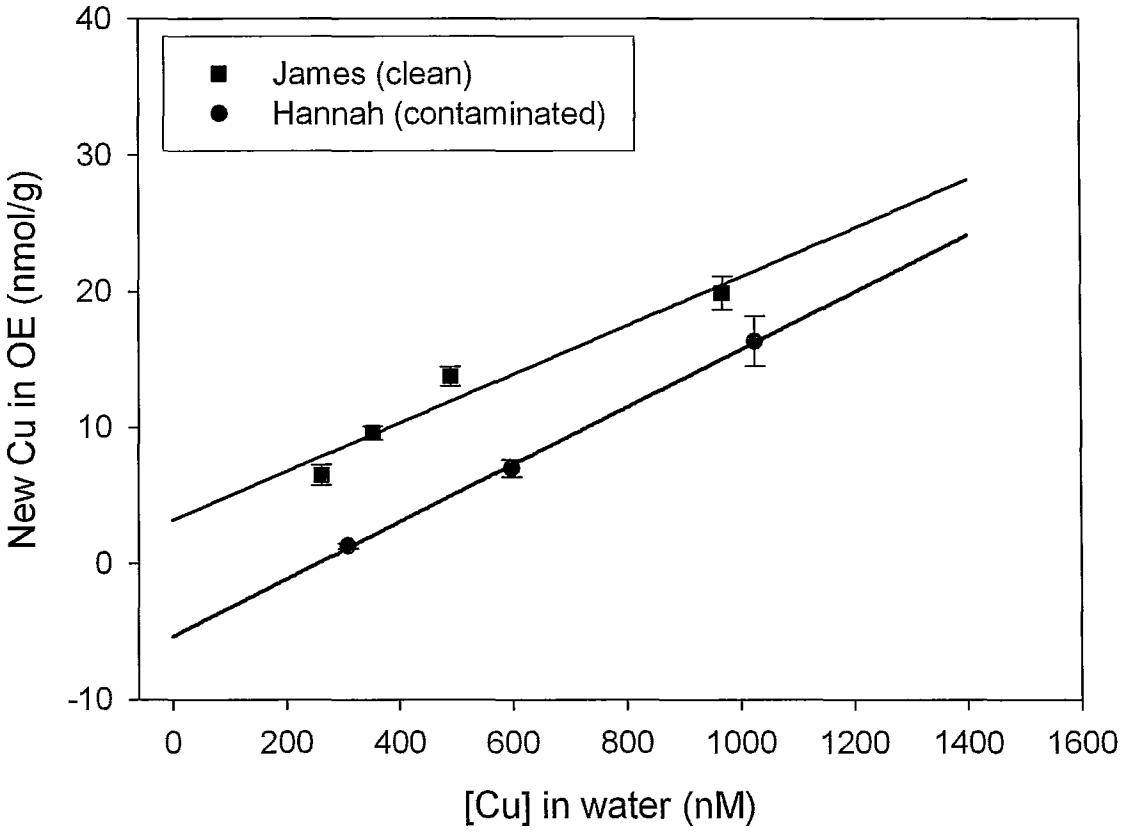

Figure 3-6. New Cu binding to the olfactory rosettes of wild yellow perch from James L. (reference L.) and Hannah L. (metal contaminated) across a range of waterborne Cu concentrations in synthetic softwater after a short-term 3 hour Cu exposure. Points represent means ± S.E.M (n = 6-7).

CHAPTER 4

OVERALL CONCLUSIONS

The overall goal of this thesis was to begin development of a chemosensory-based biotic ligand model for Cu and Cd in a laboratory fish species (fathead minnows) and examine whether similar results occur in a wild fish species (yellow perch). This goal was accomplished by utilizing a variety of experimental techniques in the laboratory including radiolabelled metal binding studies, electrophysiology, histological techniques, and behavioural assays.

Taken together, the results of this thesis indicate that the olfactory epithelium of laboratory raised FHM is a viable biotic ligand to use in producing a cbBLM due to its rapid and saturable metal binding characteristics, changes in binding characteristics under different ambient water chemistries, and its inhibited function due to sub-lethal metal toxicity. Furthermore, the production of a cbBLM for Cd in chronically metal-exposed wild fishes may be possible due to the saturable OE-Cd binding kinetics observed in wild YP. However, for a cbBLM to be designed for chronically metal-exposed wild YP, the observed disruption of olfactory function in chronically metal-exposed wild YP needs to be related to the observed OE-metal binding.

Further research is needed in order to better understand the relationship between metal binding dynamics and other water chemistry parameters that can influence metal-ligand binding such as Na^+, Mg^{2+}, DOC, and pH in acutely metal-exposed lab fish species. Furthermore, the mechanism of metal toxicity in chronically metal-exposed wild fishes needs to be better understood in order to determine what part of the olfactory

system is disrupted causing an inhibition of behavioural responses to olfactory stimuli. Further research is needed to understand the relationship between the observed neurophysiological and behavioural dysfunctions and how they translate to population-level effects in wild fish species living in metal-contaminated aquatic environments. Once this is accomplished a cbBLM (Fig. 4-1) would be an excellent tool to predict chronic sub-lethal metal toxicity in wild fish populations and would ultimately improve ecological risk assessment.

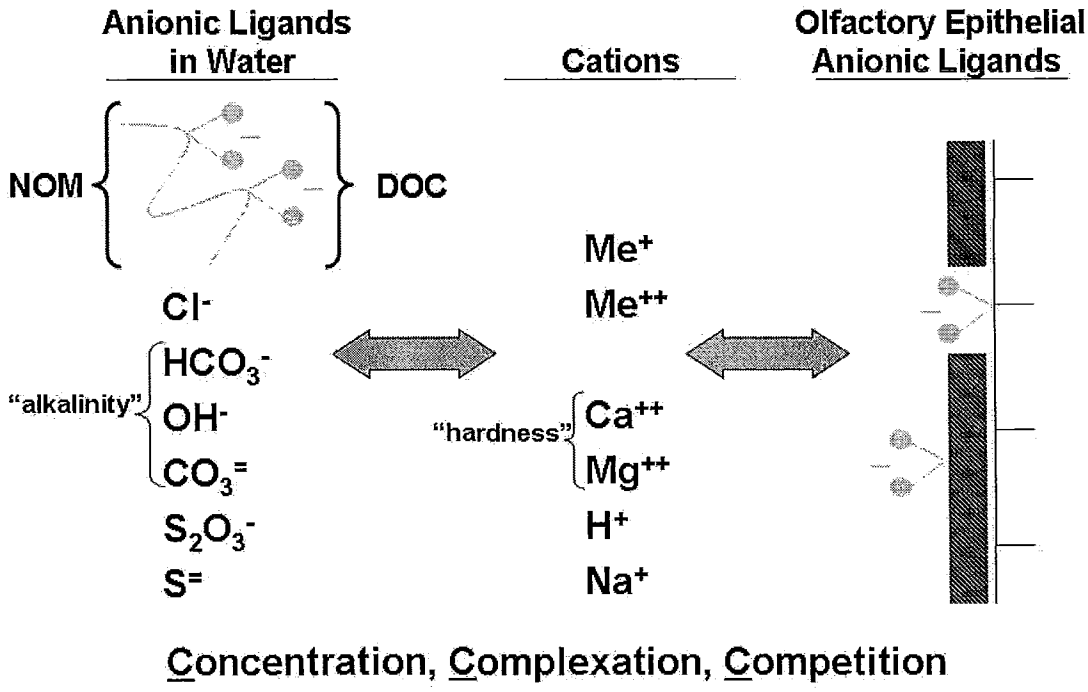

Figure 4-1. Illustration of a conceptual chemosensory-based biotic ligand model (cbBLM). Figure taken from Pyle et al., 2006.

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