THE EFFECTS OF COPPER, CALCIUM, AND NICKEL ON THE OLFACTORY RESPONSE OF FATHEAD MINNOWS: FROM NEUROPHYSIOLOGY TO BEHAVIOUR

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ABSTRACT

The olfactory system is essential for a fish to be successful in an ecological context, and has been demonstrated to be sensitive to a variety of toxicants. The current biotic ligand model (BLM) for copper is an acute-toxicity model based on the gill that is used to predict site-specific safe copper concentrations. Recently, work has been done to develop a BLM based on a more sensitive tissue, namely the olfactory epithelium. The work presented in this dissertation determines that a number of the assumptions of the current acute-toxicity gill-based BLM do not hold for the olfactory epithelium. Two techniques were employed for the work contained within this dissertation, a neurophysiological measure of olfactory acuity, electro-olfactography (EOG), and measurement of behavioural responses. For all experiments, fathead minnows (*Pimephales promelas*) and yellow perch (*Perca flavescens*) were used as these species are ubiquitous in waterways across Canada. Fathead minnows were exposed to low, ecologically-relevant concentrations of copper for varying exposure durations in hard and soft water. While it was determined that there was a significant of inhibition of olfactory function as measured by EOG, there was recovery, at least partially, of EOG function with increased exposure duration. It was also determined that not only does calcium have no protective effect against copper-induced olfactory dysfunction at the olfactory epithelium as it does at the gill, but calcium induces its own response. The response to calcium in fathead minnows was further investigated. Fathead minnows had a strong olfactory-dependent avoidance response to calcium. The reduction in EOG response caused by calcium was demonstrated to be due to cross-adaptation with the odourant used, namely L-arginine. Different olfactory sensory neuron (OSN) classes

within the olfactory epithelium respond specifically to different odourants. Exposures of fathead minnows or yellow perch to copper demonstrated that there was a specific impairment of ciliated OSNs, while exposure to nickel resulted in impairment of microvillous OSNs. Behavioural work with fathead minnows using an anti-predator cue demonstrated that copper impairs the response to an anti-predator cue, while nickel does not. These observations demonstrate that ciliated cells are responsible for mediating response to anti-predator cue, which is the first time response to a specific chemosensory cue has been directly connected with a specific OSN class. The work presented in this dissertation represents a significant advancement in our understanding in how copper impairs the olfactory system of fish, which will aid in the construction of models and regulations to protect fish populations.

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LIST OF ABBREVIATIONS

ANOVA - Analysis of variance

BLM - Biotic ligand model

CALA - Canadian Association for Laboratory Accreditation

cbBLM - Chemosensory-based biotic ligand model

CCC - Criterion continuous concentration

CCME - Canadian Council of the Ministers of the Environment

CMC - Criterion maximum concentration

CS - Coho salmon

DOC - Dissolved organic carbon

EEG - Electroencephalogram

EOG - Electroolfactogram

FHM - Fathead minnow

gbBLM - Gill-based biotic ligand model

HA - Humic acid

ICP-AES - Inductively coupled plasma atomic emission spectroscopy

LUCAS - Lakehead University Centre for Analytical Services

OE - olfactory epithelium

OSN - Olfactory sensory neuron

RBT - Rainbow trout

TCA - Taurocholic acid

USEPA - US Environmental Protection Agency

CHAPTER 1: Introduction

1.1 General Introduction

Aquatic animals may use a wide variety of sensory systems to obtain information about their environment. Fish, for example, utilize vision, hearing, olfaction, detection of vibrations, gustation, and electroreception to gather information. Through their olfactory system, fish receive information about their environment via odourants from both biotic and abiotic sources, including the environment, other animals, predators, and conspecifics (Lurling and Scheffer, 2007). These odourants are used to mediate a wide variety of behaviours including food searching behaviours (Valentincic et al., 2011), predator avoidance (Chivers and Smith, 1993), migratory movements (Yamamoto et al., 2010), shoaling (Ward et al., 2008), mate selection and mating (Stacey et al., 2003), detection of cations (Atchison et al., 1987; Chapter 3), and recognizing kin (Green et al., 2008). Without an intact olfactory system, a fish’s ability to detect and respond to odourants that mediate essential life processes is impaired.

Many things may interfere with the ability of a fish to detect odourants, including the presence of toxicants such as organic contamination or metals (Scott and Sloman, 2004; Tierney et al., 2010). The disruption of the ability to detect odourants means that fish may not be properly perceiving their environment, resulting in a reduced ability to find food, avoid predators, or select an appropriate mate (Lurling and Scheffer, 2007). While the loss of a fish’s ability to detect these molecules may not directly result in death, it can lead to ecological death (Scott and Sloman, 2004). Ecological death is the inability of an individual to function in an ecological context due to impairment of normal behavioural responses to odourants. The effect of contamination on the olfactory

system of fish is of great interest as it represents a very sensitive endpoint, far more sensitive than lethal endpoints (Pyle and Wood, 2007).

Different toxicants may have differing effects on the olfactory system. For example, specific metals are taken up into the olfactory system of fish, while others are not (Scott and Sloman, 2004). The olfactory system can be divided into distinct sections, the olfactory epithelium, the olfactory nerve and bulb, and the brain. The olfactory epithelium is bathed in mucous and is in constant contact with the external environment where it can be bound by odourants in the water. Upon binding of odourants, an action potential may be initiated that is conducted by the olfactory neuron to the olfactory bulb. There is some processing of the signal in the olfactory bulb, which is then transmitted to the telencephalon via two olfactory tracts (Hamdani and Døving, 2007). The telencephalon and the rest of the brain then processes the integrated signals from the olfactory epithelium and bulb, and can mount a behavioural response (Hamdani and Døving, 2007). A disruption can occur anywhere along the olfactory system, which means that any investigation into the effects of toxicants on the olfactory system of fish would ideally take into account multiple levels of organization.

1.2 Metals

When life first started to evolve on Earth, the anoxic atmosphere meant that most metals were sequestered in sediment as metal sulphides. As a result of the anoxic environment, life evolved in the presence of a relatively low abundance of metals (Clarkson, 1995). The evolution of photosynthesis by plants resulted in a dramatic increase in atmospheric oxygen concentration, resulting in increased dissolved oxygen

in aquatic environments (Dupont et al., 2010). Increased oxygen concentrations changed the redox potential of the water resulting in the dissolution of insoluble metal sulphides leading to a massive release of metals from marine sediments to overlying waters (Couture and Pyle, 2012). The release of metals into the environment, in conjunction with the increase in oxygen, resulted in an extinction event that decimated life across the planet (Sessions et al., 2009). Species that were able to survive incorporated metals into their physiology, so much so that approximately 30% of all proteins require a metal for proper function (Nielsen, 2000; Dupont et al., 2010). Metals can be viewed as either essential or non-essential, with essential metals being required by organisms for the proper function of enzymes, and non-essential metals not being required (Jeffery, 2001).

Metals have been used by humans for millennia, starting with the making of jewellery and tools. In recent times, metals have become essential for the construction of most items. There has been a near-exponential world-wide demand for metals over the last 100 years (Han et al., 2002). The increase in metal mining and use has also increased the amount of anthropogenic metal released back into the environment (Han et al., 2002). The release of metals into the environment has been recognized as a major issue, with many jurisdictions around the world setting guidelines for safe limits of various metals in waterways (Wood, 2012).

The Sudbury region in Northern Ontario represents one of the richest deposits of copper and nickel in the world, and as a result the area has been continuously mined since 1888 (Conroy and Kramer, 1995). The continuous mining in Sudbury represents not only a major supply of copper and nickel for worldwide consumption, but it also

represents a major source of copper and nickel contamination to the environment (Conroy and Kramer, 1995). In an effort to reverse damage done to the Sudbury region, over 3,400 hectares of land have been limed to reverse acidification of the soil, over 3,200 hectares of land has been fertilized, over 3,100 hectares of land has been seeded, and over 9 million trees have been planted since 1978 (Regreening Program, 2011). Lakes in the Sudbury region have not only been contaminated with copper and nickel, but they have been acidified due to sulphur dioxide release (Yan et al., 1995). While the most effective way to reduce acidification of lakes is to remove the release of sulphur dioxide at the source, many lakes in the Sudbury region have also been limed (Yan et al., 1995). In an effort to aid in the reclamation process, it is vital to understand what effects copper and nickel have on aquatic animals. As the olfactory system is vital to the ability of a fish to thrive in an ecosystem, the effect of copper and nickel on a fish's olfactory system is important to understand. The effect of copper on the olfactory system of fish has been investigated in salmonids and some cyprinid species (McIntyre et al., 2008; Green et al., 2010). However, much is still unknown concerning copper-induced olfactory dysfunction, namely the relation between different levels of biological organization (i.e., neurophysiology and behaviour), and how other cations affected this dysfunction. In terms of olfaction, nickel is an understudied metal, and as such represents an important metal to study.

1.2.1 Copper

Copper is believed to be the first metal used by humans, with the first instance of use of copper occurring in or around 9000 BC (Stanczak, 2013) . As humanity

progressed the manner in which copper was extracted and used changed, with the oldest known copper smelting site being located in Eastern Serbia dating to 7000 years ago (Radivojević et al., 2010). In modern times, copper is used in a multitude of products, resulting in an increase in production of copper by 25 fold between 1900 and 2000 (Han et al., 2002). The 2011 world-wide production is estimated at 16.1 million metric tons, with Canadian production of 940,000 metric tons (United States Geological Survey, 2012). In 2010, the mining sector (including mining and mineral-processing) in Canada produced 2.8% of the GDP, or $34.7 billion, and accounted for 2.1% of Canada's total employment (Natural Resources Canada, 2013). Copper, specifically, accounted for $3.8 billion in revenue in 2010 in Canada (Natural Resources Canada, 2013).

Not only is copper important to the economy and society in general, copper is essential to life as we know it. Copper is an essential metal, and due to its redox potential it is found in a variety of enzymes, most notably in cytochrome c oxidase (Solomon and Lowery, 1993). The redox properties of copper that are essential for cytochrome c oxidase function also lead to the production of reactive oxygen species (ROS) when the concentration of copper in a cell is too high (Harris and Gitlin, 1996). There is, therefore, a "sweet spot" for the concentration of copper within cells; if the concentration is too low, copper deficiency results, or if the concentration is too high, copper toxicity from ROS-induced cellular damage occurs (Grosell, 2012). While copper is primarily obtained through dietary sources it fish, it can also be taken up by the gills (Kamunde et al., 2002). At high concentrations, copper will induce death in fish, most likely through impairment of cation exchange at the gill (Grosell, 2012). Copper

has been shown to impair gill Na^{+}/K^{+}-ATPase, which results in an imbalance in the ionic nature of plasma due to loss of sodium and potassium (Laurén and McDonald, 1985; Laurén and McDonald, 1987). The loss of ionic balance in blood plasma results in an increase in blood viscosity and subsequent death due to cardiovascular collapse (Wilson and Taylor, 1993; Kamunde et al., 2003).

There are two regulations that apply in Ontario concerning acceptable copper concentrations in waterways, both of which are based on toxicity testing. The Ontario Provincial Water Quality Objective is set at 5 μgL^{-1}, while the Canadian Council of the Ministers of the Environment (CCME) utilizes a hardness-adjustment equation (Ontario Ministry of Environment and Energy, 1994; Canadian Council of the Ministers of the Environment, 1999). The hardness equation used by the CCME is:

$$(1.1) \quad \text{copper guideline} = e^{0.8545[\ln(\text{hardness})]-1.465} * 0.2\ \mu gL^{-1}$$

based on the fact that increased hardness (driven primarily by the calcium concentration in the water) is protective of copper-induced toxicity in fish (Niyogi and Wood, 2004). In the United States, the USEPA uses a biotic ligand model (BLM) to predict site-specific safe copper concentrations for short and long-term exposure (USEPA, 2007). The European Union has also adopted a BLM for predicting safe copper concentrations (Grosell, 2012). A BLM incorporates site-specific water quality parameters and interactions at a physiologically-sensitive biotic ligand (e.g., a fish gill) to predict a safe copper concentration for that site (Paquin et al., 2002). The biotic ligand used for the current copper BLM in fish is the surface of the gill, and predicts site-specific copper regulations based on acute testing (Niyogi and Wood, 2004). The precursors to this model were the free-ion activity model (FIAM), which predicts the speciation of copper

based on pH, temperature, and other factors, and the gill surface interaction model (GSIM), which predicts the interactions of copper and other cations at the gill surface (Paquin et al., 2002). These two models laid the foundation for the current copper BLM for fish.

Questions have been raised about the use of an acute gill-based BLM for determining site-specific copper regulations because the concentration of copper found in the environment (pristine or contaminated) rarely, if ever, approaches concentrations that would induce acute toxicity (Pyle and Wood, 2007). The disconnect between copper concentrations found in the environment and those that led to acute toxicity has lead to the development of chronic models based on other endpoints and tissues. An example of this is the olfactory epithelium, which can be impaired in fish at low, environmentally-relevant concentrations of copper (Baldwin et al., 2003; Green et al., 2010; Sandahl et al., 2004; Sandahl et al., 2006). The olfactory epithelium is so sensitive, in fact, that the concentration of copper that affects olfaction can be at or below the current regulations for copper. It is due to this increased sensitivity of the olfactory system over gill function that work has been done to construct a model based on the olfactory epithelium to produce site-specific regulations for aquatic copper concentrations (Pyle and Wood, 2007; Meyer and Adams, 2010). Recent progress into the development of a chemosensory-based BLM (where the olfactory epithelium replaces the gill as the biotic ligand) assumes that copper binding dynamics on the olfactory epithelium are the same as at the gill. The assumption that the gill and olfactory epithelium are interchangeable in terms of a biotic ligand is untenable because

recent evidence demonstrates that calcium does not offer the same protective effect at the olfactory epithelium as it does at the gill (Green et al., 2010; Dew et al., 2012).

1.2.2 Nickel

The first use of nickel by humans has been traced to ancient Syria in 3500 BC where it was used to make copper alloys (Braidwood et al., 1951). . In current times, the majority (approximately 60%) of nickel mined is used in stainless steel (Pyle and Couture, 2012). Between 1900 and 2000, there has been a 110 fold increase in the world wide production of nickel, with a current world wide production of approximately 1.8 million tons in 2011 (Han et al., 2002; United States Geological Survey, 2012). In Canada, production topped 200,000 tons, making Canada the fourth largest producer of nickel in the world in 2011 (United States Geological Survey, 2012). Approximately $5 billion of the economy of Canada is due to nickel mining and related industries (Natural Resources Canada, 2013).

Nickel is essential for a number of enzymes found in bacteria, most of which are related to the use or production of gasses (Ragsdale, 2009). While no enzymes requiring nickel have been found in humans or other animals, circumstantial evidence indicates that nickel is, in fact, an essential metal (Pyle and Couture, 2012). Nickel is taken up by fish through both waterborne and dietary sources, and nickel toxicity can be consistently predicted based on how much nickel is in or on gill tissue (Meyer et al., 1999). The ability to predict nickel toxicity based on metal load in or on gill tissue means that, like copper, nickel most likely exerts an effect at the gill surface (Meyer et al., 1999). The naturally-occurring concentration of nickel in waterways (due to natural

sources such as erosion) ranges from 0.1 - 10 μgL^{-1}, while contaminated waterways can have 50 - 2000 μgL^{-1} nickel (Chau and Kulikovsky-Cordeiro, 1995). The Ontario Provincial Water Quality Objective for nickel is 25 μgL^{-1} (Ontario Ministry of Environment and Energy, 1994). As the toxicity of nickel is affected by hardness, the CCME guidelines set safe nickel concentrations using the following hardness-adjustment equation:

(1.2) $\text{nickel guideline } (\mu gL^{-1}) = e^{0.76[\ln(\text{hardness})]} + 1.06$

with a minimum limit of 25 μgL^{-1} (Canadian Council of the Ministers of the Environment, 2012). In the United States, both the acute and chronic limits for nickel are determined using hardness-corrected equations (USEPA, 1996). For determining a chronic limit, the equation is:

(1.3) $\text{chronic nickel guideline } (\mu gL^{-1}) = e^{0.846(\ln \text{hardness})} + 0.0584$

for an acute limit, the equation is:

(1.4) $\text{acute nickel guideline } (\mu gL^{-1}) = e^{0.846(\ln \text{hardness})} + 2.255$ (USEPA, 1996).

The European Union sets the safe nickel limit at 20 μgL^{-1} but allows members to take other factors such as hardness into account when setting limits (Pyle and Couture, 2012). While work has been done to develop BLMs for nickel, no model has been adopted for determining site-specific regulations (Niyogi and Wood, 2004).

1.3 Measuring olfaction

As discussed above, the olfactory system can be broken down into three distinct sections, the olfactory epithelium, the olfactory nerve and bulb, and the brain (Hamdani

and Døving, 2007). Each element of the olfactory system is important for detecting and mounting a response to odourants in water.

1.3.1 Olfactory epithelium

The olfactory epithelium comprises a variety of cell types including support cells, mucous cells, basal cells, and olfactory sensory neurons (OSNs) (Zielinski and Hara, 2006). Basal cells replenish damaged cells, mucous cells produce protective mucous, and the OSNs have receptors that are bound by odourants in water (Hamdani and Døving, 2007). There are three different classes of OSNs: crypt, microvillous, and ciliated cells (Hansen et al., 2003). Some odourants will bind to receptors on one specific class of OSNs, some odourants are capable of binding to receptors on multiple classes of OSNs, as discussed in Chapter 4. Receptors on OSNs are coupled to G-proteins, and once bound the associated G-proteins are activated and induce an olfactory signalling pathway (Firestein, 2001). There are two known olfactory signalling pathways in OSNs, one that uses cAMP as a secondary messenger, one that uses IP3 (Ache and Young, 2005). Production of the secondary messenger results in the opening of channels in the OSN, allowing an influx of cations which can result in the production of an action potential (Firestein, 2001). The bulk response of the olfactory epithelium (i.e., movement of cations into the OSNs) can be directly measured using electro-olfactography (EOG) (Scott and Scott-Johnson, 2002; Baldwin et al., 2005). It is important to note that an EOG does not measure whether or not an action potential forms, it measures whether or not the olfactory epithelium responds to a cue. In this

way, an EOG can be viewed as a surrogate for olfactory acuity (i.e., to what extent the olfactory epithelium can perceive and respond to odourants).

1.3.2 Olfactory nerve and bulb

The olfactory nerve is composed of the extensions of the OSNs from the olfactory epithelium, and terminates at the olfactory bulb where it synapses with mitral cells in the bulb (Firestein, 2001). The response of the olfactory nerve and bulb to a cue being presented to the olfactory epithelium can be measured using an electro-encephalogram (EEG) (Hara et al., 1976). In this case, the EEG can be used to directly measure the action potential travelling down the olfactory nerve and into the bulb.

1.3.3 Brain

The processing of the signal transmitted by the olfactory bulb to the brain is complex. Two tracts (the medial and lateral olfactory tracts) connect the olfactory bulb to the telencephalon, where the signal is processed. How the signal processing works is unclear, evidence indicates there is both a spatial and temporal aspect to the coding (Laberge and Hara, 2001). Although EEGs of the brain can measure the response of the brain to a chemical cue, behavioural responses are more commonly used. A behavioural response to a cue can be viewed as a manifestation of the processing of the brain, and as such is an effective method to determine if a toxicant is causing a dysfunction in the brain. There are many olfactory-mediated behaviours in fish as described above, all of which can be measured in a laboratory setting through various techniques, as reviewed by Tierney (2011).

1.4 Impairment of olfaction

1.4.1 General impairment

The olfactory system of fish is sensitive to a wide variety of pesticides, organic contaminants, and metals (Scott and Sloman, 2004; Lurling and Scheffer, 2007; Tierney et al., 2010). The reduction or loss of olfaction is not a lethal endpoint, that is, it will not directly kill the fish. However, a decreased ability to avoid a predator, find food, or to find an appropriate mate decreases the ability of a fish to function in an ecological context. The work presented in this dissertation focuses on the effect of copper on the olfactory system, and to a lesser extent, the effect of nickel.

1.4.2 Impairment by copper

1.4.2.1 Neruophysiological effects of copper

Copper impairs the olfactory system of fathead minnows (*Pimephales promelas*) (Green et al., 2010), coho salmon (*Oncorhynchus kisutch*) (Baldwin et al., 2003; Sandahl et al., 2004; McIntyre et al., 2008), atlantic salmon (*Salmo salar*) (Winberg et al., 1992), chum salmon (*Oncorynchus keta*) (Sandahl et al., 2006), goldfish (*Carassius auratus*) (Kolmakov et al., 2009), and rainbow trout (*Oncorhynchus mykiss*) (Baldwin et al., 2011). When a fish is exposed to copper, the copper does not move into neural tissue (i.e., the olfactory neurons, nerve, olfactory bulb, or brain) but instead localizes in the melanosomes of the lamina propria (Julliard et al., 1995). When adult fish are exposed to a relatively low concentration of copper (5-15 μgL^{-1}), removal of the copper from the olfactory epithelium results in recovery of olfactory acuity over a short time

frame (Baldwin et al., 2003; Green et al., 2010). Recovery is also seen during continuous exposure with copper in fathead minnows, up to a concentration of 14 μgL^{-1} (Dew et al., 2012). Julliard et al. (1995) showed that rainbow trout exposed to 20 μgL^{-1} for 15, 30, or 60 days underwent rounds of cellular death and regrowth in their olfactory receptor cells. The mechanism for cellular death was shown to be apoptosis (Julliard et al., 1996). Taken together, it appears that there are two mechanisms for copper-induced olfactory dysfunction in adult fish based on the concentration of copper used, one that is quickly reversed when the copper is removed, and another that induces long-term damage that takes more time to repair.

1.4.2.2 Behavioural effects of copper

Fish can detect and respond to the presence of copper using olfaction. However, at higher concentrations, copper can interfere with a fish's ability to respond to other chemosensory stimuli. The behavioural response to copper is concentration dependent. At very low concentrations fish are attracted to copper, while at higher concentrations fish avoid copper (Brown et al., 1982). Hansen et al. (1999) demonstrated that chinook salmon lost the ability to detect and avoid copper when they were allowed to acclimate to 2 μgL^{-1} copper. The avoidance of copper by fish may be a mechanism by the fish to avoid elevated concentrations of copper sufficient to cause harm. Interestingly, an avoidance response can also be seen with a number of other cations, including chromium, nickel, cadmium, iron, and zinc (Atchison et al., 1987)

The majority of investigations into how copper affects olfactory-mediated behaviours have focused on the effect of copper on the predator avoidance of different

fish species. Fathead minnows exposed to 10 μgL^{-1} during embryonic development did not respond to conspecific skin extract up to 14 weeks post hatch (Carreau and Pyle, 2005). Beyers and Farmer (2001) demonstrated that Colorado pikeminnows (*Ptychocheilus lucius*) had reduced response to anti-predator cues due to copper treatment. Copper has also been shown to impair anti-predator response in fathead minnows as discussed in Chapter 4. In an ecological context, as copper would be expected to impair the olfactory system of both predator and prey, an inability of prey to detect predators may be compensated for by an inability of predators to detect prey. To investigate this, McIntyre et al. (2012) exposed both juvenile coho salmon and one of their natural predators, cutthroat trout (*Oncorhynchus clarkii*), to ecologically-relevant concentrations of copper (5-20 μgL^{-1}) and ran a series of predator-prey trials. Their work showed that more coho salmon were attacked under a copper exposure than controls, meaning that the loss of olfaction in this model benefits the predator, most likely as they rely more on visual rather than olfactory cues. In terms of other behaviours, rainbow trout were shown to no longer show preference for their holding water when exposed to copper (Saucier and Astic, 1995). Unfortunately, the effects of copper on other olfactory-mediated behaviours of fish have not been investigated.

1.4.3 Impairment by nickel

1.4.3.1 Neurophysiological effects of nickel

Very little research has been done to investigate the effect of nickel on the olfactory system of fish, with the work detailed in Chapter 4 being the first measures of nickel-induced olfactory dysfunction. In other model systems, nickel was shown to

induce damage in olfactory tissue in rats and cause olfactory dysfunction in humans (Evans et al., 1995; Sunderman, 2001). In addition, Tallkvist et al. (1998) demonstrated that nickel is taken up into the neural tissue of the olfactory system and transported down the olfactory nerve. Unlike a fish model which indicated that nickel does not pass the blood brain barrier, experiments with a rat model indicate that there is movement of nickel from the olfactory nerve to the brain (Henriksson et al., 1997). Other metals have been shown to either enter the olfactory nerve and not pass the blood-brain barrier (e.g., cadmium), or pass into the brain (e.g., manganese mercury, and zinc) (Scott and Sloman, 2004). The movement of nickel into the olfactory nerve indicates that nickel-induced olfactory dysfunction occurs via a different mechanism than copper-induced olfactory dysfunction, as copper does not pass into the olfactory nerve. If nickel is capable of passing into the brain as are other metals, it implicates the olfactory system as a vehicle for transport of potentially dangerous metals to the brain. Once in the brain, these metals may exert long-term effects on behaviour.

1.4.3.2 Behavioural effects of nickel

Fish have been shown to avoid nickel at concentrations as low as 23.9 μgL^{-1} (Giattina et al., 1982). Beside the work detailed in Chapter 4, only two other behavioural experiments have been performed using nickel as a toxicant. Nickel was shown to have no effect on agonistic interactions between conspecifics (Sloman et al., 2003). Exposure of Nile tilapia (*Oreochromis niloticus*) to relatively high concentrations of nickel (1 500 - 5 000 μgL^{-1} resulted in increased aggressive behaviour between fish as well as

increased discomfort movements and respiratory behaviour (Alkahem, 1994). Little is known about how nickel affects the behavioural response to odourants in fish.

1.5 Objectives

The objectives of this work are threefold:

(1) to determine aspects of copper toxicity at the olfactory epithelium;

(2) to determine the olfactory response to calcium at multiple levels of organization, and;

(3) to measure specific OSN classes in the olfactory epithelium to determine if OSNs have a differential susceptibility to copper or nickel, and to determine if any OSN specific effects by each toxicant translates to the behavioural level of organization

Unlike the gill, where calcium has been shown to be protective, evidence demonstrates that calcium is not protective of copper-induced olfactory dysfunction (Green et al., 2010). The results detailed in Green et al. (2010) could have been influenced by the time points used in that study. The work detailed in Chapter 2 demonstrates how different exposure lengths influence copper-induced olfactory dysfunction, as well as directly measures whether or not calcium is protective against copper. In addition, the work in Chapter 2 shows whether or not a simple reparameterization of a gill-based BLM, as was done in Meyer and Adams (2010), is sufficient to make predictions on site-specific safe copper concentrations based on the olfactory epithelium.

The results of the work detailed in Chapter 2 served as the basis for the work done in Chapters 3 and 4. In Chapter 2, calcium was shown to have an effect on the olfactory system by itself. While different species of fish have been shown to react to calcium as an odourant, no work has been done to elucidate the effect of calcium across multiple levels of organization (Bodznick, 1978; Hubbard et al., 2000). The work presented in Chapter 3 details measurements of the olfactory response of fathead minnows to calcium at the neurophysiological and behavioural levels of biological organization.

While it is known that different classes of OSNs will respond to specific cues, no work has been done to determine if copper-induced olfactory dysfunction is due to copper impairing all of the OSNs found within the olfactory epithelium, or if copper differentially impairs different OSN classes. Work by Kolmakov et al. (2009) demonstrated that after exposure with a very high concentration of copper, different classes of OSNs recover at different rates, indicating that there is, in fact, a differential effect of copper across OSN classes. The work in Chapter 4 details measurements into whether or not copper differentially affects ciliated and microvillous cells, and if so determines if this differential effect can be connected to a specific behavioural deficit. Chapter 4 also details work done measuring the effect of nickel on EOG and behavioural responses, for comparison against copper.

The work presented here expands our understanding of how copper impairs the olfactory system of fathead minnows, how calcium interacts with copper at the olfactory epithelium, and how specific OSN classes are impaired by different toxicants. The work

detailed herein represents a significant advancement in our understanding of how the olfactory system of fish is affected by copper and nickel.

CHAPTER 2: Effects of continuous copper exposure and calcium on the olfactory response of fathead minnows

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Summary

The current gill-based Biotic Ligand Model (gbBLM) is an acute-toxicity model used to predict site-specific safe copper (Cu) concentrations. Recent efforts to develop a chronic BLM has focused on the olfactory epithelium. To further this effort, the current study looked at the effect of varying Cu concentration and exposure duration on Cu-induced olfactory dysfunction, and whether calcium (Ca) protected against Cu-induced impairment as it does at the gill. Fathead minnows (*Pimephales promelas*) were treated with five Cu concentrations for varying exposure durations in hard and soft water. A neurophysiological technique, electro-olfactography (EOG), was employed to determine the level of olfactory dysfunction. While at the low, ecologically-relevant Cu concentrations tested there was significant inhibition of EOG function, over time there was at least a partial recovery of olfactory function, despite the continuous Cu exposure. Calcium did not appear to protect against Cu-induced olfactory dysfunction; and even alone, Ca appeared to interfere with the olfactory response to the amino acid L-arginine. Safe copper concentrations as predicted by the gbBLM, chemosensory-based BLMs, the USEPA BLM, and hardness-adjustment equations based on the exposure waters were not entirely protective against olfactory dysfunction.

2.1 Introduction

The Biotic Ligand Model (BLM) is a powerful predictive toxicological model that is used to predict site-specific acute metal toxicity to freshwater fishes and invertebrates (Di Toro et al., 2001; Paquin et al., 2002; Niyogi and Wood, 2004). The BLM is based on the complex relationship among metal binding to a physiologically-sensitive binding site (e.g., fish gills), metal speciation as it relates to bioavailability, and acute toxicity (Erickson et al., 1996; Playle, 1998). Questions about the ecological relevance of the acute, gill-based BLM (gbBLM) have emerged given model assumptions that metals are only taken up via a waterborne source and that metal concentrations typically found in contaminated waters are rarely sufficient to induce acute toxicity (Kamunde and Wood, 2004). Consequently, research attention has shifted focus to the development of chronic BLMs in order to improve the ecological relevance of BLM predictions (Kamunde and Wood, 2004).

To address some of these concerns, Pyle and Wood (2007) and Meyer et al. (2007a) proposed the olfactory epithelium as an alternative biotic ligand to the gill in support of a chemosensory-based BLM (cbBLM). Meyer and Adams (2010) proposed an early version of such a model for coho salmon (*Oncorhynchus kisutch*) exposed to copper (Cu). The cbBLM is a chronic BLM that considers metal binding at the olfactory epithelium instead of the gill and its relationship to olfactory dysfunction instead of mortality (Pyle and Wood, 2007). Such a model is not confounded by dietary metal exposure. The predicted effects have been shown to occur at environmentally relevant metal concentrations, and are ecologically relevant because olfaction mediates a range of antipredator, feeding, and reproductive behaviours. However, Meyer and Adams

(2010) changed only the Cu-sensitivity parameter [i.e., they replaced the medial lethal accumulation (LA50) in the gbBLM with a lower median inhibitory accumulation (IA50) of Cu that optimized the fit to olfactory impairment data in McIntyre et al. (2008)] without changing the binding constants for Cu, Ca, Mg, and Na to the biotic ligand. Therefore, that early cbBLM ignored potential differences in binding affinities for cations between branchial and olfactory epithelia.

Green et al. (2010) published the first empirical olfactory tissue Cu-binding constant for fathead minnows (*Pimephales promelas*). They showed that olfactory dysfunction occurred at approximately 160 nM (10 μgL^{-1}), as demonstrated by both behavioural and neurophysiological assays. Calcium (Ca), which protects gill epithelium against Cu binding and subsequent toxicity, did not appear to be protective against olfactory intoxication following low-concentration Cu exposure. The data reported in Green et al. (2010) showed neurophysiological impairment with exposure to 160 nM Cu at 1 h (their Figure 2), but they compared that result to Cu and Ca co-exposures for 3 h. Therefore, increasing exposure time from 1 to 3 h could have shifted the concentration-response curve to the left (Figure 2.1); that is, a lower Cu concentration might have been required to produce the same degree of olfactory impairment after the longer exposure duration, as would be expected for acute lethality (Meyer et al., 2007b). Then, if Ca were protective against Cu toxicity, the left-shifted curve could be shifted back to the right at the higher Ca concentration. Because Green et al. (2010) held Cu concentration constant at 160 nM while varying Ca concentration, this Cu concentration might have been too high to demonstrate a protective effect of Ca against impairment of neurophysiological function by Cu (Figure 2.1).

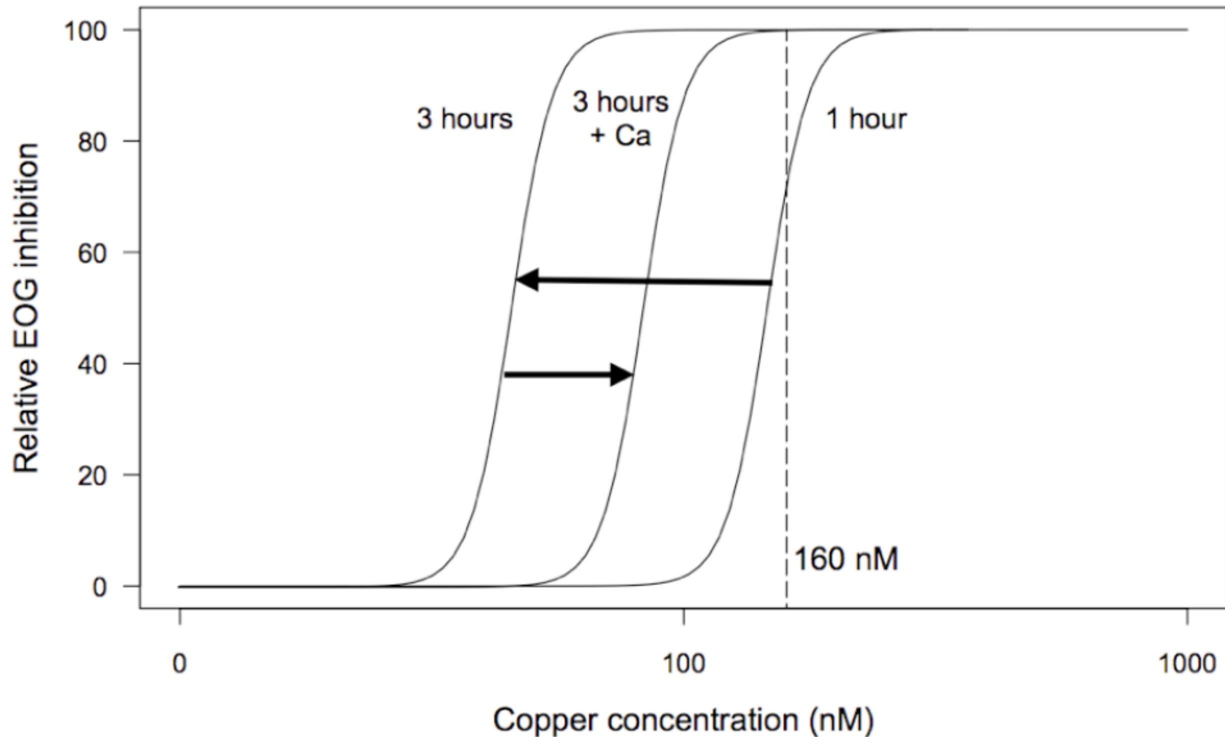

Figure 2.1. Concentration-response curves depicting the predicted theoretical reduction in the EOG response of fathead minnows by increasing time with the same exposure concentration of Cu, and predicted recovery, or “shift to the right” of the 3-h concentration-response curve with the addition of Ca. Note that in this model at 160 nM, no difference would be seen between a Cu alone and a Cu with Ca treatment at 3 h because the concentration-response curve is still at its upper asymptote of 100% inhibition in both exposures.

To further inform the development of a cbBLM, the purpose of the present study was to determine: (i) the interaction between Cu concentration and exposure duration on neurophysiological function in fathead minnows, (ii) if Ca protects against Cu-induced olfactory dysfunction in fathead minnows, and (iii) if current gbBLMs, reparameterized cbBLMs, or USEPA BLM, or hardness-adjustment equations used for setting site-specific water quality criteria would have been protective against the Cu-induced olfactory dysfunction. We exposed fathead minnows to very low, but environmentally-relevant Cu concentrations and tested their olfactory acuity using electro-olfactography (EOG), a neurophysiological assay, at time intervals bracketing those used in Green et al. (2010), and tested whether Ca could protect against Cu-induced olfactory dysfunction. We also parameterized three new cbBLMs based on the current understanding of the olfactory epithelium, and compared their predictions with standard gbBLM predictions and experimental data to determine if the current framework of the gill-based BLM can be adapted to the olfactory epithelium. For comparison, the acute (criterion maximum concentration, or CMC) and chronic criteria (criterion continuous concentration, or CCC) were also calculated using the USEPA BLM and hardness-adjustment equations (2002). Data generated by this study will be useful for the development and refinement of a cbBLM for fathead minnows.

2.2 Experimental procedures

2.2.1 Fish

Adult fathead minnows (1 - 4 g) were held in the Lakehead University Biology Aquatic Facility on a 16 h light:8 h dark photocycle in dechlorinated Thunder Bay ON municipal water (Table 2.1). All fish were held in a flow-through system at a density not

Table 2.1. Measured water quality for dechlorinated Thunder Bay municipal water. All values are mean ± SEM, except for pH which represents a range (n = 3 - 5).

Water quality parameter	Measured value
Calcium	363.6 ± 3.7 μM
Magnesium	115.4 ± 1.2 μM
Sodium	140.9 ± 2.4 μM
Temperature	19.0 ± 1 °C
Dissolved organic carbon (DOC)	1.6 ± 0.1 mgL^{-1}
pH	7.29 - 7.79
Alkalinity	50.4 ± 1.8 mgL^{-1} $CaCO_3$
Sulphate	38 μM
Chloride	<detection limit (1.4 μM)
Potassium	15.5 ± 0.3 μM

exceeding one fish per litre. Fish were fed *ad libitum* once daily, with *Artemia* spp. and commercial fish flakes (Tetra, Blacksburg, VA, USA) on alternate days. All fish were acclimated to laboratory conditions for a minimum of two weeks before being used in experiments. All experimental treatments were conducted in 4 L of aerated holding water, with a 50% water change every 24 h. All water quality measurements were performed by the Lakehead University Centre for Analytical Services (LUCAS), which is accredited through the Canadian Association for Laboratory Accreditation (CALA). All QA/QC procedures followed internal standard operating procedures of the LUCAS lab, including analysis of NIST traceable reference material standards.

2.2.2 Electro-olfactography procedure

Electro-olfactography (EOG) experiments were performed as previously described (Green et al., 2010). Fish were anaesthetized in 0.42-0.46 mM (110-120 mgL^{-1}) of pH 7.4 buffered MS-222. Water used to irrigate the olfactory epithelium had the same Cu and Ca content as the experimental treatment water detailed below, and was made just prior to the EOG measurements.

The stimulus used to induce an olfactory response was 10^{-4} M L-arginine, and was made fresh daily from powdered L-arginine (Sigma, Oakville, ON, Canada) in dechlorinated Thunder Bay municipal water (Table 2.1). For animals treated with Ca, the stimulus was prepared in dechlorinated Thunder Bay ON municipal water containing the same concentration of Ca as the experimental treatment waters. Calcium stock solutions were prepared by dissolving $Ca(NO_3)_2$ (Sigma, Oakville, ON, Canada) in dechlorinated water, and were diluted to the appropriate concentration for each experiment. A minimum of three 3-second pulses of the stimulus was delivered to the

olfactory epithelium, with a minimum of 2 minutes between each of the stimulus deliveries to minimize potential chemosensory attenuation to the stimulus.

2.2.3 Interaction between Cu concentration and exposure duration

To determine the single and combined influences of Cu concentration and exposure duration, fathead minnows were randomly assigned to one of 5 concentrations of Cu, each at 4 different exposure durations (1, 3, 24, and 96 h). A copper stock solution was prepared by dissolving $CuCl_2$ (Sigma, Oakville, ON, Canada) in dechlorinated water, and was used to make dilutions at the appropriate concentration for each experiment. The concentrations of Cu selected bracketed the Ontario water quality guidelines for Cu (78.7 nM or 5 μgL^{-1}) and represent concentrations that are found in clean and Cu-contaminated lakes (Ontario Ministry of Environment and Energy, 1994; Pyle et al., 2005). The two lowest concentrations (0 and 31.5 nM) are reported as nominal concentrations because all measurements of Cu concentration in the 0 nM exposure waters were below the detection limit (31.5 nM), two measurements of the 31.5 nM (2.0 μgL^{-1}) exposures were at the detection limit, and two were below ($n = 4$). All the other Cu concentrations (mean ± SEM, 72.7 ± 6.6, 106.2 ± 3.9, and 204.6 ± 17.0 nM; $n = 4 - 5$) were measured by LUCAS using inductively coupled plasma atomic emission spectroscopy (ICP-AES). All exposure waters used to treat fish were made up immediately before exposures began, and, if needed, immediately before any water changes.

2.2.4 Effect of Ca on Cu intoxication

To determine the effect of Ca on Cu-induced olfactory dysfunction, fish were randomly assigned to each of four experimental treatments (control (nothing added), Cu

added, Ca added, and Cu and Ca added) for two exposure durations (3 and 96 h; Table 2.2). The concentration of Cu in the Cu-added treatment approximately matched the second highest concentration used in the first experiment, and the concentration of Ca in the Ca-added treatment approximately matched the highest concentration of total Ca used in Green et al. (2010). The olfactory acuity of each animal was measured using the EOG technique outlined above. All measurements of Cu and Ca were performed via ICP-AES. All exposure waters used to treat fish were made up immediately before exposures began, and, in needed, immediately before any water changes.

To establish if the effect of Cu and Ca coexposures on EOG response to the stimulus represents an additive interaction between Cu and Ca, we selected an effects addition model as Ca and Cu were expected to act dissimilarly. To this end, the additivity equation (eq 2) from Norwood et al. (2003) was adapted by substituting mean relative EOG amplitudes for survival proportion. Consequently, the expected EOG amplitude of a mixture of Cu and Ca can be calculated as follows:

$$(2.1) \quad EOG_{Mixture} = EOG_{Cu} \times EOG_{Ca}$$

where EOG_{Cu} is the mean relative EOG amplitude (i.e., the proportional amplitude relative to the mean no-Cu control response) of fish when exposed to Cu alone, and EOG_{Ca} is the mean relative EOG amplitude of fish when exposed to Ca alone. The product ($EOG_{Mixture}$) is the predicted value for the mean relative EOG amplitude of fish given a Cu and Ca co-exposure, if the interaction between Cu and Ca is response-additive. The predicted $EOG_{Mixture}$ was then compared with the measured mean relative EOG response of fish given a Cu and Ca co-exposure (EOG_{Cu+Ca}). If the value for EOG_{Cu+Ca} is similar to $EOG_{Mixture}$, there is a response-additive interaction between Cu

Table 2.2. Measured Cu and Ca concentrations for control, Cu alone, Ca alone, and the Cu and Ca combined treatments. All values are in nM for Cu and in μM for Ca, and are presented as mean ± SEM, n = 4.

Experimental Treatment	Cu (nM)	Ca (μM)
Control	52.5 ± 5.3	424.8 ± 8.3
Cu alone	173.1 ± 16.2	426.0 ± 36.8
Ca alone	65.3 ± 7.0	1,117.0 ± 19.4
Cu and Ca combined	173.1 ± 18.6	1,112.4 ± 23.4

and Ca. If EOG_{Cu+Ca} is substantially lower than $EOG_{Mixture}$, the interaction is classified as more than response-additive. If EOG_{Cu+Ca} is substantially higher than $EOG_{Mixture}$, the interaction is antagonistic (relative to response-additivity). Equation 2.1 was used to determine the expected EOG values for a mixture of Cu and Ca based on the EOG values for the individual metal exposures at the 3 and 96 h time points.

2.2.5 Data handling and statistical analysis

The raw EOG amplitude in response to 10^{-4} M L-arginine for each animal was determined by measuring the difference between the baseline and the maximum response recorded following each stimulus delivery. The average response from a minimum of three measurements per fish was determined. Each averaged EOG amplitude was then corrected by subtracting any response measured to the appropriate blank. For the first set of experiments, the blank was dechlorinated municipal water, for the second set of experiments the blank contained the same concentration of Ca as the water to which each fish was exposed. Results are presented as relative EOG responses, expressed as a proportion of the response measured for the 0 nM Cu exposures. The values at each time point/Cu concentration or time point/Cu and Ca concentration combination were then averaged. As such, all reported results are blank-corrected mean relative EOG amplitudes (with associated standard error of the mean).

All statistical analyses were performed using R, version 2.13.0 (R Development Core Team, 2012), with graphics made using the sciplot package (Morales, 2011). Mean differences in relative EOG responses were considered statistically significant when $p \leq 0.05$. A two-way analysis of variance (ANOVA) was used to examine the single or combined influences of Cu concentration and exposure duration. A 2-way ANOVA was

also used to examine the single or combined influence of treatment and exposure duration for the Cu, Ca, and Cu/Ca coespoxures. A Tukey-Kramer test was performed to determine differences between individual groups for the Cu, Ca, and Cu/ca coexposures at $p \leq 0.05$.

2.2.6 BLM modelling

Predicted median lethal concentrations (LC50s) and median inhibitory concentrations (IC50s) for olfactory impairment from a total of six BLMs (two gbBLMs, four cbBLMs) were compared to empirical results to determine if the current BLMs could be used or adapted to determine safe Cu concentrations. All BLMs were based on the HydroQual Cu BLM version 2.2.3 (HydroQual Inc., 2007). The six BLMs were the rainbow trout gbBLM (RBT-gbBLM), the coho salmon cbBLM (CS-cbBLM) as parameterized by Meyer and Adams (2010), the fathead minnow gbBLM (FHM-gbBLM), and three fathead minnow cbBLMs (FHM-cbBLM 1, FHM-cbBLM 2, and FHM-cbBLM 3) parameterized based on current understanding of the olfactory epithelium.

The three FHM-cbBLMs were made by adjusting the parameter file as follows. For the first model, FHM-cbBLM 1, the parameter file was changed by deleting sections dealing with Ca binding to the ligand (but retained the effects of Ca binding to DOC), as recent evidence shows that Ca offers little to no protection against Cu-induced olfactory dysfunction (Baldwin et al., 2003; McIntyre et al., 2008; Green et al., 2010*; and this study)*. For FHM-cbBLM 2 the binding constant for Cu was adjusted to 6.7, as this is the empirical binding constant of Cu for the olfactory epithelium of fathead minnows (Green et al., 2010). Both modifications for FHM-cbBLM 1 and FHM-cbBLM 2 were used in FHM-cbBLM 3.

The water quality parameters in Table 2.1 were used as inputs to produce the BLM predictions. Running the BLMs in 'toxicity mode' produced 'LC50' values, as EOG inhibition is not a measure of lethality the LC50 estimates from cbBLMs were considered to be IC50 values for EOG inhibition. As the humic acid (HA) content of the dechlorinated tap water was not known, two different concentrations, 10% HA and 50% HA were used for comparison. HydroQual (HydroQual Inc., 2007) recommends 10% HA when the exact HA content is not known. The 50% HA content was used as it is representative of humic acid content in DOC from Ontario lakes (Al-Reasi et al., 2011). For the experimentally-derived values, concentration response curves of EOG inhibition and IC50 values for the 24 and 96 h Cu exposures were determined using the drc package in R (Ritz and Streibig, 2005). As the lowest concentration tested was below the detection limit, 1/2 the detection limit (15.75 nM) was used with the 0 inhibition level.

According to Meyer and Adams (2010), the HydroQual BLM uses the same algorithms as the USEPA BLM, therefore the HydroQual BLM was set to water quality criteria (WQC) mode and the CMC and CCC values were determined for the exposure waters (based on Table 2.1). The acute US EPA Criterion Maximum Concentration (CMC) specifies the highest average concentration of a toxicant in ambient water to which an aquatic community can be exposed briefly without resulting in an unacceptable adverse effect. For regulatory compliance, the CMC should not be exceeded in a 1-h averaging period more than once every three years. The chronic US EPA Criterion Continuous Concentration (CCC) specifies the highest average concentration of a toxicant in ambient water to which an aquatic community can be exposed indefinitely without resulting in an unacceptable adverse effect. For regulatory

compliance, the CCC should not be exceeded in a 4-d averaging period more than once every 3 years. Values for CMC and CCC were also calculated based on hardness-adjustment Cu-criteria equations (USEPA, 2002). As these concentrations represent predicted safe Cu concentrations, the appropriate comparisons to experimental results would be the inhibitory concentration shown to cause no impairment to the animals. While there is currently no empirical evidence in the literature showing what the safe inhibitory concentration is, the IC20 has been proposed as a surrogate because variability in EOG responses is typically approximately 20% (Meyer and Adams, 2010). The IC20 value for the 96 h exposures was calculated for comparison to the CCC value using the drc package in R (Ritz and Streibig, 2005). Any IC20 prediction based on the 1 h exposures would be highly suspect as the lowest degree of inhibition seen in the data was approximately 60%. For this reason all that can be said is the CMC is comparable to a value less than the lowest concentration that showed an effect after a 1 h exposure.

2.3 Results

2.3.1 Interaction between Cu concentration and treatment duration

Impaired olfaction resulting from the initial Cu exposure at all concentrations was followed by a recovery, at least partly, by 96 h. There was a statistical interaction in EOG function between Cu concentration and exposure duration ($F_{(12,83)}$ = 5.83, p < 0.001; Figure 2.2). At Cu concentrations of 31.5 and 72.7 nM, there was a strong initial reduction in EOG amplitude followed by a gradual increase over time resulting in a full recovery at 96 h. At the next highest Cu concentration (106.2 nM), the same pattern of

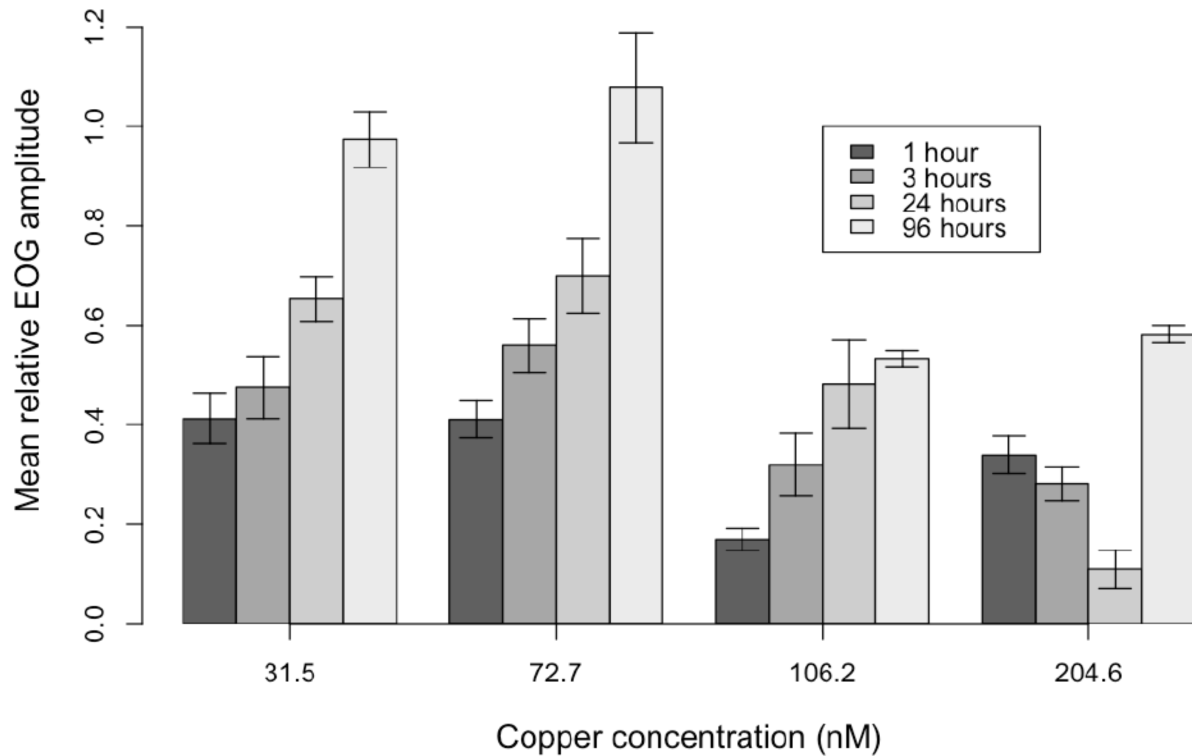

Figure 2.2. Interaction of exposure duration (h) and Cu concentration (nM) on mean EOG (± SEM) response of fathead minnows in response to 10^{-4} M L-arginine (n = 3 - 6 animals per bar). As time increases there is an increased recovery of EOG function for all concentrations, and as concentration increases there is a decrease in EOG function at each sampling point.

recovery was seen, except that the initial inhibition of EOG function was greater and the recovery was not complete after 96 h. Interestingly, the pattern of recovery for the highest concentration of Cu (204.6 nM) was unlike all of the other concentrations. Olfactory function, as measured by EOG, gradually decreased over 24 h, but showed significant recovery at 96 h. The amount of recovery at 96 h with the animals treated with the highest concentration of Cu was approximately the same as with the 106.2 nM Cu exposure concentration.

2.3.2 Effect of Ca on Cu intoxication

Elevated waterborne Ca offered no protection against the inhibition of olfaction caused by elevated waterborne Cu. There was a statistical interaction between treatment and exposure duration ($F_{(2,13)} = 25.65$, $p < 0.03$; Figure 2.3). There was a reduction in EOG function across all treatments (Cu, Ca, Cu and Ca) at both sampling points (3 and 96 h). The Cu-alone exposures showed a trend similar to that seen in the first experiment (Figure 2.2), in that there was an increase of EOG function between 3 and 96 h. Interestingly, when fish were treated with only Ca, EOG function decreased by almost the same percentage at the 3 and 96 h time points. Co-treatments with both Cu and Ca showed two separate patterns: at 3 h the relative EOG response was significantly lower than that with Ca alone, but was not significantly different from the Cu alone treatment, while at 96 h the relative EOG response for the combined treatment was significantly lower than both of the individual treatments.

The interaction between Cu and Ca differed depending on the exposure duration. In the short-term exposure (3 h), there was a response-additive relationship between Cu and Ca because the values for $EOG_{Mixture}$ and EOG_{Cu+Ca} were similar (Table 2.3). For

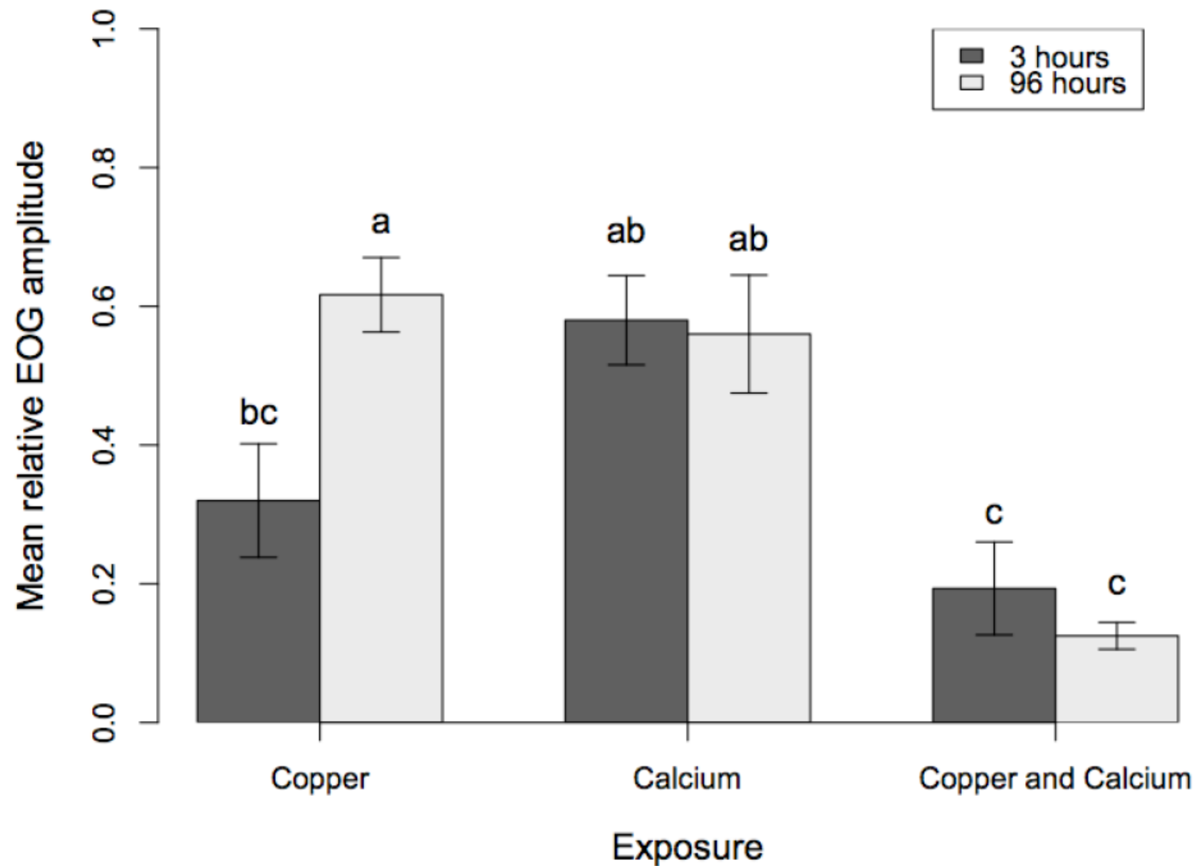

Figure 2.3. Individual (copper treatment has 173.1 nM Cu, calcium treatment has 1,117.0 nM Ca) and combined effect of Cu and Ca (173.1 nM Cu and 1,112.4 nM Ca) exposure on the mean relative EOG response (± SEM) of fathead minnows to 10^{-4} M L-arginine (n = 3 for each bar). Different letters above bars indicate a significant difference ($p \leq 0.05$)

Table 2.3. Comparison of the measured mean relative EOG response of fathead minnows to 10^{-4} M L-arginine for co-exposures of Cu and Ca (EOG_{Cu+Ca}) to the predicted mean relative EOG response of fathead minnows ($EOG_{Mixture}$) based on the mean relative EOG responses for individual treatments of Cu and Ca.

Exposure Duration	EOG_{Cu+Ca}	$EOG_{Mixture}$
3 hours	0.192	0.186
96 hours	0.126	0.347

the long-term exposure (96 h), EOG_{Cu+Ca} was much lower than $EOG_{Mixture}$, indicating a more-than-additive relationship with respect to the assumption of response additivity. Therefore, the relationship between Cu and Ca appears to shift from additive to more-than-additive inhibition over time.

2.3.3 BLM predictions and experimental IC50s

To test whether or not the current Cu BLM framework could be applied to the olfactory epithelium, IC50 predictions from two gbBLMs and four cbBLMs were compared to experimentally derived values (Table 2.4). The BLMs were run with HA content set to either 10% or 50% HA, and all LC50 and IC50 predictions when models were based on 50% HA were higher. Currently, the only chemosensory-based BLM that has been parameterized is the CS-cbBLM proposed by Meyer and Adams (2010). For a comparison the RBT-gbBLM was also used in the current study, as this source file was what was used to construct the CS-cbBLM (Meyer and Adams, 2010). The IC50s predicted by the CS-cbBLM were less than those predicted by the RBT-gbBLM, which was expected as the parameterization of the model assumes that the olfactory epithelium is more sensitive than the gill to Cu. Both the RBT-gbBLM LC50s and the CS-cbBLM IC50s were higher than the experimentally derived IC50s. In terms of fathead minnow models, when the FHM-gbBLM predictions were compared to the three different FHM cbBLMs, clear differences were seen. The prediction for FHM-cbBLM 1 was lower than the prediction for FHM-gbBLM; this was expected as the protective effect of Ca was removed from the model. For the second model (FHM-cbBLM 2), it was expected that as the binding constant for Cu was reduced, a higher IC50 would be predicted as compared to the FHM-gbBLMs results. The lower the binding constant of

Table 2.4. Comparison of LC50s and IC50s predicted by six BLM models to experimental IC50s. The two gbBLMs used the default parameters specified by HydroQual {HydroQual Inc., 2007, Biotic Ligand Model User's Guide and Reference Manual, version 2.2.3}; all cbBLMs were parameterized as discussed in the Experimental Procedures section. The experimental values were derived from the concentration response curves in SI-Figure 1, bracketed values represent 95% confidence intervals. All concentrations are in μM.

Name	Fish Species	Endpoint	10% HA	50% HA
RBT-gbBLM	Rainbow trout	LC50	1.89	2.45
CS-cbBLM	Coho salmon	IC50	0.57	1.08
FHM-gbBLM	Fathead minnow	LC50	2.28	2.96
FHM-cbBLM 1	Fathead minnow	IC50	1.84	2.49
FHM-cbBLM 2	Fathead minnow	IC50	4.71	5.58
FHM-cbBLM 3	Fathead minnow	IC50	3.44	4.23
Experimental - 24 h	Fathead minnow	IC50	0.087 (0.063-0.111)	
Experimental - 96 h	Fathead minnow	IC50	0.213 (0.103-0.323)	

Cu, the less Cu will be binding to the biotic ligand and causing an effect. The FHM-cbBLM 2 did, in fact, predict a higher IC50 value than the FHM-gbBLM, as expected. The third model, FHM-cbBLM-3 predicted an IC50 between the other two FHM-cbBLMs. Regardless of which fathead minnow model was used, all predicted IC50s were higher than the experimental results. In fact, the closest prediction to experimental was with FHM-cbBLM 1 (at 10% HA), which predicted an IC50 over 8.5x higher than the experimental value for 96 h and over 21x higher than the experimental value for 24 h.

To determine if the USEPA BLM and hardness-adjustment equations predicted CMCs and CCCs protective of the olfactory epithelium, CMC and CCC criteria were compared to experimentally measured values (Table 2.5). For comparison to the CMC and CCC values, the appropriate comparison would be IC20s for 1 and 96 h. Unfortunately the data were not appropriate to predict a 1 h IC20, and all that can be stated is that the IC20 is below the lowest concentration tested, namely <31.5 nM. The 96 h IC20 value was calculated to be 94.6 nM (Figure 2.4). The results show that the lowest prediction for a CMC value (the USEPA BLM set to 10% HA) is over 3x higher than the 1 h experimental value. For the CCC, the hardness-adjustment equation predicted a Cu concentration that was below the 96 h IC20, the USEPA BLM set to 10% HA predicted a Cu concentration that was lower than the 96 h IC20, and when set to 50% HA, the USEPA BLM predicted a CCC that was approximately 60% higher than the 96 h IC20.

Table 2.5: Comparison of USEPA BLM predictions for CMC and CCC at two concentrations of humic acid (HA) to the hardness-adjusted values and experimentally derived IC20 values. The values predicted for the CMC are compared with the lowest concentration used, and the values predicted for the CCC are compared to the 96 h IC20. All values are in nM.

	CMC predictions and 1 h experimental results	CCC predictions and 96 h experimental results
gbBLM (10% HA)	96.5	60.0
gbBLM (50% HA)	246.4	153.1
Hardness adjusted	105.9	75.2
Experimental IC20	<31.5	94.6

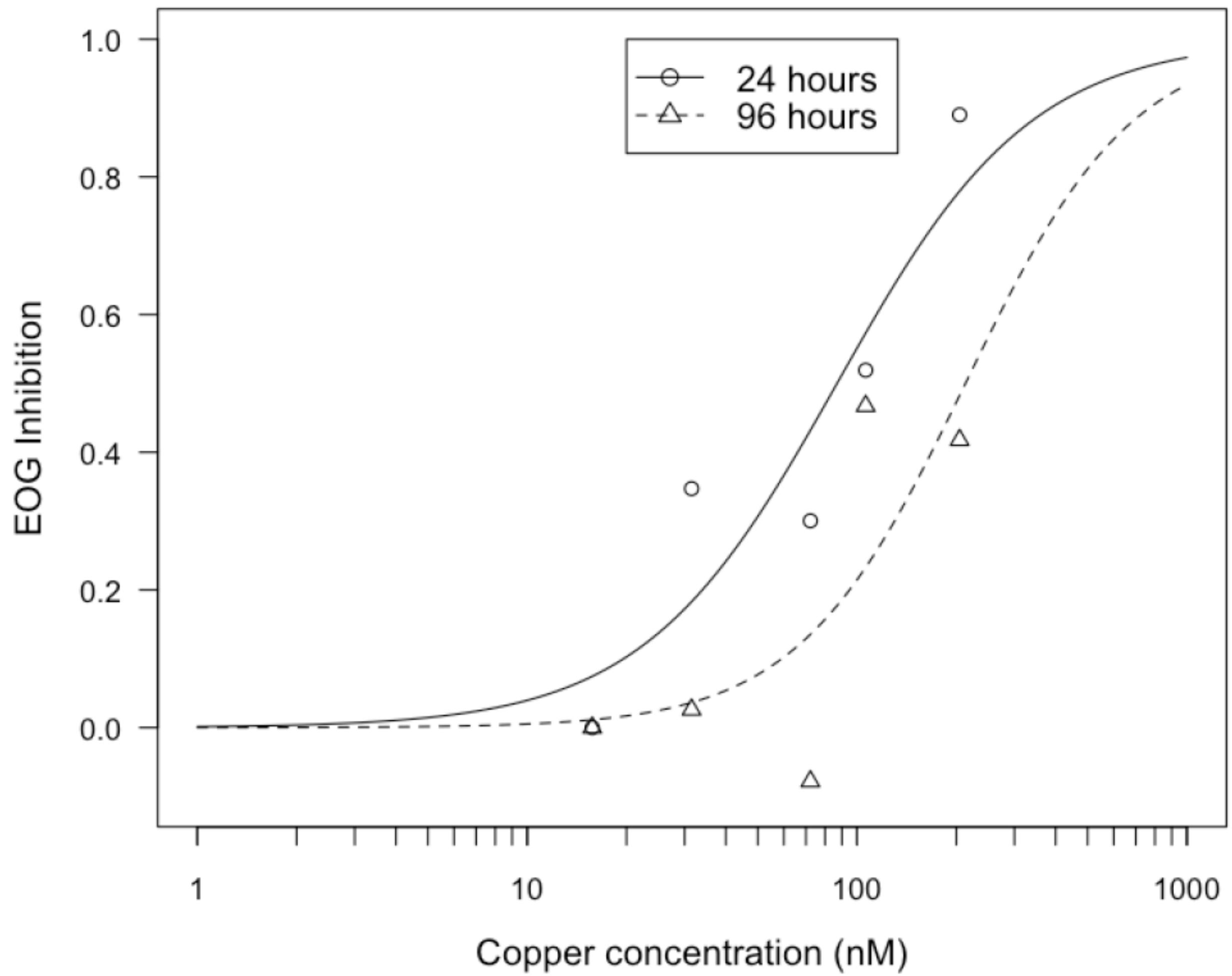

Figure 2.4. Concentration-response curve of EOG inhibition at the 24 and 96 h Cu exposures shown in Figure 2.2.

2.4 Discussion

This study demonstrates that Cu exposure at environmentally-relevant concentrations (in this case below the analytical detection limit of 31.5 nM) can inhibit a fish's olfactory function. Although short-term Cu exposure effectively inhibited the EOG response at all concentrations tested, continuous exposure to Cu for up to 96 h resulted in at least partial EOG recovery. This pattern of recovery illustrates that olfactory sensory neurons (OSNs) have a capacity to recover from Cu-induced inhibition while still being exposed to Cu. Similar exposures using a behavioural endpoint have also shown recovery over time during continuous copper exposure (Saucier and Astic, 1995; Beyers and Farmer, 2001). The degree of recovery seen in the current study is dependent on exposure concentration, because the fish exposed to the two lowest concentrations were able to fully recover their EOG function by 96 hours. Fish exposed to the two highest Cu concentrations did not fully recover, indicating that the ability of the OSNs to recover within 96 h was exceeded. At the longest exposure durations the effect appeared to be concentration-dependent in agreement with a previous study (Sandahl et al., 2004). Previous studies have shown that removal of contaminants such as Cu (Baldwin et al., 2003; Sandahl et al., 2006) or pesticides (Tierney et al., 2006) results in recovery of OSN response to stimuli. No studies to date have shown recovery during continuous exposure to a contaminant. A study comparing yellow perch (*Perca flavescens*) from metal-contaminated and clean lakes showed higher EOG response in fish from metal-contaminated lakes than those from clean lakes (Mirza et al., 2009). The same study showed that even though EOG response was intact, behavioural response to an antipredator cue was impaired (Mirza et al., 2009). The current study suggests

that fish continuously exposed to Cu-contaminated water may recover from short-term neurophysiological (EOG) deficits via some unknown mechanism; whether or not they recover from corresponding behavioural deficits remains unknown. Work that may elucidate this mechanism was done by Tilton et al. (Tilton et al., 2008) In their study zebrafish (*Danio rerio*) was exposed to Cu at concentrations similar to ours for 24 h, and microarrays were performed using their entire olfactory system (olfactory epithelium, nerve, and bulb). In the present study, there was some recovery at 24 h, so the gene expression patterns discussed in their paper may hold clues into this mechanism of recovery. An alternative explanation of these results is that the differences in EOG response between time points is not due to recovery, but due to the length of time it takes DOC and Cu to come to equilibrium (Ma et al., 1999). This could mean that the longer a fish is left in the Cu exposure waters, the less Cu it is being exposed to as more is being bound by the DOC, resulting in less effect on the olfactory epithelium. We do not believe that this is occurring in this study, as the irrigation water used for the EOG experiments was made up just prior to performing the experiments (from a common stock), and therefore animals across all time points were exposed to water of the same age and concentration during the EOG measurements. Earlier studies have shown that when fish have been exposed to Cu in the irrigation water, impairment can occur within 10 min of exposure (Green et al., 2010). In addition, investigations on the accumulation of Cu at the gill showed that the age of DOC-Cu mixtures has no effect on Cu accumulation (Hollis et al., 1996). This result conflicts with the work by Ma et al., (1999) who showed a difference. However, the latter study was done to test an acute end point on an invertebrate, while the work in Hollis et al. (Hollis et al., 1996) measured

accumulation of Cu in a fish tissue. As the current study is with fish and measures a subacute end point, it is likely that the age of DOC-Cu mixtures does not explain why there is a greater EOG recovery with longer Cu exposures. Further research is required, especially to elucidate the potential effects of varying water chemistry parameters on the effect concentrations.

As was seen in previous studies, the addition of Ca offered no protection from Cu-induced olfactory dysfunction (Baldwin et al., 2003; Green et al., 2010). In our experiments, we held the Cu and Ca concentrations constant and varied the exposure durations, meaning we do not know the full spectrum of responses across mixture scenarios. However, at the concentrations used, the combined effect of Cu and Ca was different depending on the exposure duration. A 3 h co-exposure to Ca and Cu yielded an additive effect on EOG response, while a Ca and Cu 96 h co-exposure to Ca and Cu showed a more than additive effect. The additive interaction between Cu and Ca at 3 h indicates that both Ca and Cu are exerting their effect on olfaction through independent mechanisms. The more than additive interaction between Ca and Cu after a 96 h exposure is most likely due to Ca interfering with the ability of the OSNs to recover from the Cu treatment. This means that not only does Ca fail to protect against Cu-induced olfactory dysfunction, but it also prevents recovery from Cu intoxication. These results seem to conflict with a study published by Bjerselius et al., (1993) which showed that the effect of short term exposures (4 min) with high concentrations of Cu (10 µM) on EOG response was reduced by the addition of Ca (up to 4 mM). However, those authors also showed adding enough magnesium (Mg) to the water to match the ionic strength found in the 4 mM Ca exposure resulted in the same protective effect.

Therefore, this apparent protective effect is most likely due to a general protective mechanism involving the ionic strength of the exposure water, and not a specific protective effect of Ca. In fact, the conclusion of the authors was that the reduced effect of Cu was most likely explained due to a lower Cu^{2+} activity in these solutions due to an increased ionic strength of the solution.

That exposure to Ca alone causes olfactory dysfunction in fish has had little attention in the literature. McIntyre et al. (2008) observed a small but significant negative relationship between EOG amplitude and Ca concentration (from $CaCl_2$) in juvenile coho salmon (*Oncorhynchus kisutch*). This Ca-induced reduction in EOG response could be occurng due to a variety of mechanisms. As calcium is essential in numerous steps in olfactory signal transduction, (Firestein, 2001) adding Ca may directly interfere with the olfactory response to the cue. In addition, there may be cross-adaptation between Ca and the amino acid stimulus used. Cross-adaptation occurs when one odourant activates OSNs that overlap with those activated by a second odourant, causing a decreased response to the second odourant (Hansen et al., 2003; Michel et al., 2003; Rolen et al., 2003). Calcium has been shown to be an odourant in a variety of fish species including the gilthead seabream (*Sparus aurata*) and goldfish (*Carassius auratus*) (Hubbard et al., 2000; Hubbard et al., 2002), and may interfere with the response to L-arginine due to an overlap in receptors that recognise both odourants. The fact that Ca causes a reduction in response by itself and that it offers no protection from Cu-induced olfactory dysfunction suggests that any future cbBLM should consider Ca, not as a competing cation, but as a metal capable of inducing its own independent effects on fish olfaction.

It may be argued that the counter-ion of the Ca salt may explain some of the observed Ca-induced effects, including the apparent lack of protection against Cu intoxication of olfactory epithelium, the exacerbation of the Cu effect, or the induction of a unique olfactory response. Although the current study used $Ca(NO_3)_2$ as the Ca salt [i.e., the same salt used by Green et al. (2010)], McIntyre et al. (2008) found that $CaCl_2$ produced similar effects to those reported here. Bodznik (1978) reached a similar conclusion regarding the counter anions of sodium salts.

Predictions from six different BLMs (two gbBLMs and four cbBLMs) were compared to experimentally-derived results to answer two questions: first, are LC50s predicted by the current gbBLMs lower than experimentally-derived IC50s (i.e., are they protective against Cu-induced olfactory dysfunction)?; and second, can a simple reparameterization of current gbBLMs make cbBLMs which predict IC50s lower than experimentally-derived values? To answer the first question, the RBT-gbBLM and FHM-gbBLM were used with the water quality measures of the exposure waters, assuming either 10% or 50% HA content. When compared to the experimentally-derived results, it is clear that the LC50s predicted by the current gbBLMs are higher than experimentally-derived values. To answer the second question, four cbBLMs were made based on different reparameterizations of the gbBLMs. All cbBLMs predicted IC50 values in excess of the experimentally-derived IC50s. This result indicates that a simple reparameterization of currently existing gbBLMs is not sufficient to produce a model that predicts IC50s below experimentally-derived values for the olfactory epithelium. Taken together, there is currently no BLM, gill or chemosensory based, that is capable of predicting IC50s for Cu intoxication below experimentally-derived values for the

olfactory epithelium. This is not surprising as the olfactory epithelium and gills have very different functions, and as this study has demonstrated, the interactions between ions (Cu, Ca, etc.) at the olfactory epithelium and gills appear to be quite different. Much more work must be undertaken to understand the interactions at the olfactory epithelium before a useful cbBLM can be constructed.

The USEPA BLM and hardness-adjustment equations were used to determine Cu concentrations which are considered “safe” from a regulator perspective (i.e., the CMC and CCC). For the acute criterion (CMC), all concentrations predicted were higher than the experimental value, suggesting that the USEPA BLM and hardness-adjustment equations are not protective for acute Cu-induced olfactory dysfunction. For the chronic criterion (CCC), the hardness-adjustment equation predicted a value below the experimental 96 h IC20, and the USEPA BLM predictions bracketed this result. While this means that the CCC as predicted by the hardness-adjustment equation was protective, the USEPA BLM may or may not be protective, depending on the %HA used in the calculations. Taken together, these results demonstrate that the current USEPA BLM and hardness-adjustment equations are not always protective against Cu-induced olfactory dysfunction.

In contrast to what was predicted in Figure 2.1, a comparison of the concentration response curves in Figure 2.4 clearly demonstrates that increasing the length of Cu exposure results in a decreased effect of Cu. A comparison of the IC50 value for 24 h (87 nM) with that for 94 h (213 nM) clearly shows this trend. At the concentrations used in this study, pulses of Cu into waterways may be more detrimental to olfaction in fish than increased background Cu levels.

In conclusion, this study shows that interaction of Cu and Ca at the olfactory epithelium is much more complex than expected. This work demonstrates that: (i) fathead minnows are able to at least partially recover olfactory function during continuous exposure to waterborne Cu, (ii) Ca is not protective against Cu-induced olfactory dysfunction and has its own effect, (iii) current gbBLMs and simple reparameterizations to make cbBLMs do not predict realistic IC50 values for Cu-induced olfactory dysfunction, and (iv) current models do not produce CMC values which are protective against Cu effects at the olfactory epithelium. To construct a cbBLM that will be protective of Cu-induced olfactory dysfunction, the ability of OSNs to recover and the role of Ca (and other ions) at the olfactory epithelium must be further investigated.

Prelude to chapter 3

A major finding of Chapter 2 was that calcium was not protective of copper-induced olfactory dysfunction. An unexpected associated finding was that calcium, by itself, causes a reduction in the EOG response of fathead minnows to L-arginine. While no work to date has demonstrated that calcium induces olfactory dysfunction in fish, it is known that calcium is an odourant for a variety of fish species. It is possible, then, that calcium and L-arginine are competing for olfactory receptors resulting in what appears to be olfactory dysfunction caused by calcium. The work detailed in Chapter 3 has two major lines of inquiry. First, the effect of calcium on the olfactory response of fathead minnows at multiple levels of biological organization was measured to determine if calcium is an odourant for fathead minnows. Second, cross-adaptation at the olfactory epithelium between L-arginine and calcium was measured to determine if the reduced EOG response to L-arginine in fathead minnows is due to competition between calcium and L-arginine at the olfactory epithelium.

CHAPTER 3: Smelling salt: calcium as an odourant for fathead minnows

Summary

Calcium plays an essential role in olfactory sensory neuron function. Studies with fish have demonstrated that in addition to being involved in olfactory signalling, calcium is itself an odourant. In this study we used fathead minnows (*Pimephales promelas*) and employed two different techniques; electro-olfactography (EOG), a neurophysiological technique that measures olfactory acuity at the olfactory epithelium, and a behavioural choice assay using a trough maze. The results demonstrate that calcium shares receptors with L-arginine, induces an EOG response in a concentration-dependent manner, and induces a strong avoidance behaviour. Through the use of pharmacological agents, we also showed that calcium induces an olfactory response through a currently-unknown pathway. Taken together, the results demonstrate that calcium is a potent odourant for fathead minnows. Being able to smell calcium may represent an ability to sense and avoid areas with drastic changes in ionic strength, thereby avoiding physiological stress.

3.1 Introduction

Calcium ions play a vital role in the basic function of olfactory sensory neurons (ONSs), by being involved in the formation of an electrochemical gradient across the cell membrane and in olfactory signalling (Schild and Restrepo, 1998; Firestein, 2001). Evidence shows that in addition to being a vital element in basic olfaction, calcium is recognized by the olfactory epithelium (OE) of fish as an odourant (Bodznick, 1978; Hubbard et al., 2000). Response of fish to calcium as an odourant was first demonstrated by Bodznick (1978), where sockeye salmon (*Oncorhynchus nerka* Walbaum 1792) fry produced an electroencephalogram (EEG) response at the olfactory bulb and a behavioural avoidance response when presented with calcium as a chemosensory stimulus. Subsequent work measuring EEG or olfactory nerve responses in other fish species demonstrated that estuarine and saltwater teleost species respond to decreasing calcium concentrations (Hubbard et al., 2000; Velez et al., 2009), whereas freshwater species respond to increasing calcium concentrations (Bodznick, 1978; Hubbard et al., 2002).

In addition to behavioural and EEG responses to calcium, molecular studies have demonstrated that gene transcripts for calcium receptors are expressed in Atlantic salmon (*Salmo salar* Linnaeus 1758) (Dukes et al., 2006). As well, immunocytochemistry has been used to detect the presence of Ca^{2+}-sensing receptors in goldfish (Hubbard et al., 2002) and dogfish shark (*Squalus acanthias* Linnaeus 1758) (Nearing et al., 2002). Calcium sensing receptors are found in a variety of tissues and organs in fish, the function of which is to measure extracellular calcium concentrations(Loretz, 2008). In terms of internal tissues and organs, such as kidneys,

this sensing allows for homeostatic control of ions, when present in external tissues such as gills and OSNs, their function is not as clear. In OSNs, the calcium sensing receptors could be acting as odourant receptors, be part of a homeostatic control, or both (Loretz, 2008). Even though there are calcium sensing receptors present in OSNs, it does not mean that these receptors are acting as traditional olfactory receptors.

Olfactory information is transmitted via the olfactory nerve to the brain when odour molecules bind to OSN olfactory receptors (Zielinski and Hara, 2006). There are three known types of OSNs: ciliated, microvillous, and crypt cells (Hamdani and Døving, 2007). While all three OSN types have been shown to respond to a variety of odourants, each class of OSN shows some specificity to certain odourant classes. Ciliated cells, for example, are associated with response to bile salts (Hansen et al., 2003), while crypt cells are associated with mating cues (Hamdani et al., 2008; Bazáes and Schmachtenberg, 2012). While microvillous and ciliated cells both respond to amino acids (Zielinski and Hara, 2006), a recent study has shown that L-alanine is only recognized by microvillous cells (Laframboise and Zielinski, 2011). There are two known olfactory signalling cascades in fish OSNs; ciliated and crypt cells utilize a cAMP secondary messenger pathway, while microvillous cells have an IP3-dependent pathway (Hansen et al., 2003). By using pharmacological agents to modulate cAMP or IP3 production, it can be determined which class(es) of OSNs react to a specific odourant (Hansen et al., 2003). In addition to the two known olfactory pathways there is at least one additional signalling pathway because polyamines (e.g., cadaverine, putrescine, spermidine) induce an olfactory response independent of the two known cascades (Michel et al., 2003; Rolen et al., 2003).

Recently, we demonstrated that fathead minnows (*Pimephales promelas* Rafinesque 1820) exposed to calcium for 3 or 96 h had a smaller olfactory response to L-arginine than untreated controls (Dew et al., 2012). We hypothesized that this decrease in response may be due to cross-adaptation between calcium and L-arginine. Cross-adaptation occurs when two odourants share receptors, such that when the OE is stimulated by one of the odourants, OSNs that would normally respond to the second odourant cannot because they are already activated by the first odourant (Hansen et al., 2003; Michel et al., 2003; Rolen et al., 2003). Cross-adaptation, therefore, results in a reduced olfactory response to a second odourant after a first odourant has already activated OSNs required to perceive the second odourant. If calcium is shown to be cross-adaptive with another, well defined odourant, this gives further evidence that there are odourant receptors in OSNs that bind to calcium.

There are a number of gaps within the knowledge about calcium acting as an odourant for fish. It is unknown if calcium induces a response via olfactory receptors. Moreover, the olfactory signalling pathway that calcium induces is not known. The sole behavioural experiment of a fish's response to calcium used $CaCl_2$ as the chemosensory stimulus and demonstrated an aversion response (Bodznick, 1978). Their study did not consider that the counter anion could be inducing the behavioural response, nor did it demonstrate that the response was dependent on olfaction. As calcium sensing receptors have been shown to be in other tissues such as the gills, it is possible these tissues, and not the OE, are mediating the response to calcium.

In order to investigate the various gaps outlined above, we employed electro-olfactography (EOG), a technique that measures changes in the extracellular field

potential at the OE due to the addition of an odourant (Scott and Scott-Johnson, 2002; Green et al., 2010) and a behavioural assay. These two techniques were used to investigate various aspects of the response of fathead minnows to calcium. Cross-adaptation between L-arginine and calcium was investigated to determine if calcium was blocking the response to L-arginine and *vice versa*, something that would be expected if calcium was binding to olfactory receptors. In addition, EOG was employed to determine if the response to calcium was concentration dependent, and which signalling pathway mediates the olfactory response to calcium. For behavioural experiments, tests were done to determine if there was a similar avoidance to calcium in fathead minnows as was shown in sockeye salmon, if this response was concentration dependent, if the response was due to calcium and not an effect of a counter anion, and if the response to calcium did, in fact, require the olfactory system. This work fills in a number of outstanding gaps in our understanding of how calcium acts as an odourant for fish.

3.2 Experimental methods

3.2.1 Animals

Adult fathead minnows (1 - 4 g) were obtained from the USEPA and held in the Lakehead University Biology Aquatic Facility in a static renewal system at a density not exceeding one fish per litre. A 16 h light: 8 h dark photoperiod was maintained and all fish were held in dechlorinated Thunder Bay, Ontario (Canada) municipal water (Table 3.1). All fish were fed *ad libitum* once a day, with *Artemia* spp. and commercial fish flakes (Tetra, Blacksburg, VA, USA) on alternate days. All fish were allowed to acclimate to laboratory conditions for a minimum of two weeks before being used in experiments. All experiments were performed in accordance with the Canadian Council on Animal Care guidelines.

3.2.2 Electro-olfactography experiments

Electro-olfactography experiments were performed as previously described (Green et al., 2010). Fish were anaesthetized in 120 mgL^{-1} of pH 7.4 buffered MS-222 (Syndel Laboratories Inc, Qualicum Beach BC, Canada). Each olfactory stimulus was administered to the olfactory chamber a minimum of 3 times, with the order of stimulus delivery randomized for each fish to avoid any systematic bias to recorded responses as a function of delivery order. There was a delay of at least two minutes between each stimulus delivery to ensure the OE had sufficient time to recover from the previous stimulus delivery. The amplitude of each EOG response was measured by determining the difference between the baseline and the maximum response after a stimulus delivery. All measured EOG responses to each stimulus were blank corrected by

Table 3.1: Basic water quality measurements of dechlorinated Thunder Bay Municipal water. All values are mean ± s.e.m., except for pH and temperature which are presented a ranges (n = 3).

Water quality variable	Measured value
Calcium	13.91 ± 0.35 mgL^{-1}
Magnesium	2.82 ± 0.04 mgL^{-1}
Sodium	3.37 ± 0.01 mgL^{-1}
Temperature	19.5 - 20.5°C
pH	7.29 - 7.79
Alkalinity	50.4 ± 1.8 mgL^{-1} as $CaCO_3$

subtracting any response elicited to a blank stimulus. The composition of the blank stimulus depended on which experimental procedure was used, as detailed below.

3.2.3 Cross-adaptation methodology

Olfactory cross-adaptation between L-arginine and calcium was tested by first measuring the EOG response of an animal to either 1 mM L-arginine (Sigma, Oakville, ON, Canada) or 1 mM calcium (as $Ca(NO_3)_2$; Sigma). Animals were then adapted to either calcium (if response to L-arginine was initially measured) or L-arginine (if response to calcium was initially measured) alone for a minimum of 10 min. Adaptation was accomplished by irrigating the olfactory rosettes with water containing either 1 mM of L-arginine or 1 mM of calcium , as appropriate. After the 10 min. adaptation period, the L-arginine-adapted animals were tested for their olfactory response (using EOG) to 1 mM calcium and the calcium-adapted animals were tested for their olfactory response to 1 mM L-arginine. The initial and post-adaption response to each cue were corrected using an appropriate blank consisting of the irrigation water. The percent unadapted response for each animal was calculated by dividing the post-adaption response by the initial response for each cue. Any reduction in EOG response during the co-exposure would positively indicate for cross-adaptation.

3.2.4 Calcium concentration curve

To test if calcium induces a concentration dependent EOG response in fathead minnows, four different concentrations of calcium (0.1, 1, 10, and 100 mM nominal concentrations) were made from stock solutions of $Ca(NO_3)_2$ in dechlorinated water. Calcium concentrations were measured using inductively coupled plasma atomic emission spectroscopy (ICP-AES) by the Lakehead University Centre for Analytical

Services (LUCAS). The LUCAS laboratory is accredited through the Canadian Association for Laboratory Accreditation (CALA). Measured concentrations of calcium were 92-106% of nominal concentrations (Table 3.2). The EOG response to each of the four calcium solutions as well as a blank containing no added calcium was measured. Each response was blank corrected and averaged for each concentration.

3.2.5 Transduction cascade determination

To test whether or not calcium stimulated either of the two known olfactory signalling pathways, we applied either forskolin to stimulate the cAMP pathway or U-73122 to inhibit the IP3 pathway. By inference, a reduced olfactory response (relative to controls) to a standard chemosensory cue following a forskolin treatment suggests that the cue was primarily perceived by ciliated or crypt cells, both of which act by way of a cAMP signalling pathway. Similarly, a reduced olfactory response following a U-73122 treatment suggests that the cue was mainly perceived by microvillous cells, which make use of an IP3 signalling pathway.

For determining the transduction cascade induced by calcium, EOG was performed with 1 mM calcium (from $Ca(NO_3)_2$) made up in dechlorinated water daily. Each OE was exposed to the cue and a blank consisting of dechlorinated water. The EOG response to calcium was blank-corrected to give the unadapted response for each cue. One of two pharmacological agents was then added; either 1 μM U-73122 (Santa Cruz Biotechnology, Santa Cruz, California, USA) or 1 μM forskolin (Santa Cruz Biotechnology), both of which were made up in dechlorinated water. After the addition of the pharmacological agent, the response to calcium mixed with the agent was then measured, as well as the response to a blank containing the agent. This value was

Table 3.2: Comparison of measured and nominal calcium concentrations. All values are mean ± s.e.m. The first four concentrations represent calcium solutions made using $Ca(NO_3)_2$, the fifth concentration represents a solution made using $CaCl_2$.

Nominal Concentration (mM)	Measured value (mM)
0.1	0.11 ± 0.00
1.0	1.05 ± 0.00
10	10.24 ± 0.01
100	96.62 ± 0.26
10 ($CaCl_2$)	9.10 ± 0.03

blank-corrected and divided by the initial unadapted response for each cue to give the percent unadapted response. Only one pharmacological agent was used on any given fish.

3.2.6 Behavioural assay method

For all behavioural choice assays, opaque trough mazes were used (Figure 3.1). Each trough measured 69 cm x 14 cm x 16 cm (L x W x H). The bottom of each trough was covered in white corrugated plastic (Coroplast, Granby, Québec, Canada), which was sealed to the sides and bottom of the maze using aquarium grade sealant. Lines were drawn on the corrugated plastic, splitting the bottom into three separate areas. A plexiglass diffusion chamber was placed into both ends of the trough maze. These diffusion chambers measured 7.5 cm x 13 cm x 15.5 cm (L x W x H), and had 32 - 0.5 cm diameter holes drilled through each side of the chamber 1 cm from the bottom, which allowed for even stimulus release into the maze environment. The trough was filled with 10 L of dechlorinated water at 19-20°C. A bottomless translucent plastic box (the acclimation chamber) measuring 19 cm x 13 cm x 11 cm (L x W x H) was placed onto the middle section on the bottom of the maze (the acclimation zone). This plastic box was attached to a string that could be pulled from behind a blind to allow for the box to be raised without the fish being able to see the observer. At the beginning of each trial a fathead minnow was placed into the acclimation chamber, 50 mL of the stimulus for the trial was added to one of the diffusion chambers and 50 mL of dechlorinated water (blank) was added to the diffusion chamber on the opposite end. The stimulus was allowed to diffuse for 20 minutes, which also allowed the fish to acclimate to maze conditions. After this 20 minute period, the acclimation chamber was lifted off the bottom

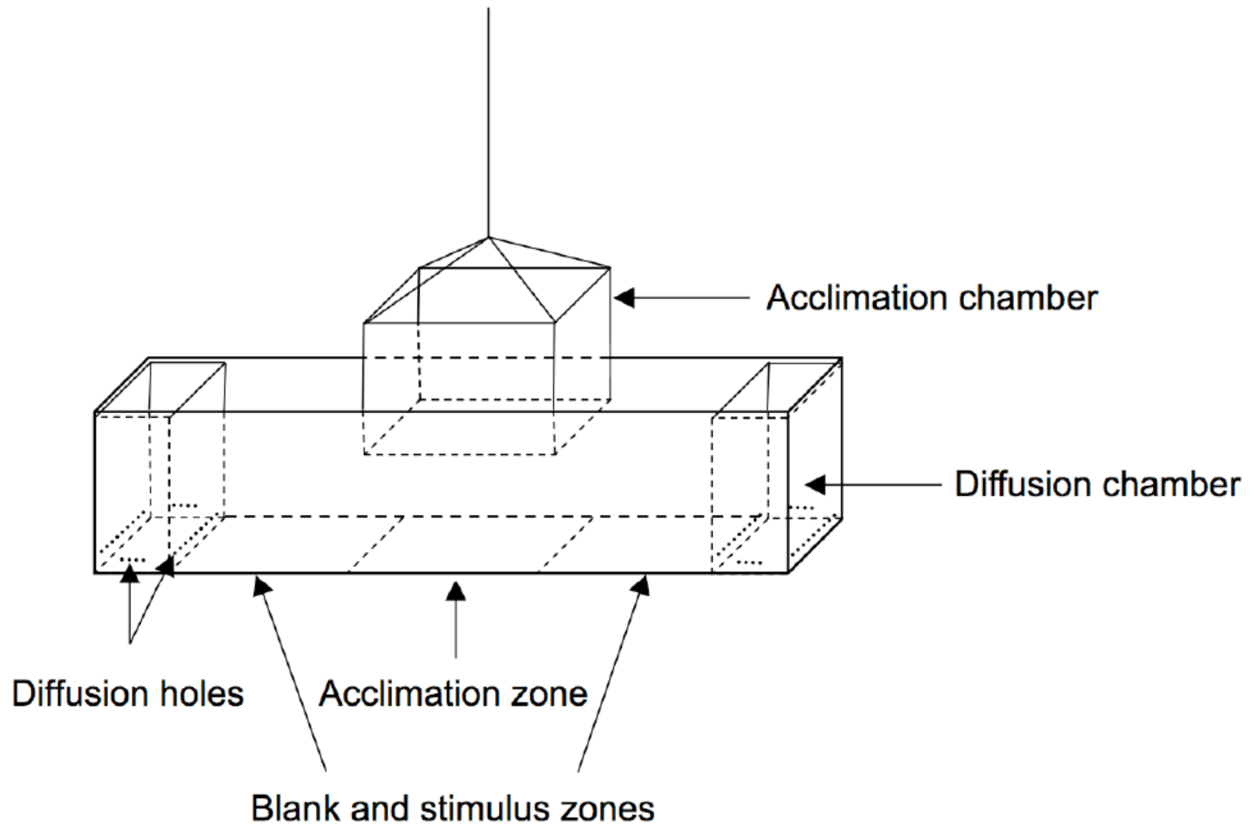

Figure 3.1: Diagrammatic representation of the trough maze used for behavioural trials.

to just below the surface of the water to ensure that the surface of the water was not disturbed. Each fish was monitored using a web camera (RocketFish, Richfield MN, USA) attached to a MacBook computer. The position of the fish in the maze, either the stimulus arm, blank arm, or acclimation zone, was recorded every 10 seconds for a total of 8 minutes. The number of times each fish was in each location was tallied and multiplied by 10 to derive time spent in each arm.

3.2.7 Behavioural response to calcium

To measure the response of fathead minnows to calcium, the behavioural assay method detailed above was used with the four different concentrations of calcium that were used in the calcium concentration curve EOG experiment (Table 3.2).

To eliminate the possibility that the behavioural response to calcium was due to the counter ion of the calcium salt being used, $Ca(NO_3)_2$, a second calcium salt, $CaCl_2$ (Sigma), was used to make a nominal 10 mM calcium solution (9.1 mM measured, Table 3.2). This calcium solution was then used as the stimulus in a behavioural experiment.

It is possible that the response to calcium was mediated by some other tissue or sense besides olfaction. To determine if the behavioural response was olfactory-mediated, behavioural trials were performed with anosmic fish. Fathead minnows were made anosmic by the application of Vetbond tissue adhesive (3M, St. Paul, MN, USA) to their olfactory chambers. Fish were first lightly anaesthetised in water containing 120 mgL^{-1} MS-222 buffered to pH 7.5. The snout of each fish was dried and excess water was removed from each naris using a folded KimWipe (Kimberly-Clark, Dallas, TX, USA). Using a pipette tip, 2-3 µL of Vetbond was added to each naris. The Vetbond was

allowed to dry (5-10 s) and the fish was placed into a tank of aerated water to recover. For a control, a second group of fish were treated in exactly the same manner, except 2-3 μL of dechlorinated water was added to each olfactory chamber instead of the Vetbond adhesive. Fish were allowed to recover overnight, after which their response to 10 mM calcium (using $Ca(NO_2)_3$ as the calcium salt) was measured.

3.2.8 Statistical analysis

All statistical analyses were conducting using R, version 2.13.0 (R Development Core Team, 2012). All graphics were made using the sciplot package (Morales, 2011). For the cross-adaptation study and transduction cascade determination, the percent unadapted response of each cue was analysed using a one-sample t-test against 100%, with a Benjamini-Hochberg p-value correction to compensate for multiple comparisons. As the calcium concentration curve experiment represented multiple measurements on the same fish, a repeated measures analysis of variance was used to detect significant differences among groups. This was followed by a Tukey's test to determine differences among individual groups. For behavioural trials, outliers were detected using a Grubbs test followed by a paired t-test (when data were parametric) or paired Wilcox test (when data were nonparametric) to determine if there was a difference between time spent in the stimulus and blank treatment arms. In all cases significance was declared when $p \leq 0.05$.

3.3 Results

3.3.1 Cross adaptation of calcium and L-arginine

A previous study (Dew et al., 2012) showed that treatment with calcium for 3 or 96 h caused a reduction in the EOG response elicited by L-arginine in fathead minnows.

In order to determine if the decrease in response was due to cross-adaptation (due to calcium and L-arginine sharing olfactory receptors in OSNs), the cross-adaptation methodology detailed above was used. When calcium was irrigated across the OE and L-arginine was used as a cue, there was a significant reduction in EOG (t_2=-9.582, $p<0.03$; Figure 3.2). There was an approximately 40% lower EOG response after calcium was added than before. When L-arginine was irrigated across the OE and calcium was used as a cue, there was a significant decrease of approximately 40% in the EOG response to calcium (t_2=-5.818, $p<0.03$; Figure 3.2).

3.3.2 Concentration curve for calcium

In order to determine if calcium induces a concentration-dependent EOG response, four different concentrations of calcium (0.1, 1.0, 10, and 100 mM) were used as stimuli. There was a 6.7 fold increase in EOG response when 1.0 mM calcium was used as the stimulus as compared 0.1 mM calcium, a 1.8 fold increase when 10 mM calcium was used as the stimulus as compared to 1 mM calcium, and a 1.8 fold increase when 100 mM calcium was used as the stimulus as compared to 10 mM calcium ($F_{3,11}$=4.287, $p<0.0001$; Figure 3.3A).

The response induced by each of the four concentrations of calcium was extracted and compared, as shown in Figure 3.3B. While the EOG response increases as concentration of calcium increases, it is important to note that at the two highest concentrations of calcium used there was a small to moderate positive waveform in the trace immediately before the depolarization. This initial positive waveform appeared to delay the depolarization as compared to the two lower concentrations of calcium.

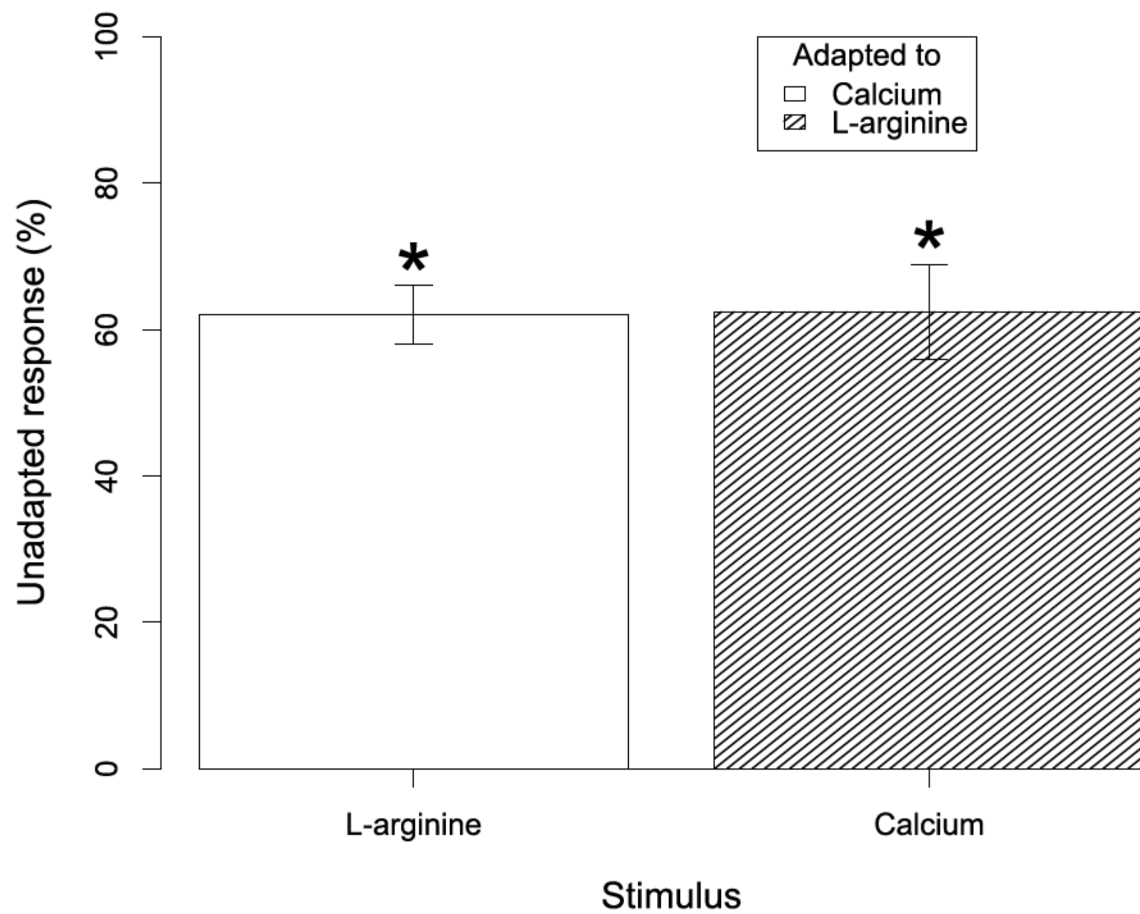

Figure 3.2: The percent unadapted response (±s.e.m.) of L-arginine when calcium is used as the adapting agent, and *vice versa*. An asterisk denotes significant difference from 100% using a one-sample t-test when $p \leq 0.05$.

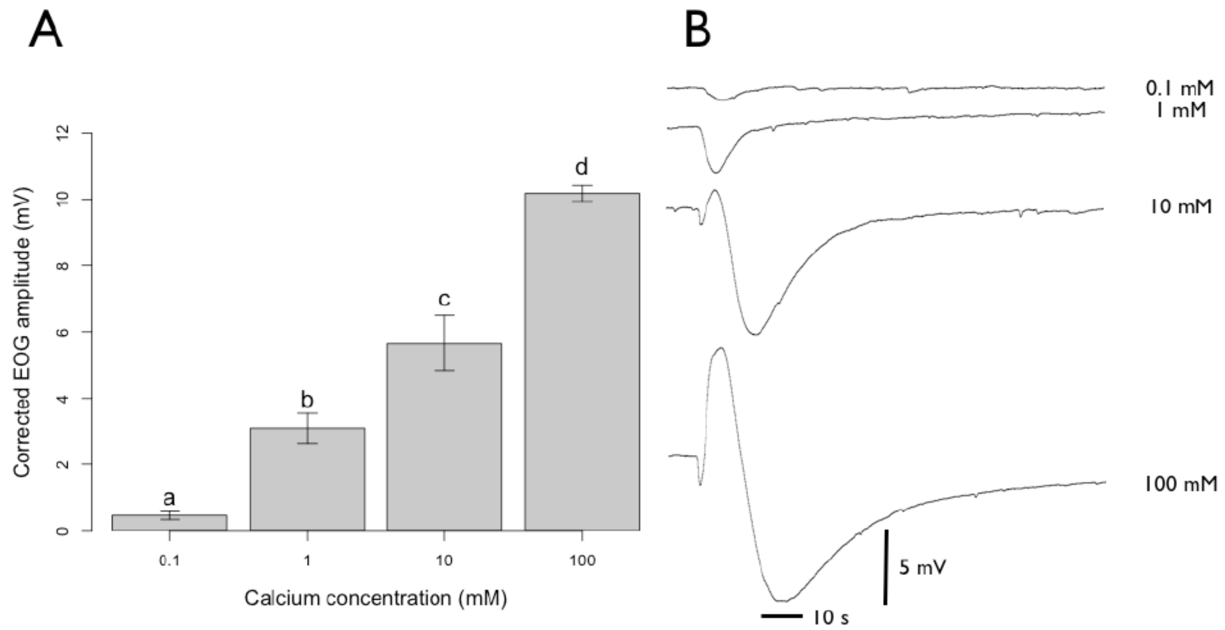

Figure 3.3: Concentration-dependent response of the EOG response (±s.e.m.) of fathead minnows to calcium. Bars with different letters are significantly different from each other when $p \leq 0.05$.

3.3.3 Transduction cascade determination

When treated with forskolin, there was no change in the EOG response of the OE to calcium (t_3=0.227, p>0.8), as shown in Figure 3.4. The addition of U-73122 also had no effect on the EOG response of the OE to calcium (t_3=0.297, p>0.8) as shown in Figure 3.4.

3.3.4 EOG trace comparison

Examples of the raw trace of the response of the OE to 1 mM L-alanine, 1 mM L-arginine, 1 mM calcium, and 0.1 mM TCA were compared, as is shown in Figure 3.5. For all four compounds, the depolarization of the tissue occurred at approximately the same time after the addition of the compound. The recovery back to baseline for calcium, TCA, and L-alanine occurred rapidly, while the recovery back to baseline after L-arginine was used as a cue was slower.

3.3.5 Behavioural response to calcium

A trough behavioural maze was used to determine how fathead minnows respond to elevated calcium concentrations. At the three highest concentrations of calcium tested, fathead minnows spent significantly more time in the arm containing the blank. This avoidance of calcium was seen when 100 mM (V=104, p<0.002), 10 mM (V=104, p<0.002), or 1 mM (V=87, p<0.04) calcium was used (Figure 3.6). However, there was no significant difference between time spent in the arm containing the blank or 0.1 mM calcium (V=59, p=0.71; Figure 3.5).

To ensure that the behavioural response was to calcium and not the counter ion of the calcium salt used, the behavioural response to a second calcium salt, calcium chloride ($CaCl_2$), was measured. When 10 mM calcium chloride was used as the

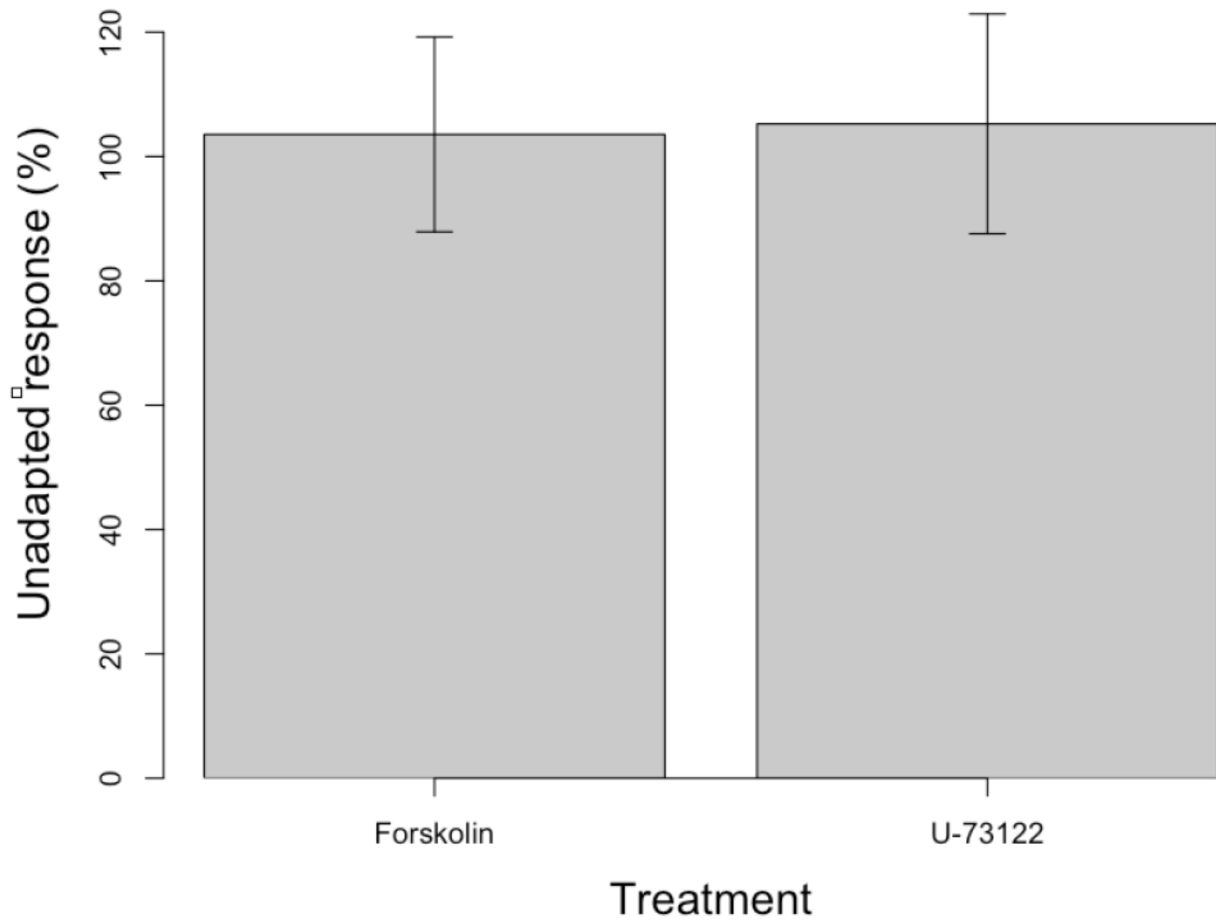

Figure 3.4: The change in EOG response (±s.e.m.) to calcium under a forskolin or U-73122. Neither bar is significantly different from 100% using a one-sample t-test.

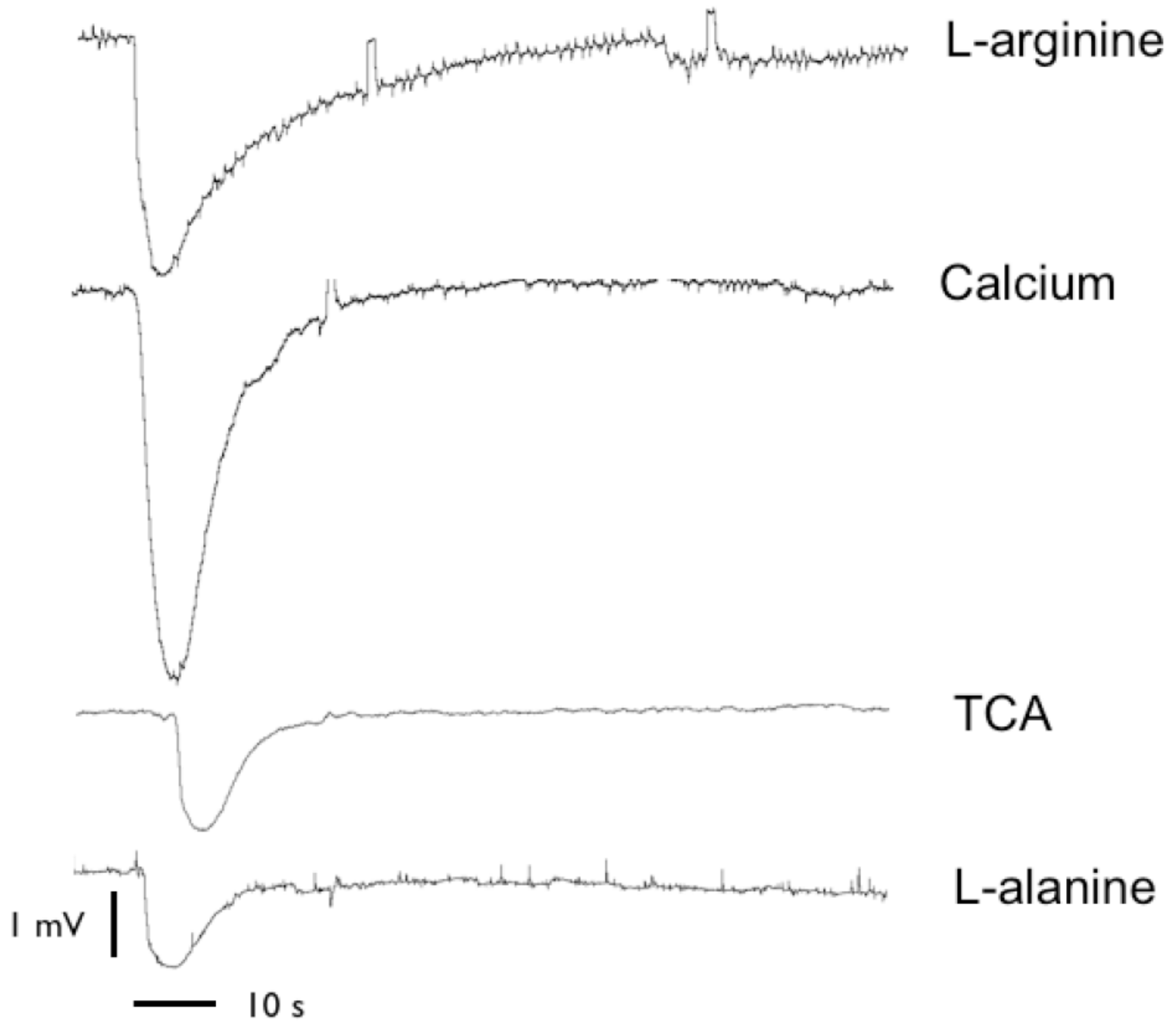

Figure 3.5: EOG traces for 1 mM L-arginine, 1 mM calcium, 0.1 mM TCA, and 1 mM L-alanine.

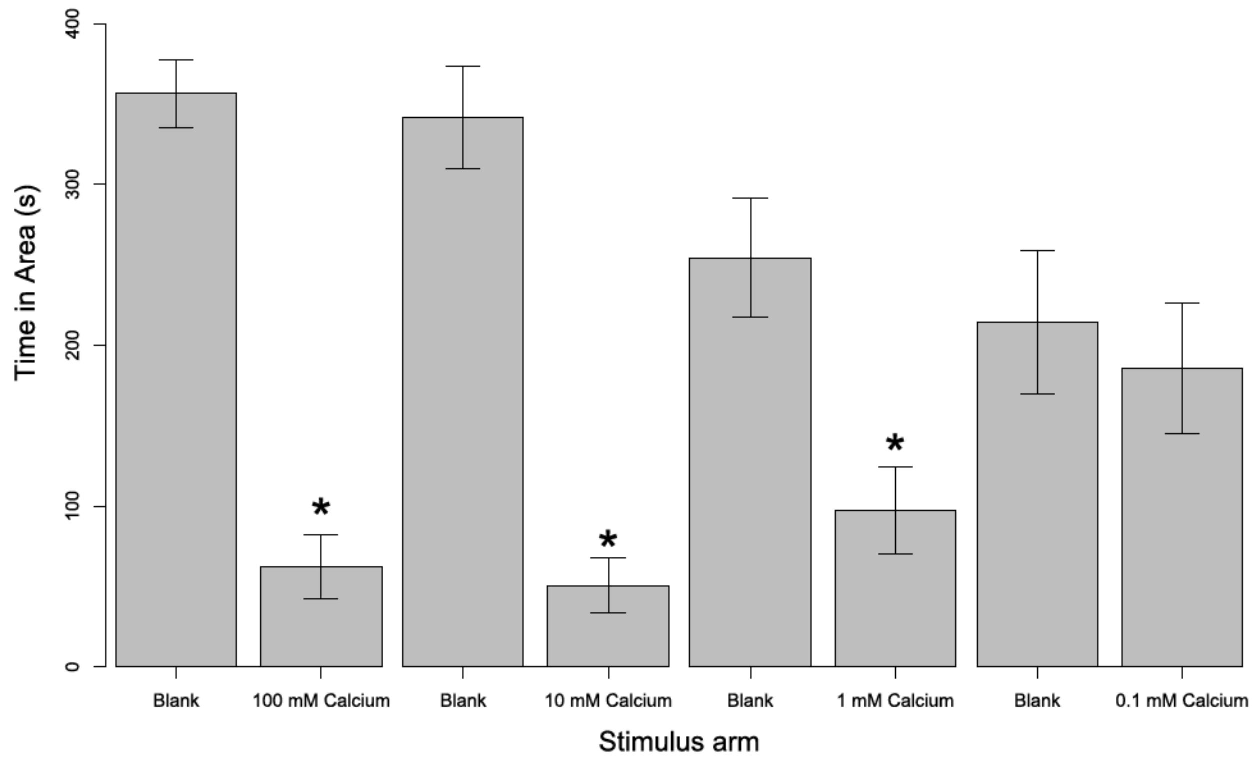

Figure 3.6: Avoidance response (±s.e.m.) of fathead minnows to calcium when various concentrations of calcium were used. An asterisk denotes significant difference from the corresponding blank stimulus arm when $p \leq 0.05$.

stimulus, fathead minnows spent more time in the arm given a blank than the stimulus ($V=63$, $p<0.01$; Figure 3.7). This response was in line with the response seen when calcium nitrate was used.

In order to demonstrate that the behavioural response of fathead minnows to calcium was mediated by olfaction and not another sense, fish were made anosmic using tissue glue. When the behavioural response of fathead minnows to 10 mM calcium was measured, anosmic fish did not show a preference for either end of the maze ($t_{12}=0.347$, $p=0.73$; Figure 3.8A). However, when fish were given a mock anosmic treatment, they spent significantly more time in the arm of the trough were the blank was added, as was seen in previous trials ($V=98$, $p<0.01$; Figure 3.8B).

3.4 Discussion

The current study is the first to demonstrate cross-adaptation between calcium and a second odourant molecule. Cross-adaption has been seen in fish for other stimuli such as polyamines and amino acids, but not calcium (Hansen et al., 2003; Michel et al., 2003; Rolen et al., 2003). A study by Hubbard et al. (2002) showed that removal of calcium from the surrounding water for goldfish resulted in a small, but significant, increase in response to L-serine. While the authors argued that their results were likely due to a hyperexcitability of fish OSNs, as shown in a different study (Parker et al., 2000), it is also possible that their observations were consistent with cross-adaptation between calcium and L-serine. Additionally, an investigation into whether or not calcium was cross-adaptive with sodium demonstrated that there was, in fact, no cross-adaptation between the two ions (Hubbard and Canario, 2007). The addition of calcium to the OE of sockeye salmon did not affect the EEG response to L-alanine, most likely

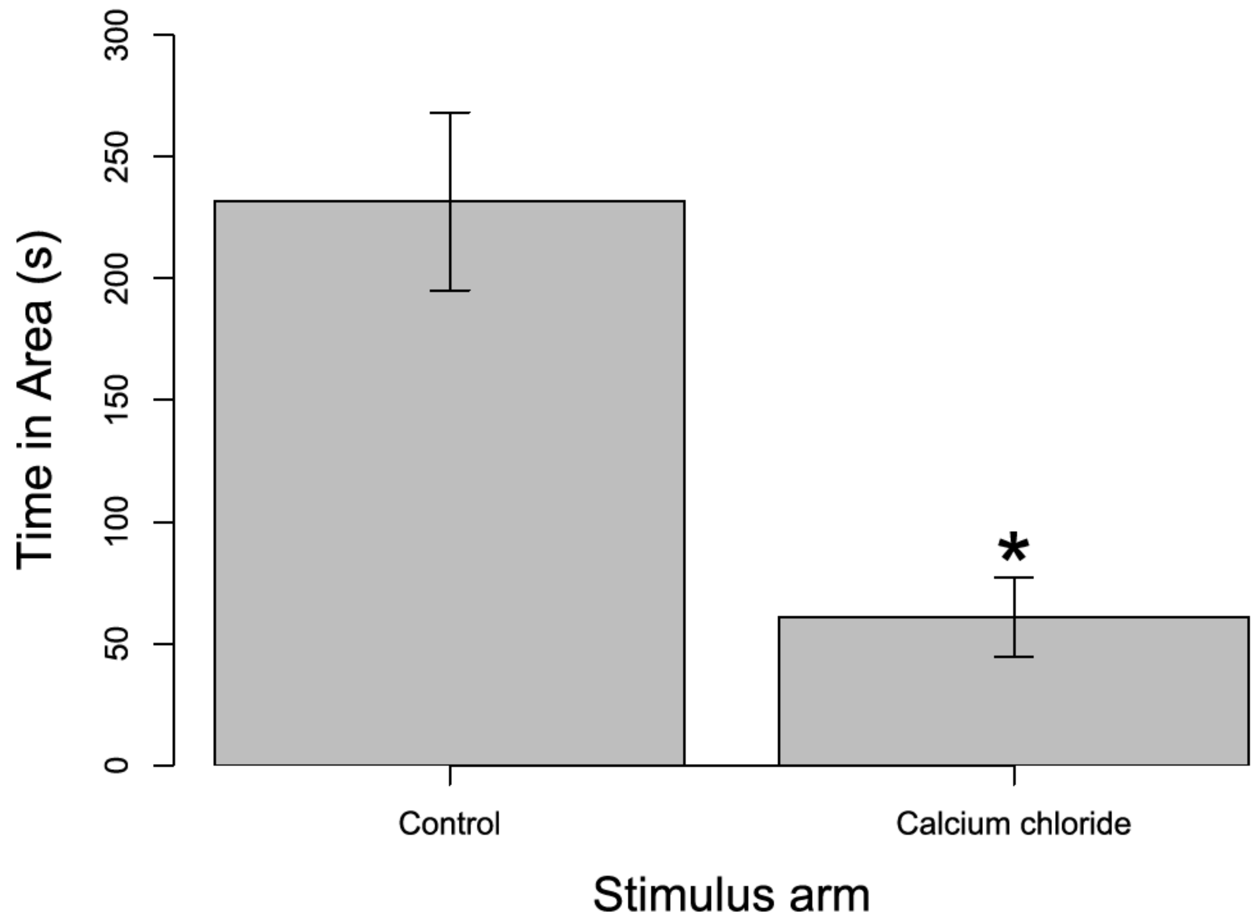

Figure 3.7: Avoidance response (±s.e.m.) to calcium chloride. An asterisk denotes significant difference from the control stimulus arm when $p \leq 0.05$.

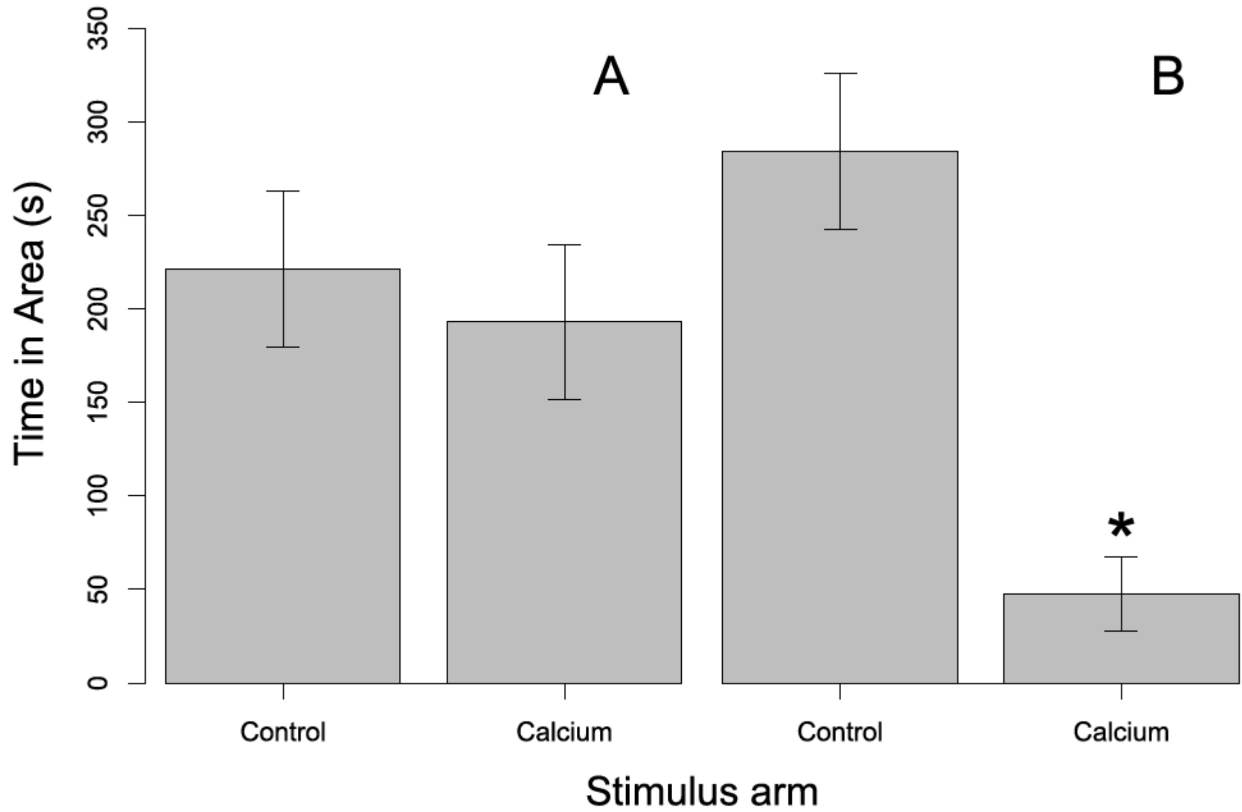

Figure 3.8: Behavioural response to calcium of anomsic (A) or intact olfaction (B) fathead minnows to calcium. An asterisk denotes significant difference from the control stimulus arm when $p \leq 0.05$.

because calcium and L-alanine do not share olfactory receptors (Bodznick, 1978). The fact that calcium and L-arginine are cross-adaptive provides further evidence that calcium is binding to olfactory receptors in OSNs as calcium is capable of blocking the action of L-arginine, a well defined odourant.

It can be argued that any response of the OE to calcium as measured by EOG is not, in fact, a measure of the reaction of the OE to calcium, but merely due to a change in the conductivity of the water bathing the OE. What looked like an EOG response would in fact be a temporary shift in the baseline that shifts back after the calcium has washed from the OE. However, when the OE is nonreactive or when it is removed altogether, the addition of calcium induces a waveform in the opposite direction of an EOG. This positive waveform is most likely due to the fact an EOG measures extracellular field potential, and the addition of calcium increased the positive charge present in the OE. This positive waveform was seen in the EOG trace for the two highest concentrations of calcium (as shown in Figure 3.3B), in that both had a positive waveform immediately prior to the expected negative waveform. The positive aspect of the trace was most likely excess calcium that did not bind to odourant receptors. A depolarization was subsequently measured, either due to excess calcium washing off the OE, or the inward current became greater than the effect of calcium and its associated positive charges in the extracellular environment. Therefore, while calcium has an effect on the conductivity of the water bathing the OE, it cannot be mistaken for an EOG response as the effect of calcium is to produce a positive waveform. In addition, a comparison of the various compounds used as odourant demonstrate that calcium has a similar waveform to other, well defined odourants. While the amplitude of

each of the EOG responses to the various odourants are not identical, the timing of the initiation of the EOG and the time to recover the baseline are all similar. In summary, the comparison of the traces of the various odourants used further supports that calcium is acting as an odourant, and that EOG is a valid technique to investigate the response of the OE to calcium.

This study utilized both neurophysiological and behavioural assays to answer a variety of questions about calcium sensing in fish. The neurophysiological evidence (via EOG measurements) demonstrated that the OE of fathead minnows respond to calcium in a dose-dependent manner, as would be expected of an odourant. Other studies have demonstrated that calcium induces a dose-dependent response using a different neurophysiological endpoint (EEG responses at the olfactory bulb) to calcium in sockeye salmon (Bodznick, 1978). Multi-unit extracellular recordings from the olfactory nerve of an estuarine species, gilthead seabream (*Sparus aurata* Linnaeus 1758), showed that this species was sensitive to changes in calcium concentrations that it is likely to encounter in its natural habitat (Hubbard et al., 2000). Furthermore, goldfish (*Carassius auratus* Linnaeus 1758) EEG responses to calcium were shown to be concentration-dependent (Hubbard et al., 2002). Interestingly, the cumulative evidence demonstrates that estuarine and salt water species of fish demonstrate a response when presented with a cue containing less calcium than what they are acclimated to, while the addition of increased calcium induces a response in freshwater fish. The current study demonstrates that fathead minnows, a freshwater species, reacts to increasing calcium with an increase in a neurophysiological response, which supports the previous work with freshwater fish. In addition, this is the first study to demonstrate

an EOG response to calcium in a fish species. As EOG experiments are considerably easier to perform than EEG or olfactory nerve measurements, this represents a robust option to determine the effects of calcium on fish olfactory neurophysiology.

In this study, calcium was shown to induce an olfactory response in a non-cAMP, non-IP3 mediated manner. Ciliated and crypt cells have been shown to use a cAMP-mediated pathway (Ronnett and Moon, 2002; Zielinski and Hara, 2006). The addition of forskolin activates adenylyl cyclase in ciliated and crypt OSNs, resulting in an increase in cAMP and a decreased response to any odourant that activates these neurons (Hansen et al., 2003; Michel et al., 2003; Rolen et al., 2003). The only odourant which caused a lower EOG response due to forskolin treatment was TCA, indicating that TCA is specific to ciliated and/or crypt cells. However, due to the fact that crypt cells have been shown to be non-responsive to TCA (Vielma et al., 2008), it can be assumed that TCA is only inducing a response in ciliated cells. Cell staining experiments have also demonstrated the specificity of TCA to ciliated cells (Døving et al., 2011). The addition of U-73122 inhibits the activity of phospholipase C, which prevents IP3 from forming and is associated with microvillous cells (Zielinski and Hara, 2006). The only odourant that had a lower EOG response due to the addition of U-73122 was L-alanine, indicating that the response to L-alanine is specific to microvillous cells. The specificity of L-alanine to microvillous cells and TCA to ciliated cells was also demonstrated in round goby (*Neogobius melanostomus* Pallas 1814) (Laframboise and Zielinski, 2011). Interestingly, our data show that calcium induces an olfactory response through a non-cAMP, non-IP3 pathway. The use of a pathway independent of the two known pathways has also been demonstrated for two polyamines, spermine and agmatine (Michel et al., 2003). The

response of the olfactory system of fish to calcium, spermine, and agmatine indicates that there is a third olfactory signalling pathway present in the cells of the OE. It is possible that there is an additional OSN type or a subset of a currently known OSN class that utilizes this novel pathway. Elucidating this alternative pathway and which OSN class is involved would broaden our understanding of how fish perceive their environment.

The behavioural endpoint tested in this study clearly demonstrates that fathead minnows avoids increased concentrations of calcium. This effect was seen when using multiple salts (calcium nitrate and calcium chloride) providing evidence that the response is due to calcium and not a the counter ion of the calcium salt used. Anosmic fish did not react to the presence of calcium, while olfactory-intact fish did. This result demonstrates that the response to calcium is, in fact, mediated by the olfactory system, and not by some other sense. An avoidance response to calcium has only been demonstrated once before (Bodznick, 1978), and until this study it has not been demonstrated to be olfactory in nature. In addition to calcium, fish have been shown to avoid a number of other cations, including copper, chromium, nickel, cadmium, iron, and zinc (Atchison et al., 1987). Interestingly, a recent paper by Kennedy et al. (2012) demonstrates that at the higher concentrations used (10^{-3} and 10^{-4} M), chinook salmon (*Oncorhynchus tshawytscha* Walbaum 1792) avoided L-histidine, a positively charged amino acid. The general avoidance of increased concentrations of cations by fish indicates that fish may have the ability to not only detect differences in salinity based on calcium and sodium, but may be able to detect cations in general. The ability to detect

dissolved cations would allow fish to avoid areas that may cause osmotic stress or lead to situations where fish become exposed to potentially toxic metals.

Three hypotheses for why fish detect calcium in water have been proposed. Bodznick (1978) proposed that the ability of sockeye salmon fry to detect calcium was that it helped in the recognition of natal waters. Hubbard et al. (2002) hypothesized that this was a sort of "advance warning system", to let an estuarine fish tell when it had reached the limits of its tolerance (Hubbard et al., 2000). A third hypothesis is that this ability allows a fish to maintain homeostasis of ion content surrounding OSNs (Hubbard et al., 2002). As fathead minnows do not migrate and have no need to recognize drastic changes in salinity, it is most likely that this phenomenon represents a homeostatic control. The ability to sense and respond (through avoidance) to areas that may put a fish into osmotic shock represents a fascinating use of olfaction as an osmoregulatory sense.

In summary, this work demonstrates that calcium is a potent odourant to fathead minnows at the neurophysiological and behavioural levels. This work also demonstrates that calcium competes with a known odourant (L-arginine) implying that calcium does, in fact, bind to olfactory receptors. In terms of behaviour, this work is the first to demonstrate that the avoidance of calcium by fish is olfactory dependent and that calcium, and not a counter anion, is driving the avoidance behaviour. The ability to sense and avoid calcium represents a fascinating ability of fish to recognize areas that may prove physiologically detrimental to the fish.

Prelude to chapter 4

Chapter 2 detailed work that showed how time influenced copper-induced olfactory dysfunction. Specifically, at the concentrations tested, as the exposure duration increased so did the EOG response to L-arginine in fathead minnows. While there was a full recovery of olfactory function as measured by EOG at the two lowest concentrations tested, there was only a partial recovery at the two highest concentrations tested. These results led to a new line of inquiry: namely, does the partial recovery of EOG function seen at the highest concentrations tested represent a partial recovery of the olfactory epithelium as a whole, or are there elements of the olfactory epithelium that recover while others do not? To investigate whether different aspects of the olfactory epithelium were differently affected by copper, odours specific to two different olfactory sensory neuron classes were used in EOG measurements. The behavioural effect of copper was also measured to determine if the behavioural and neurophysiological levels of biological organization could be connected.

Chapter 4: Effects of copper and nickel on olfaction in fish: connecting specific neuron impairment with a behavioural deficit

Summary

The olfactory system of fish (and many other vertebrates) comprises a variety of cell types, including different classes of olfactory sensory neurons (OSNs). Two odourants, L-alanine and TCA have been demonstrated to specifically activate either ciliated or microvillous OSNs. We used this fact to determine the effect of copper and nickel on these two classes of neurons. While at the highest concentration tested, copper impaired both microvillous and ciliated OSNs, at lower concentrations copper only impaired ciliated OSNs. Nickel, on the other hand, specifically affected microvillous OSNs at all concentrations tested but had no effect on ciliated OSNs. This effect was found in both fathead minnows (*Pimephales promelas*) and yellow perch (*Perca flavescens*). Fathead minnows exposed to copper failed to avoid a conspecific alarm cue relative to controls, whereas those exposed to nickel could respond to the same cue. These results demonstrate that fathead minnows perceive conspecific, damage-released alarm cue by ciliated, but not microvillous, OSNs. The connection of an EOG response with an ecologically-relevant behavioural response in fish benefits environmental risk assessment as it allows the use of a relatively simple laboratory experiment to predict behavioural deficits in fish.

4.1 Introduction

The olfactory epithelium of fish comprises a variety of cell types, including olfactory sensory neurons (OSNs) (Zielinski and Hara, 2006). These neurons are in direct contact with the external environment and when bound by odour molecules transmit vital information about the external environment to the brain (Hamdani and Døving, 2007). There are three types of OSNs found in the olfactory epithelium of fish; crypt, ciliated, and microvillous (Zielinski and Hara, 2006), which can be distinguished by morphological differences as well as by their responses to various odourant molecules (Hansen et al., 2003). The response of specific classes of OSNs to different odourants can be measured by exploiting a difference in the olfactory signalling pathways in different OSN types, namely that crypt and ciliated OSNs have a cAMP-mediated olfactory signalling pathway while microvillous OSNs have an IP3-based pathway (Hansen et al., 2003; Michel et al., 2003; Rolen et al., 2003). By taking advantage of this specificity of pathways to OSN classes, it was demonstrated that in the round goby (*Neogobius melanostomus*), taurocholic acid (TCA), a bile salt, induces a response specific to OSNs with a cAMP-mediated pathway (i.e., ciliated and/or crypt OSNs), while L-alanine induces a response specific to OSNs with an IP3-mediated pathway (i.e., microvillous OSNs) (Laframboise and Zielinski, 2011). Crypt OSNs in Pacific jack mackerel (*Trachurus symmetricus)* do not respond to a mixture of bile salts including TCA, therefore using a bile salt as a cue will omit any response from crypt OSNs (Vielma et al., 2008). The specificity of TCA to ciliated OSNs was similarly found in crucian carp (*Carassius carassius*) (Døving et al., 2011). These results lead to the conclusion that L-alanine specifically induces an olfactory response in microvillous

OSNs, while TCA specifically induces a response in ciliated OSNs. This specificity of odourant to an OSN class means that the status of either ciliated or microvillous OSNs can be measured to determine if they are affected differently by different toxicants.

Odour molecules binding to receptors in OSNs initiate a signal transduction cascade whose net effect is to propagate an electrical signal to the brain where the signal can be processed and the animal can mount an appropriate behavioural response (Laberge and Hara, 2001). An example of such an appropriate behaviour to a chemosensory stimulus is avoidance behaviour in response to conspecific, damage-released alarm cue. This response was first observed by von Frisch (1938) in fathead minnows responding to Schrekstoff (alarm cue). When fathead minnows are attacked and their skin is ruptured, a chemical cue is released to the water that warns nearby conspecifics of the predation event (Smith, 1992). The response of any fathead minnows perceiving this cue is to engage in various alarm behaviours such as freezing, dashing, increased use of shelter, and avoidance (Kats and Dill, 1998). The perception of alarm cues, as well as food, mating, and migratory cues, confer an adaptive benefit to the receiver and are vital to survival. Thus, any impairment of this ability to detect and avoid predators may prevent a prey fish from avoiding predation (Carreau-Green et al., 2008).

A variety of contaminants (e.g., metals, pesticides) are known to impair the olfactory acuity of fish (Scott and Sloman, 2004; Pyle and Mirza, 2007; Tierney et al., 2010). Copper is a trace metal and an environmental contaminant commonly associated with mining, smelting, and refining activities. The effect of copper on the olfactory system of fish has been studied at the neurophysiological and, to a lesser extent, the

behavioural level. At the neurophysiological level, copper has been shown to reduce olfactory acuity in fathead minnows with exposures ranging from 10 minutes to 96 hours (Green et al., 2010; Dew et al., 2012). Coho salmon (*Oncorhynchus kisutch*) also show decreased olfactory acuity when exposed to low concentrations of copper (McIntyre et al., 2008). The behavioural effects of copper mirror those seen at the neurophysiological level as low concentrations of copper inhibit the antipredator response of coho salmon (McIntyre et al., 2012) and Colorado pikeminnows (*Ptychocheilus lucius*) (Beyers and Farmer, 2001). These results demonstrate that copper can impair the olfactory system at multiple levels of biological organization.

Copper and nickel have been continuously mined since 1888 in the Sudbury region in Northern Ontario, Canada. The mining of copper and nickel in the Sudbury region has lead to widespread environmental damage, to the extent that since 1978 over 3,400 hectares of land have been limed to reverse soil acidification, and over 9 million trees have been planted (Regreening Program, 2011). Lakes in the region have also been affected, both by being contaminated with copper, nickel, and other contaminants, and by being acidified due to sulphur dioxide release (Yan et al., 1995). Work has been done to neutralize the lakes in the Sudbury region, both by decreasing sulphur dioxide release from mining and smelting activities, as well as by directly liming lakes (Yan et al., 1995). In an effort to aid in the reclamation process, it is vital to understand what effects copper and nickel have on aquatic animals. As the olfactory system is vital to the ability of a fish to thrive in an ecosystem, the effect of copper and nickel on a fish's olfactory system is important to understand. While the effect of copper on the olfactory system of fish has been investigated as discussed above, much is still

unknown concerning copper-induced olfactory dysfunction. Little work has been done relating different levels of biological organization (i.e., neurophysiology and behaviour), and no work has determined the effects of copper on specific OSN classes in the olfactory epithelium. In terms of olfaction, nickel is an understudied metal, and as such represents an important metal to study.

Previously we determined that very low concentrations of copper inhibit olfactory acuity in fathead minnows (Dew et al., 2012). However, it remains unclear whether copper has a general effect on all OSNs, or if each OSN class is impaired differentially. To determine if copper specifically affects one or more OSN class, we first confirmed that L-alanine activates microvillous OSNs and TCA activates ciliated OSNs in fathead minnows using electro-olfactography (EOG), a neurophysiological technique that measures olfactory acuity. We then exposed fish to increasing concentrations of copper and measured their OSN-specific EOG response to TCA (ciliated OSNs) or L-alanine (microvillous OSNs). Nickel was used as a second contaminant to determine if the effect of copper was a generalized effect of metal exposure, or specific to copper. The effect of copper and nickel on EOG response in wild yellow perch (*Perca flavescens*) was then tested, using native lake water to make up the exposure water. This comparison of lab reared fish with wild-caught fish in their native water demonstrates whether or not any OSN-specific effects of copper and nickel occur in wild fish populations.

Little work has been done to directly compare the neurophysiological and behavioural levels of biological organization in fish. To connect the effect of copper and nickel exposures on neurophysiological responses with effects on behavioural responses, the effect of copper and nickel on response to an anti-predator cue was

measured in fathead minnows. As discussed above, the response to an anti-predator cue confers an adaptive benefit to the receiver and is vital to survival. In addition to determining how copper and nickel affects the anti-predator response of fathead minnows, by measuring both the effect of copper and nickel on EOG and response to an anti-predator cue, a connection can be made between the neurophysiological and behavioural levels of biological organization.

4.2 Experimental methods

4.2.1 Animals

Adult (1.8 - 4.1 g) fathead minnows were obtained from the USEPA (Duluth, MN) and housed in static renewal or re-circulatory systems in the Lakehead University Biology Aquatic Facility. Fish were held in dechlorinated Thunder Bay, Ontario municipal water (Table 4.1) with a 16 h photoperiod. Fish were fed *ad libitum* once daily with *Artemia* spp. *ad libitum*, and were allowed to acclimate for a minimum of two weeks prior to being used in experiments. Alkalinity was determined as previously described (Pyle et al., 2005). All water samples were collected in tubes that were rinsed a minimum of three times with the water to be sampled. For metal analysis, samples were acidified using concentrated trace metals grade nitric acid (Fisher Scientific, Toronto, ON, Canada) and filtered through a 0.45 μM filter. Metal concentrations and dissolved organic carbon were measured by ALS Environmental (Thunder Bay, ON, Canada), a laboratory accredited by the Canadian Association for Laboratory Accreditation (CALA). Metal concentrations were measured using inductively coupled plasma mass spectrometry in accordance to all CALA QA/QC guidelines.

Table 4.1: Basic water quality measurements of dechlorinated Thunder Bay Municipal water and water from Geneva Lake. All values are mean ± SEM, except for pH and temperature which are presented a ranges (n = 44 for dechlorinated municipal water, n = 4 for Geneva Lake water).

Water quality variable	Dechlorinated municipal water	Geneva Lake water
Calcium (mgL^{-1})	14.3 ± 0.3	2.7 ± 0.1
Magnesium (mgL^{-1})	2.9 ± 0.0	0.7 ± 0.1
Sodium (mgL^{-1})	3.6 ± 0.1	1.8 ± 0.1
Temperature (°C)	19 - 21	25 - 26
pH	7.29 - 7.79	6.85 - 6.98
Alkalinity (mgL^{-1} as $CaCO_3$)	50.4 ± 1.8	21.5 ± 0.9
Dissolved organic carbon (DOC) (mgL^{-1})	3.3 ± 0.1	4.9 ± 0.5

Yellow perch (5.2 - 8.0 g) were collected from Geneva Lake in the Sudbury ON region by angling. All fish were acclimated to laboratory conditions for 24 h using water collected from Geneva Lake (Table 4.1). Water collection and analysis was as previously described (Azizishirazi et al., 2012).

4.2.2 Electro-olfactography experiments

Electro-olfactography experiments were performed as previously described (Green et al., 2010). Two cues were used to measure the response of microvillous and ciliated OSNs. The EOG response to 10^{-3} M L-alanine (MP Biomedicals, Solon OH, USA) was used as a surrogate measure for the response of microvillous OSNs, whereas the EOG response to 10^{-4} M TCA (Fisher Scientific, Toronto ON, Canada) was used as a surrogate for the response of ciliated OSNs. Cues were made fresh daily in dechlorinated Thunder Bay, ON municipal water for fathead minnows and in Geneva Lake water for yellow perch. Cues were delivered to the olfactory chamber in 2 s pulses and in randomized order to ensure olfactory attenuation to any given cue was minimized. Moreover, consecutive cue deliveries were separated by a minimum of 2 min where the olfactory chamber was irrigated with water containing no cue. The EOG response to each cue was measured three times per fish. The EOG response to a blank (dechlorinated municipal water for fathead minnows or Geneva Lake water for yellow perch) was also measured. Water irrigating the olfactory epithelium during each EOG was matched to the exposure water for that fish. The EOG response was determined by measuring the change in amplitude from the baseline to the maximum response to the cue. The EOG response to the respective blank was then subtracted from each EOG

response, and the relative, corrected EOG response was calculated by dividing each response by the response measured from control animals.

4.2.3 Transduction cascade determination

To confirm whether L-alanine activates microvillous OSNs and TCA activates ciliated OSNs in fathead minnows, we applied either forskolin (Santa Cruz Biotechnology, Santa Cruz, California, USA) to stimulate the cAMP pathway or U-73122 (Santa Cruz Biotechnology, Santa Cruz, California, USA) to inhibit the IP3 pathway. By inference, a reduced olfactory response (relative to controls) to a standard chemosensory cue following a forskolin treatment suggests that the cue was primarily perceived by ciliated or crypt OSNs, both of which act by way of a cAMP-signalling pathway. Similarly, a reduced olfactory response following a U-73122 treatment suggests that the cue was mainly perceived by microvillous OSNs, which make use of an IP3-signalling pathway.

To determine OSN specificity, the initial EOG response to either L-alanine, TCA, or a dechlorinated water blank was first measured. One of two pharmacological agents was then added; either 1 μM U-73122 or 1 μM forskolin, both of which were made up in dechlorinated water. After the addition of the pharmacological agent, the response to each of the cues mixed with the agent was then measured, as well as the response to a blank containing the agent. This value was blank-corrected and divided by the initial unadapted response for each cue to give the percent unadapted response. Only one pharmacological agent was used on any given fish.

4.2.4 Behavioural experiments

Trough mazes measuring 69 cm x 14 cm x 16 cm; L x W x H were used to measure the behavioural response of fathead minnows to a chemosensory stimulus. Each maze was identical to the one represented in Figure 3.1, except that diffusion chambers were not used. At the beginning of each trial 10 L of dechlorinated Thunder Bay municipal water was added to each trough. Each trough was divided into three sections, the middle section was the acclimation zone, with two arms extending distally from each side of the acclimation zone. A bottomless, plastic acclimation chamber (19 cm x 13 cm x 11 cm; L x W x H) was placed into the acclimation zone in each trough, where a subject fathead minnow was placed to acclimate to the maze without having access to the maze arms. At the beginning of each trial 10 mL of the stimulus was added to a randomly selected arm (the stimulus arm), and 10 mL of a blank (water matched to the maze) was added to the other arm (the blank arm) of each trough. Curtains were placed around the troughs to prevent any motion from the researcher affecting the behaviour of the subject fish. Fish behaviours were recorded by a web camera (RocketFish, Richfield MN, USA) attached to a MacBook computer from behind the curtain. After a 15 min acclimation period after each stimulus was added to the distal end of each arm as appropriate, the acclimation chambers were lifted via a string from behind the curtain making sure to not break the surface of the water as dripping water can affect fish behaviour. Initial dye tests determined that 15 min was sufficient time to allow the cue to diffuse throughout the arm. The position of each fish was monitored in five separate mazes once every 10 s for 8 min. At the end of the trial the number of

instances the fathead minnow was in each arm was multiplied by 10 to give time in blank arm and time in stimulus arm.

The stimulus for each behavioural trial was made fresh daily by preparing a skin extract from either a fathead minnow or red swordtail (*Xiphophorus hellerii*). The red swordtail was used to produce a control for the stimulus, i.e., the smell of damaged fish tissue that does not contain the fathead minnow alarm cue. Fish were euthanized using 200 mgL^{-1} MS-222 (Syndel Laboratories Inc, Qualicum Beach BC, Canada) buffered to pH 7.5. The skin on both sides of the fish was removed and any muscle or other tissue was removed from the skin. The surface area of the skin was measured before being placed into a Petri dish containing 1 mL of dechlorinated water. The skin was chopped with dissection scissors for 10 min, and then added to a flask. The Petri dish was repeatedly rinsed with dechlorinated water into the flask to ensure all materials were removed from the Petri dish. Dechlorinated water was added to the flask to bring the final concentration of skin extract to 1 cm^2 per 100 mL. The skin extract in the flask was mixed and allowed to settle for at least 10 min to allow any suspended tissue to sink to the bottom. The top 80% of the volume was used to prevent tissue from being introduced during a trial. Initial behavioural experiments were performed to determine the response of fathead minnows to both the fathead minnow and swordtail skin extracts. For the response of fathead minnows to be considered a response to fathead minnow alarm cue, the fathead minnows would be expected to react to conspecific alarm cue, but not cue from an allopatric heterospecific such as swortdtail.

4.2.5 Exposures

Copper and nickel stock solutions were made using $CuSO_4 \cdot 5(H_2O)$ (Fisher Scientific, Toronto ON, Canada) and $NiSO_4 \cdot 6(H_2O)$ (Fisher Scientific, Toronto ON, Canada), respectively. All exposure waters were made immediately prior to use by using an appropriate dilution of the stock solutions. Exposure water for fathead minnows was made up in dechlorinated Thunder Bay municipal water and all exposure water for yellow perch was made up in Geneva Lake water. All fish were exposed in tanks at a density not exceeding 1 fish per L. A daily 50% water change was performed in each exposure tank.

For EOG experiments, fathead minnows were exposed to either copper (nominal 5, 10, or 20 μgL^{-1}), nickel (nominal 50, 100, or 500 μgL^{-1}), or control water for 48 h. For copper exposures of fathead minnows, there was a background concentration of 6.76 ± 0.89 μgL^{-1} copper in all exposure waters, including the control, which was subtracted from the total copper measured in each sample to give the amount added for each exposure (Table 4.2). Yellow perch were exposed to nominal concentrations of 25 μgL^{-1} copper, 500 μgL^{-1} nickel, or to a control. Corrected measured values were determined as described above, and are found in Table 4.2.

Initial behavioural experiments were performed comparing the response of fathead minnows to an extract of fathead minnow skin or swordtail skin. In subsequent experiments, fathead minnows were exposed to either nominal 5 μgL^{-1} copper, nominal 100 μgL^{-1} nickel, or control water. Corrected measured values were determined as described above and are found in Table 4.2. All exposures were for 48 h prior to measuring the response of fathead minnows to conspecific alarm cue.

Table 4.2: Nominal and corrected measured copper and nickel concentrations for exposures of fathead minnows (FHM) and yellow perch (YP) prior to EOG and behavioural measurements. All values were corrected by subtracting the concentration of copper or nickel in the control exposure water. All values are mean ± SEM, n = 3-4.

Metal	Nominal concentration (μgL^{-1})	Corrected measured concentration (μgL^{-1})
FHM - exposures for EOG		
Copper	5	2.8 ± 0.4
	10	6.0 ± 0.5
	20	18.6 ± 1.5
Nickel	25	24.3 ± 1.4
	100	96.7 ± 0.6
	500	503.0 ± 7.4
YP - exposures for EOG		
Copper	25	19 ± 4
Nickel	500	535 ± 20
FHM exposures for behaviour		
Copper	5	2.8 ± 2.7
Nickel	100	99.8 ± 1.7

4.2.6 Statistical Analysis

All statistical analyses were performed using R (R Development Core Team, 2012). An analysis of variance (ANOVA) was used to determine if there was a difference among the corrected EOG responses for the various copper or nickel exposures of fathead minnows. When a difference was detected, a Tukey's test was employed to determine differences between groups. An independent-samples t-test was then used to determine if the corrected EOG response at each concentration of copper or nickel was significantly different from corrected EOG response with the control exposure. A Benjamini-Hochberg p-value adjustment was used to compensate for increasing experiment-wise error owing to the multiple t-test comparisons. For the yellow perch EOG experiments, an independent-samples t-test was used to compare the EOG values under copper and nickel exposures to control values. Any data not conforming to parametric assumptions were transformed using a square-root transformation to recapture parametric assumptions.

All behavioural trials were analysed using a paired t-test comparing time spent in the blank versus stimulus arm. A Benjamini-Hochberg p-value adjustment was used to compensate for multiple comparisons. Any data not conforming to parametric assumptions were transformed using a square-root transformation to recapture parametric assumptions.

4.3 Results

4.3.1 EOG experiments

Exposure of fathead minnows to forskolin resulted in a 49% reduction in response to TCA ($t_3 = -9.150$, $p < 0.01$; Figure 4.1A), but not L-alanine ($t_3 = 0.229$, p =

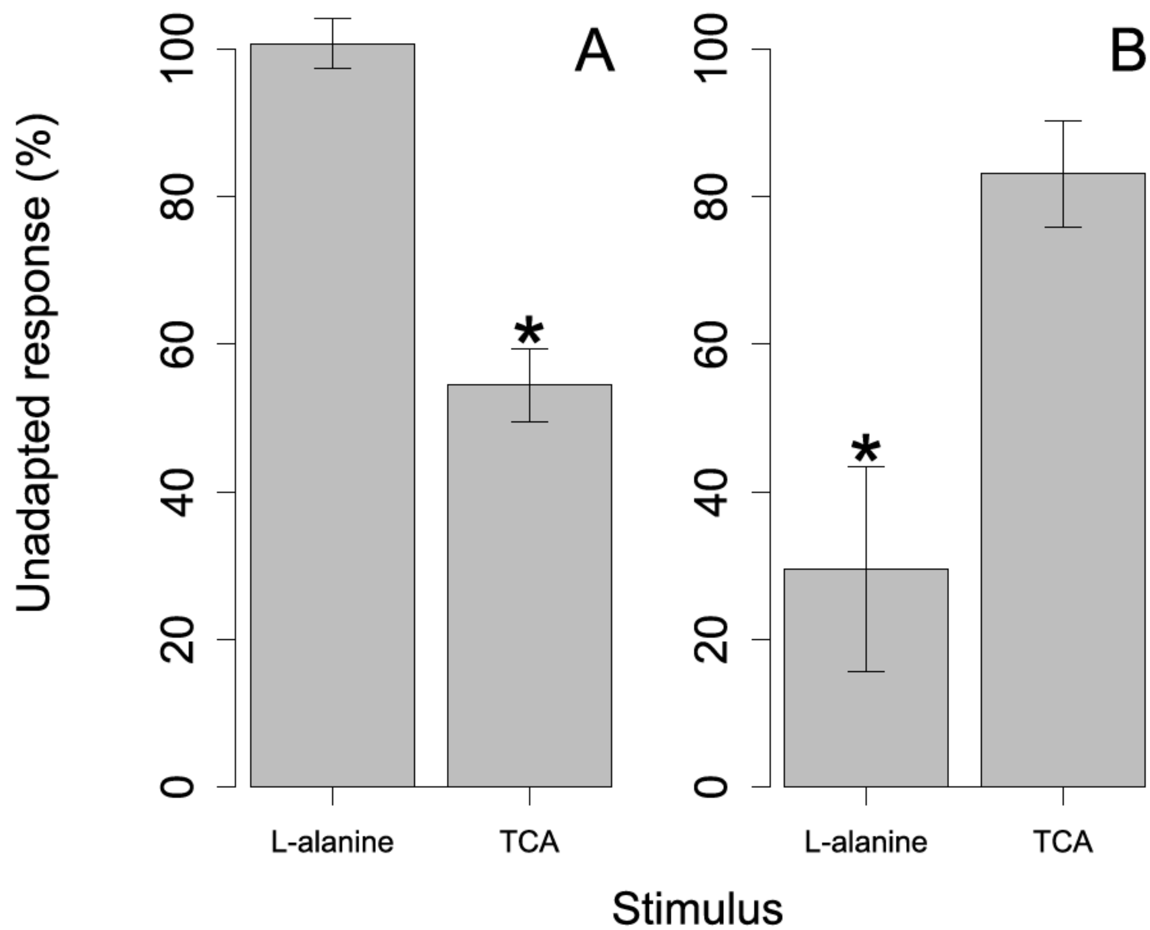

Figure 4.1: Percent unadapted response (mean ± SEM) to L-alanine and TCA under forskolin (A) or U-73122 (B) exposure in fathead minnows (n=4). An asterisk above a bar denotes a significant difference from 100% ($p \leq 0.05$).

0.84; Figure 4.1A). The opposite trend was seen when U-73122 was added, in that there was a reduced response to L-alanine (t_3 = -5.101, $p < 0.05$; Figure 4.1B), but not TCA (t_3 = -2.33,$p = 0.15$; Figure 4.1B).

Fathead minnows exposed to copper for 48 h showed a concentration-dependent decrease in EOG response to L-alanine relative to controls, ranging from no reduction at 5 µg/L to an 83% reduction at the highest copper concentration tested ($F_{2,10}$ = 20.79, $p < 0.001$; Figure 4.2A). Only those fish exposed to the highest copper concentration showed a significantly impaired EOG response to L-alanine relative to the control ($t_{3.83}$ = 4.19, $p < 0.05$). However, the same 48 h copper exposure resulted in a significant reduction in the fathead minnow's EOG response to TCA at every exposure concentration tested (Figure 4.2B). Although the EOG response to TCA was significantly higher in fish exposed to 5 µg/L copper relative to those exposed to 10 or 20 µg/L ($F_{2,10}$ = 7.76, $p < 0.001$), the response was significantly reduced by 73 - 91% relative to the control at every copper concentration tested ($p < 0.001$).

The EOG responses to L-alanine and TCA in fathead minnows exposed to nickel for 48 h (Figure 4.3) showed completely opposite effects to those observed after a 48 h copper exposure. There was no concentration-dependent reduction in EOG response to either chemosensory stimulus in nickel-exposed fathead minnows. However, the EOG response to L-alanine was reduced by 53 - 58% relative to controls ($p < 0.05$; Figure 4.3A). There was no significant reduction in EOG response to TCA at any of the nickel exposure concentrations ($p > 0.05$; Figure 4.3B).

When wild yellow perch were tested for their EOG responses to L-alanine or TCA after being exposed to copper or nickel, they showed similar patterns to those

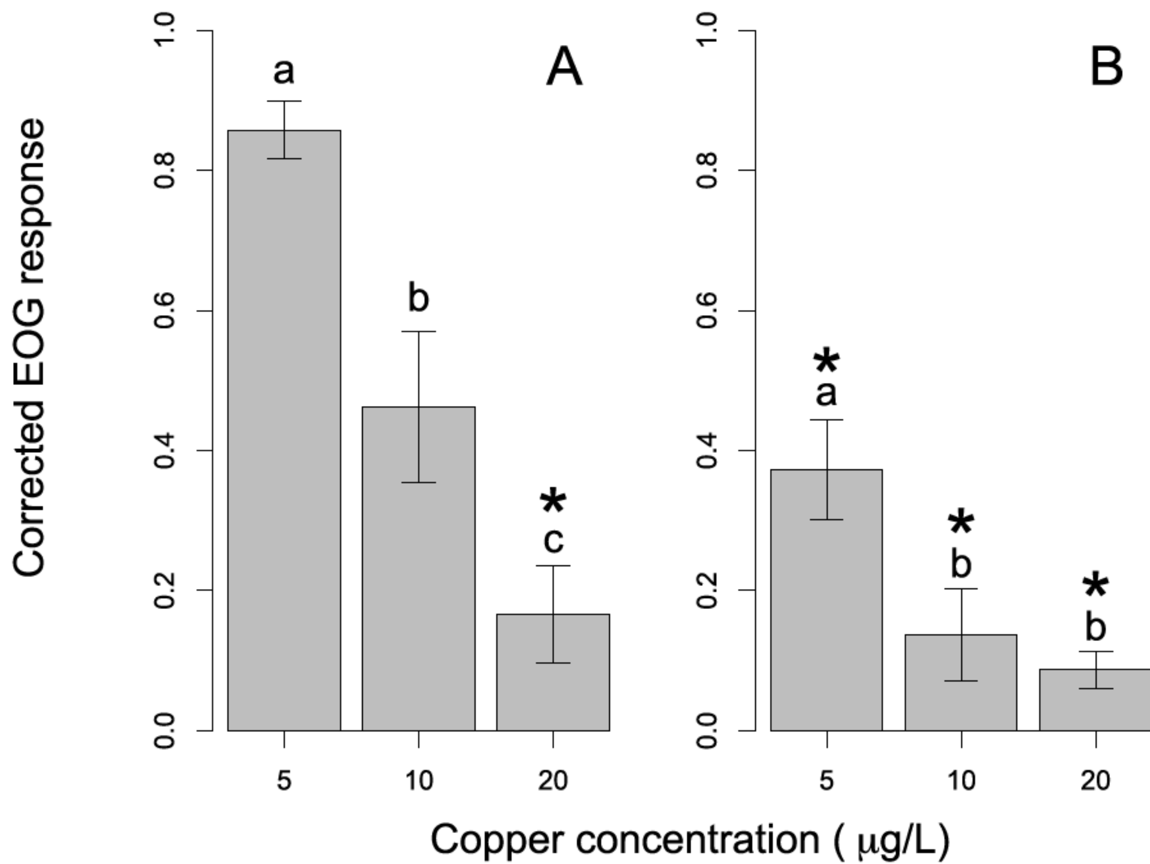

Figure 4.2: Corrected EOG response (mean ± SEM) to L-alanine (A) and TCA (B) of fathead minnows treated with copper (n=4-5 for each bar). L-alanine causes an EOG response specific to microvillous cells while TCA causes an EOG specific to ciliated cells. Different letters above bars denotes a significant difference ($p \leq 0.05$). An asterisk above a bar denotes a significant difference from control exposure ($p \leq 0.05$).

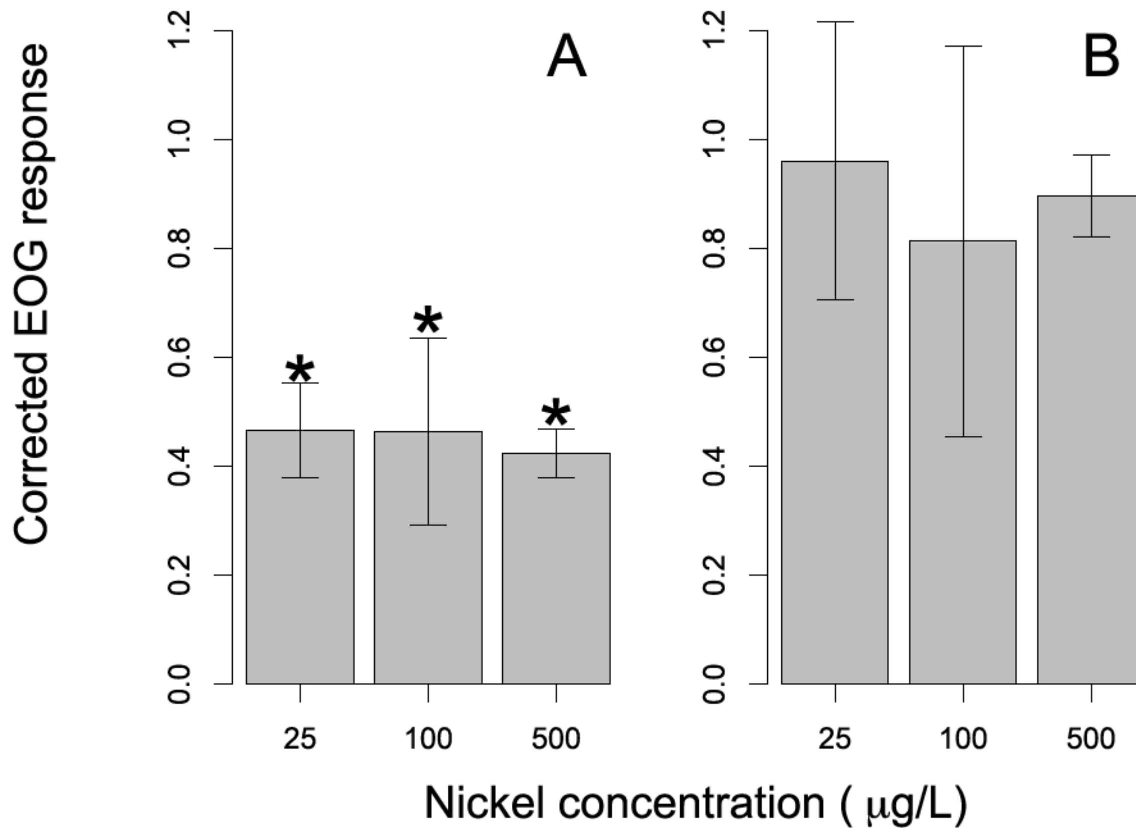

Figure 4.3: Corrected EOG response (mean ± SEM) to L-alanine (A) and TCA (B) of fathead minnows treated with nickel (n=4-5 for each bar). Different letters above bars denotes a significant difference ($p \leq 0.05$). An asterisk above a bar denotes a significant difference from control exposure ($p \leq 0.05$).

observed in fathead minnows (Figure 4.4). Yellow perch exposed to 20 µg/L copper showed a 69% reduction in their response to TCA relative to a control ($t_{4.73} = 4.15$, $p < 0.05$). However, their response to L-alanine remained intact ($t_{3.74} = 0.20$, $p = 0.85$). On the other hand, yellow perch exposed to nickel for 48 h showed an unimpaired response to TCA ($t_{3.71} = -0.26$, $p = 0.85$), whereas their EOG response to L-alanine was impaired by 63% relative to controls ($t_{4.84} = 4.52$, $p < 0.05$).

4.3.2 Behavioural experiments

When given a choice between an arm containing a skin extract made from fathead minnows or dechlorinated water, fathead minnows avoided the arm with the skin extract, spending 3.3 times more time in the arm containing the blank ($t_8 = 3.42$, $p < 0.02$; Figure 4.5A). Fathead minnows did not distinguish between the blank and stimulus end when the stimulus was from a red swordtail control ($t_9 = 0.543$, $p = 0.6$, Figure 4.5B).

Fathead minnows avoided a fathead minnow skin extract when given a control ($t_{14} = 2.81$, $p < 0.05$: Figure 4.6A) or nickel exposure ($t_{11} = 2.71$, $p < 0.05$; Figure 4.6C), but did not avoid alarm cue when exposed to copper ($t_{11} = -0.12$, $p = 0.92$; Figure 4.6B).

4.4 Discussion

The specificity of odourants in fathead minnows, namely that L-alanine induced an IP3-mediated olfactory signalling pathway and that TCA induced one based on cAMP, is in agreement with work done on round goby (Laframboise and Zielinski, 2011). Microvillous OSNs utilize IP3-mediated signalling, while ciliated and crypt OSNs have a pathway based on cAMP (Zielinski and Hara, 2006). As other studies have

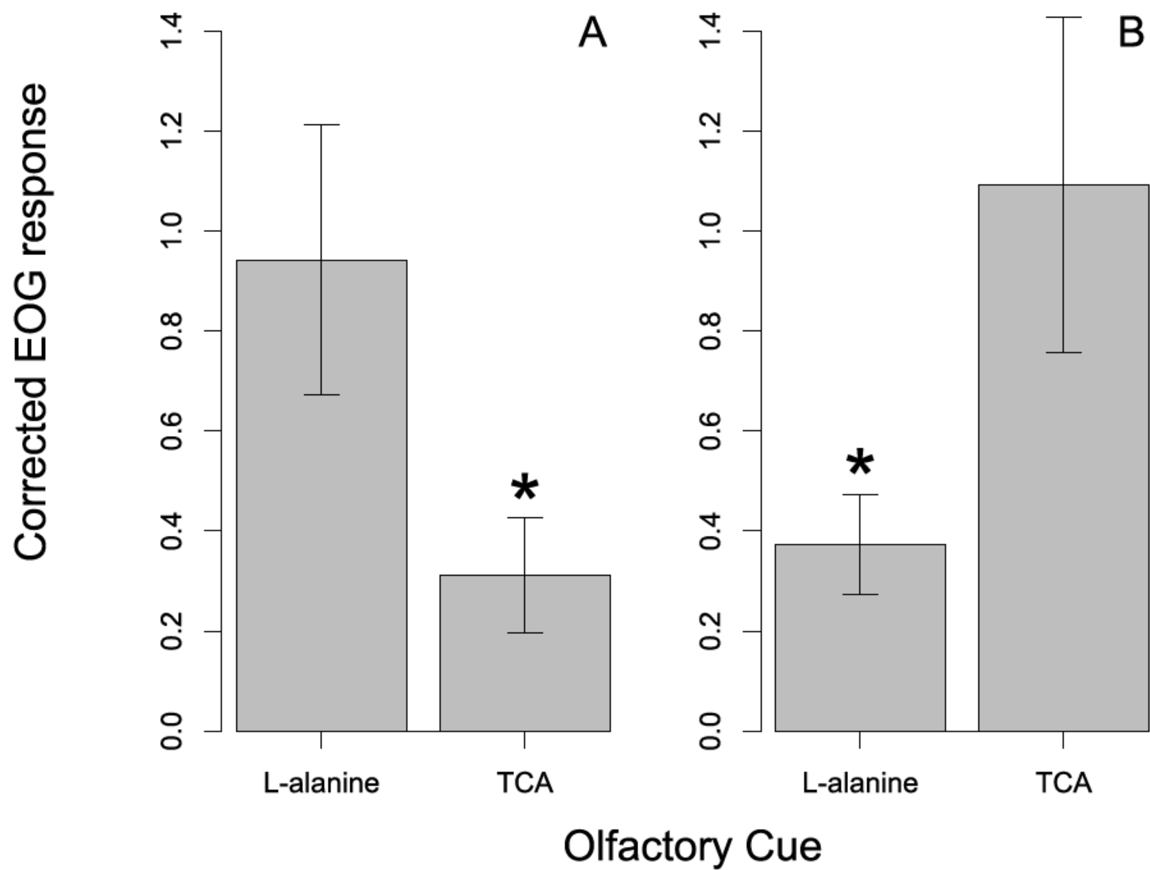

Figure 4.4: Corrected EOG response (mean ± SEM) of yellow perch exposed to 20 μgL^{-1} copper (A) or 500 μgL^{-1} nickel (B) to L-alanine or TCA (n=4 for each bar). L-alanine causes an EOG response specific to microvillous cells while TCA causes an EOG specific to ciliated cells. An asterisk above a bar denotes a significant difference from control exposure ($p \leq 0.05$).

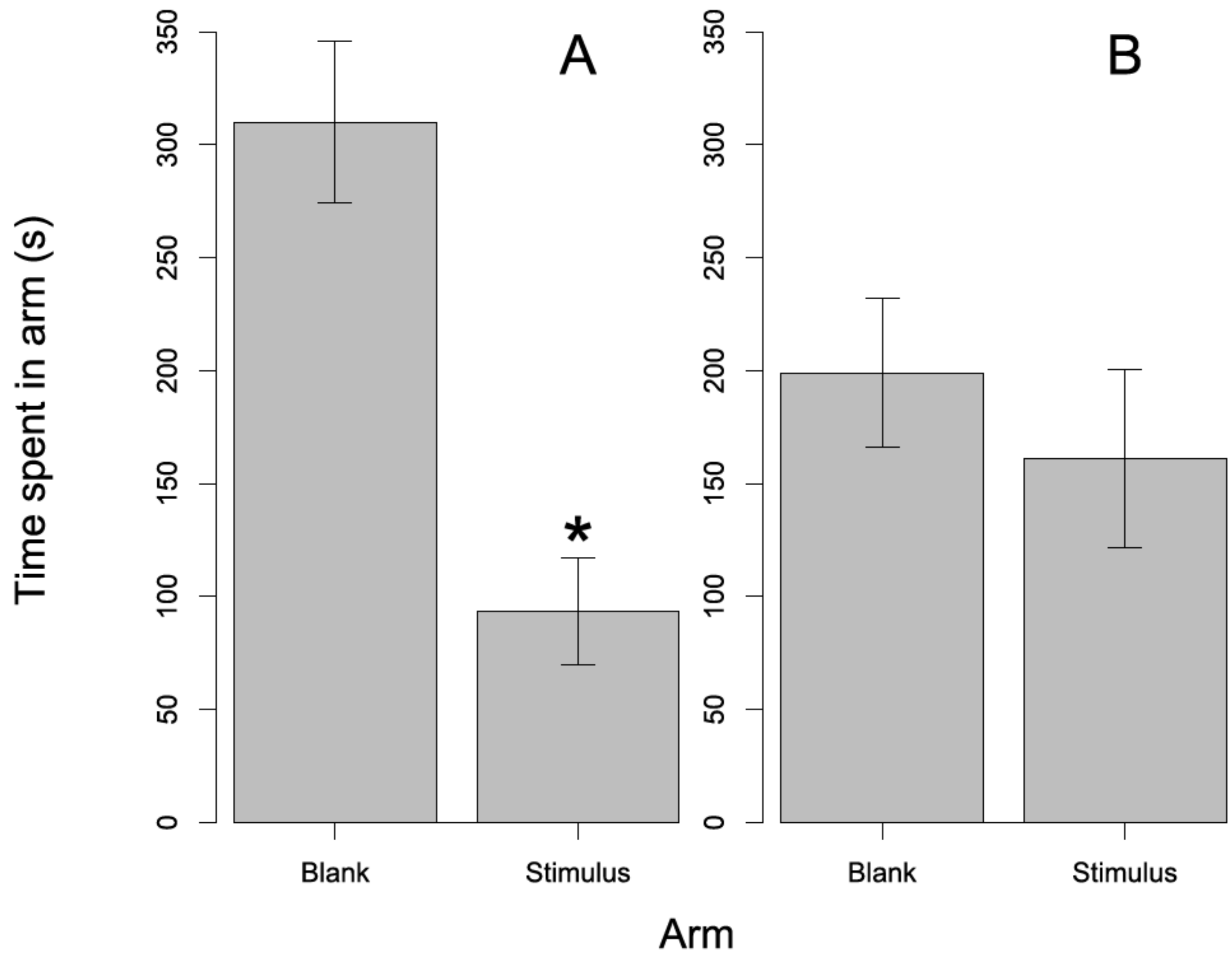

Figure 4.5: Time spent by fathead minnows in the blank and stimulus arm (mean ± SEM) when the stimulus was fathead minnow skin extract (A) or swordtail skin extract (B), n=9-10 for each bar. An asterisk above a bar denotes a significant difference between the time spent in the stimulus arm versus the time spent in the blank arm ($p \leq 0.05$).

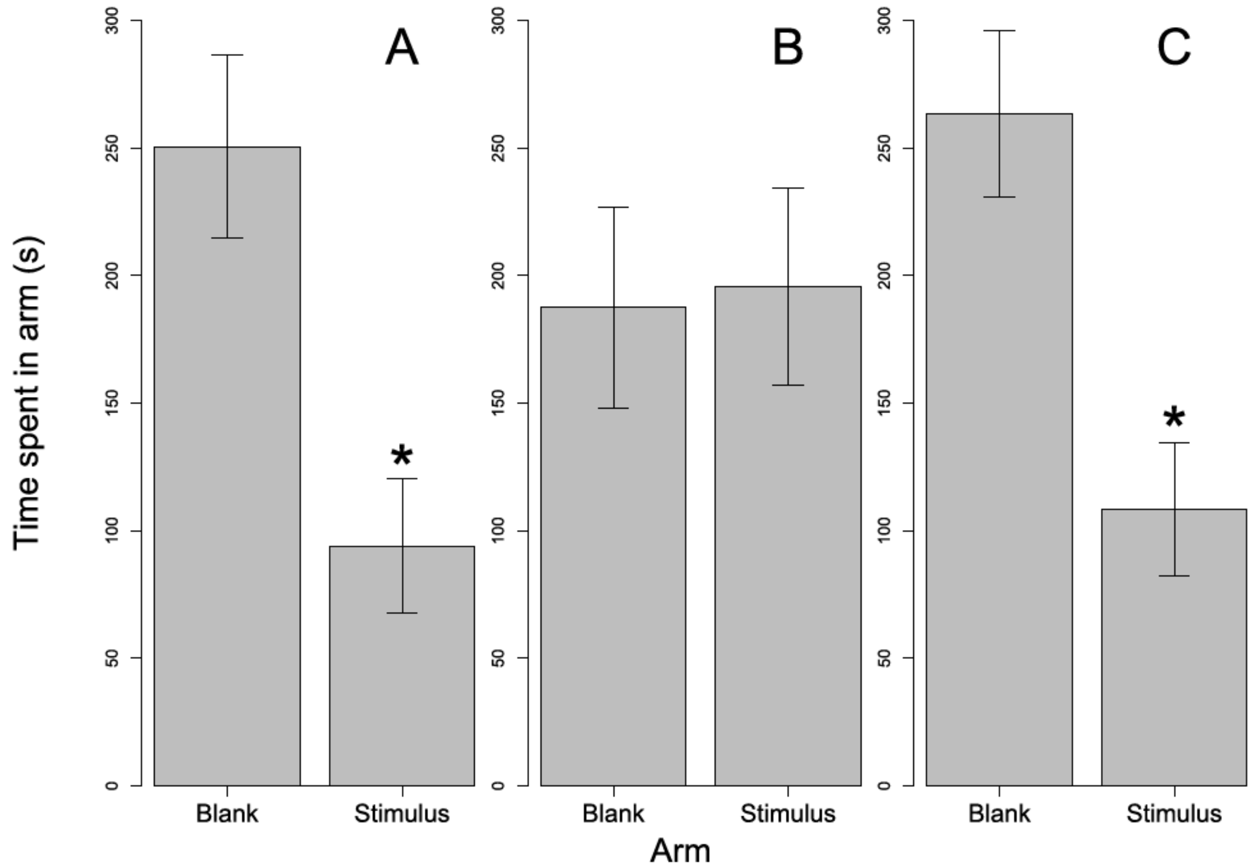

Figure 4.6: Time spent by fathead minnows in the blank (dechlorinated water) and stimulus (fathead minnow skin extract) arm (mean ± SEM) under a control, 20 μgL^{-1} copper or 500 μgL^{-1} nickel exposure (n=12-15 for each bar). An asterisk above a bar denotes a significant difference between the time spent in the stimulus arm versus the time spent in the blank arm ($p \leq 0.05$).

demonstrated that TCA is specific to ciliated and not crypt OSNs in other species of fish, it is likely that this is the case in fathead minnows (Vielma et al., 2008; Døving et al., 2011). In fathead minnows, therefore, L-alanine induced a response specific to microvillous OSNs, while TCA induced a response specifically in ciliated OSNs.

By exploiting the specificity of L-alanine to microvillous OSNs and TCA to ciliated OSNs, we were able to demonstrate that exposure of fathead minnows and yellow perch to copper and nickel impaired OSN classes differently. For both species, at all concentrations tested, nickel impaired microvillous and not ciliated OSNs. Copper, on the other hand, had differing effects in fathead minnows depending at the concentration tested. For fathead minnows, the EOG response to TCA was reduced at all concentrations of copper tested, indicating that ciliated OSNs are susceptible to copper even at very low concentrations. Copper impaired the EOG response to L-alanine in a concentration-dependent manner, suggesting that microvillous cells were only affected by copper at the highest concentration tested. Given that copper impaired the EOG response of fathead minnows regardless of which odourant was used demonstrates that at higher concentrations copper is generally toxic to the olfactory system, affecting both ciliated and microvillous OSNs. At low concentrations of copper, only ciliated OSNs are affected. Only one concentration of copper was tested with yellow perch, which resulted in ciliated OSNs being impaired while microvillous OSNs were not. Therefore, the OSN specific effects of nickel and copper seen using laboratory-reared fathead minnows are directly comparable to the effects of nickel and copper in wild yellow perch using native lake water.

The specificity of copper to ciliated OSNs and nickel to microvillous OSNs is a cross-species phenomenon and we speculate that this is likely true for other species of fish and other contaminants. Only one other study has investigated the effect of copper on individual OSN classes. Kolmakov et al. (2009) demonstrated that one day after a short-term exposure with a high concentration of copper sulphate (16 mgL^{-1}) the structure of microvillous OSNs appeared unaffected, while ciliated OSNs were damaged in goldfish (*Carassius auratus*). The differential effect on OSN type is consistent with the data presented in this study, as copper had a stronger effect on ciliated OSNs than microvillous OSNs. The work presented here is the first to demonstrate that toxicants can target their effects on ciliated and microvillous OSNs. It is likely that other contaminants besides copper and nickel may have differential effects on specific OSN classes. More work is needed to elucidate whether or not other contaminants have OSN-specific effects as well.

While nickel is taken up into olfactory neurons in fish, it is unknown into which class of OSNs (Tallkvist et al., 1998). It is possible that nickel entered into microvillous OSNs and inhibited their function. An alternate explanation is that nickel entered into all OSN types but only impaired the IP3-mediated olfactory signalling pathway found in microvillous OSNs. However, it is also equally plausible that nickel acts through an entirely different mechanism. Copper does not enter into OSNs, but instead accumulates in the lamina propria of rainbow trout (*Oncorhynchus mykiss*) olfactory epithelium (Julliard et al., 1995). Therefore, copper appears to exert its effect from the external environment, possibly through interference with cell-surface protein channels or by directly interfering with the electrochemical gradient at the OSN surface. Copper is

known to impair gill Na^+/K^+-ATPase, a membrane bound protein that is also involved in olfactory transduction (Laurén and McDonald, 1987; Kern et al., 1991). Copper may impair this pump or another pump (e.g., a calcium pump) at the membrane of ciliated OSNs and exert an inhibitory effect consistent with the inhibitory effects observed in this study. Regardless of the mechanism(s) of action of nickel and copper, they can induce olfactory dysfunction in specific OSN classes.

Fathead minnows avoided a conspecific skin extract, but not a skin extract made from a heterospecific, indicating that the predator-avoidance response was due to a conspecific alarm cue, and not a general odour representing damaged fish skin. When fathead minnows were exposed to copper, this stereotypical avoidance response to a conspecific alarm cue was impaired. However, the response remained intact after fish were exposed to nickel. Copper has been demonstrated to impair the response of two other fish species to conspecific antipredator cues, Colorado pikeminnows (Beyers and Farmer, 2001) and coho salmon (McIntyre et al., 2012), at similar concentrations (i.e., 5 μgL^{-1}) to that added in this study. While no work has been done prior to this study investigating the effect of nickel exposure on an alarm cue response, work done with rainbow trout demonstrated that 50 μgL^{-1} nickel does not impair agnostic interactions (Sloman et al., 2003). The loss of an alarm cue response due to the addition of such a low concentration of copper means that fish may lose their ability to properly evaluate predation risk and may become more vulnerable to predation than those whose chemosensory function remains intact.

At the neurophysiological level we determined that in fathead minnows copper impaired ciliated and not microvillous OSNs at low concentrations, while nickel impaired

microvillous but not ciliated OSNs at all concentrations tested. At the behavioural level we demonstrated that copper impaired an alarm cue response, but nickel does not. This leads to the conclusion that the reception of the odour responsible for inducing an alarm cue response in fathead minnows requires intact ciliated OSNs, but not intact microvillous OSNs. These are the first data to attribute the response of fathead minnows to a chemosensory cue to a specific OSN class. It is possible that other chemosensory-mediated behaviours may also be dependent on a specific OSN class. For example, crypt OSNs in crucian carp are only at the surface of the olfactory epithelium during spawning (Hamdani et al., 2008) and crypt OSNs in rainbow trout strongly react to gonad extracts (Bazáes and Schmachtenberg, 2012). Crypt OSNs, therefore, may be essential in these species for spawning behaviours, and if crypt OSNs were impaired during the a spawning season it may result in reduced reproductive output of a population of fish. The connection of the neurophysiological and behavioural levels of organization also allows for predictions to be made of behavioural deficits based on neurophysiological experiments. For example, it is possible that other toxicants target ciliated OSNs, thereby causing an impaired ability in fathead minnows to respond to conspecific alarm cue. Other contaminants and behaviours need to be investigated to fully understand this connection between these two levels of organization and to determine if predictions of behavioural deficits can be made based on neurophysiological experiments.

The organization of the olfactory epithelium has cross-taxa commonalities. Eisthen (1997) noted that different classes of vertebrates have either ciliated OSNs, microvillous OSNs, both OSN types, or some variation of these two OSN types.

Lamprey, turtles, and marsupials have ciliated OSNs only, sharks, skates, and ratfish have microvillous OSNs only, and hagfish, ray-finned fishes, lungfish, frogs, toads, and salamanders have both OSN classes. Lizards and snakes have ciliated OSNs and OSNs with both ciliated and microvillous aspects, while birds only have OSNs with both ciliated and microvillous aspects (Eisthen, 1997). Eutherians have ciliated OSNs and brush cells (a type of microvillous OSN), with recent evidence demonstrating that microvillous OSNs containing an IP3-mediated olfactory signalling pathway are found in the olfactory mucosa of mice (Elsaesser and Paysan, 2007). Both ciliated and microvillous OSNs have also been characterized in the human olfactory epithelium (Escada et al., 2009). Based on a phylogenic analysis, it is likely that early in evolutionary development both ciliated and microvillous OSN classes were present, and over time one or the other OSN type was lost (Eisthen, 1997). This phylogenetic conservation facilitates extrapolation of function among species. To that end, Evans et al. (1995) demonstrated that the addition of nickel sulphate to the olfactory epithelium of rats reduced the number of microvillous OSNs. There was no behavioural impairment of the response of rats to ethyl acetate. This result mirrors what was found in fathead minnows in the current study, namely that nickel affects the olfactory epithelium, but not in a way that interferes with a specific olfactory-dependent behaviour. Metals, including nickel, have also been shown to impair the olfactory system of humans (Sunderman, 2001). As humans share the same basic structure of OSNs as fish, fish may represent a viable model to study the effects of metals on human olfaction.

In summary, this work demonstrates that specific classes of OSNs can be differentially affected by copper and nickel. It also demonstrates that the alarm cue

response in fathead minnows first described by von Frisch (1938) is dependent on ciliated, but not microvillous OSNs. This work is also applicable to other taxa as the basic structure of the olfactory epithelium is phylogenetically conserved. Future studies should focus on not only how other contaminants affect each class of OSN and how other behaviours tie into these effects, but also perform cross-taxa comparisons to demonstrate if these effects are common among different classes of animals.

Chapter 5: Conclusion

Fish that live in a contaminated environment, such as what is found in Sudbury, ON, face many challenges. If the contamination is not enough to kill the fish outright, the contamination may have a variety of subacute effects on the fish. One of these effects is the impairment of the fish's olfactory system. The olfactory system of a fish is essential for mediating many ecologically-relevant behaviours such as find food, finding a mate, and avoiding predators. When the olfactory system is impaired, the fish does not necessarily die, but it no longer functions in an ecological context. By understanding how a fish is affected by contamination, we can develop environmental protection, monitoring, and remediation programs for areas that have been contaminated or are currently under development.

The work presented in this dissertation represents significant advancements in our understanding of how the olfactory system of fish is affected by copper, and to a lesser extent nickel. Currently, the gill-based biotic ligand model (gbBLM) is the focus of considerable amount of research involved in protecting fish from the effects of copper. The work presented herein demonstrates that the current gbBLM is not protective of the olfactory system of all fish. Some of the underlying assumptions of the gbBLM, such as calcium being protective of copper-induced dysfunction, while true for the gill, do not hold for the olfactory epithelium. A major part of the gbBLM is predicting how different cations are protective of copper-induced dysfunction at the gill, the work presented in Chapter 3 demonstrates that one of the important cations, namely calcium, is not only not protective, it exerts an effect independent of copper.

The work detailed in Chapter 4 demonstrates that the anti-predator response in fathead minnows require ciliated cells. This association between OSN type and a behavioural response is the fist instance of a specific OSN being demonstrated to be necessary for a particular behavioural response. The association of an OSN type with a behaviour allows for the development of a model based on the olfactory epithelium which can be used to make predictions of behavioural deficits based on impairment at the olfactory epithelium. While much more work is required before such a model would be useful, the work presented in this dissertation takes the first steps to being able to predict ecologically-relevant behavioural deficits based on direct measurement of the olfactory epithelium.

Another important advancement detailed in this dissertation is that while both copper and nickel impair olfaction in fish, they impair different aspects of the olfactory system. Copper impairs ciliated cells and response of fish to an anti-predator cue, while nickel impairs microvillous cells and has no effect on the response of fish to an anti-predator cue. Fish living in an environmental contaminated with nickel would still be able to detect an anti-predator cue, while fish living in a copper contaminated environment would not be able to detect an anti-predator cue. It is possible, then, that other olfactory-mediated behaviours may or may not be impaired by different toxicants. If that it is the case, environmental risk assessments can take into account what behaviours are important for specific fish populations. For example, if fathead minnows are in a pond with no predators, loss of a response to an anti-predator cue due to copper may not be detrimental to the population, while if another toxicant impairs mating related behaviour, it may be necessary to ensure that that toxicant is not released during spawning events.

Site specific regulations for copper and other toxicants strike a balance between protection and economic impact.

By understanding how the olfactory system of fish is affected by toxicants we can begin to develop the tools necessary to predict what is happening with fish populations in contaminated environments. By being able to predict how fish are affected by toxicants we be able to produce regulations and monitoring programs to ensure there are healthy fish populations for years to come.

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