Metal induced olfactory impairment in fish:

gene expression, protection and recovery

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ABSTRACT

To play their ecological role in an ecosystem, a fully functioning olfactory system is vital for fishes. A fish's olfactory system is very sensitive to low concentrations of contaminants. A great deal of research has been conducted on the toxicity of metals on the olfactory systems of fish. While tightly-controlled laboratory studies are necessary to reveal the mechanism of toxicity, field-based studies using more realistic conditions are also needed to produce more environmentally relevant data for regulatory needs. This research has four main areas of focus: 1) to examine the effect of copper on gene transcription in olfactory tissues of yellow perch (*Perca flavescens*); 2) to test if a modified diet can protect olfaction against copper toxicity in rainbow trout (*Oncorhynchus mykiss*); 3) to examine chemosensory-mediated behaviours and gene transcription profiles in wild yellow perch from metal contaminated lakes; and 4) to investigate the potential recovery of yellow perch with contaminant-induced chemosensory function from metal contaminated lakes. We used gene transcription (a recently developed custom made micro-array for yellow perch and real-time PCR), neurophysiological testing (electro-olfactography (EOG)), and behavioural assays (avoidance from alarm cues). In this research, yellow perch were exposed to elevated concentrations of copper for 3 and 24 hours. While 3 hours of exposure did not influence gene expression, 24-hour exposures to copper elicited a differential expression of 71 genes. Of these 71 genes, differential expression of two subunits of Na/K-ATPase was further explored using real-time PCR in a time-series study. To investigate if increased dietary sodium can protect fish's olfaction against copper-induced olfactory impairment, rainbow trout were fed with diets having elevated

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concentrations of sodium. While fish exposed to 10 µg/L Cu and fed with a normal-sodium diet had an impaired EOG response to standard olfactory cues, olfaction in Cu-exposed fish fed with Na-supplemented food (regardless of low or high levels of supplementation) remained intact. However, subsequent feeding trials found no evidence to support that Cu-exposed fish preferentially chose high-Na food. In addition, in this study olfactory impairment of fish from one clean and two metal contaminated lakes were compared using gene expression, EOG and behavioural I-maze choice experiments. While behavioural testing and EOG confirmed the impairment of olfaction in fish from metal contaminated lakes, the micro-array was not able to detect differential gene expression. To investigate if impaired olfaction of fish from metal contaminated lakes has recovery potential, EOG and behavioural testing methods were employed. Yellow perch from metal contaminated lakes with impaired olfaction kept for 24 hours in water from clean lakes. The results showed that impaired EOG responses of fish from metal contaminated lakes can recover quickly (within 24 hours) in water from a clean lake. When the behavioural testing methods were employed the results showed that olfactory mediated behaviours of fish from a moderately contaminated lake recovered after 24 hours of holding in clean water. However, olfactory mediated behaviours of fish from a severely contaminated lake did not recover after 24 hours of exposure to clean water.

The data produced by this thesis significantly improves our knowledge regarding the protection against metal induced olfactory toxicity as well as the recovery potential for impaired olfaction in fish. These results could be used to draft ecologically-relevant regulations that will protect fish inhabiting sensitive fresh water ecosystems.

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DOC - Dissolved organic carbon

EOG – Electro-olfactogram

ICP-AES - Inductively coupled plasma atomic emission spectroscopy

OSN - Olfactory sensory neuron

RT-PCR: Real-time polymerase chain reaction

TCA - Taurocholic acid

1. CHAPTER 1: Introduction

1.1. Olfaction

Aquatic organisms (e.g., fish) receive a remarkable amount of information through olfaction (Laberge and Hara, 2001). Their surrounding medium, water, is an ideal solvent to dissolve and disperse odourant compounds (Wisenden, 2000), and they benefit from the perception of olfactory cues for several biological processes such as foraging (; Hara, 2006; ; Ari and Correia, 2008), reproduction (Li et al., 2002 Johnson et al., 2005; Yambe et al., 2006), assessment of potential predators (Mikheev et al.,2006; Døving, and Lastein, 2009; Giaquinto and Hoffmann, 2010;) and migration (Dittman and Quinn, 1996; Bjerselius et al., 2000; Fine et al., 2004). Fish species rely more on olfaction particularly in conditions where the visual sense is not reliable such as highly structured, deep or turbid water (Wisenden, 2000). Therefore a fully functioning olfactory system seems necessary for fish to be able to survive and play their role in their ecosystems.

From an anatomical point of view, the olfactory system consists of two olfactory rosettes, olfactory nerves, olfactory bulbs and telencephalon. Olfactory rosettes are situated in olfactory cavities at both sides of snout of different species of fish. Olfactory rosettes consist of a series of olfactory lamellae (Laberge and Hara, 2001). The olfactory sensory neurons (OSNs) start from olfactory lamella where they have dendrites in direct contact to surrounding water

in olfactory cavity (Hamdani and Doving 2007). The olfactory nerve is composed of axons of OSNs which synapse with a few secondary neurons in the olfactory bulb (Laberge and Hara, 2001). The secondary neurons terminate in the telencephalon where the olfactory signal is processed (Hamdani and Doving 2007).

1.2.   Olfactory toxicity

To receive chemical information the olfactory system has to have direct contact with odour molecules dissolved in water. As a result, the olfactory system is also subject to direct exposure to contaminants in the water. The unprotected olfactory system is very sensitive to contaminants such as metals, pesticides and hydrocarbons, at lower concentrations that are needed to cause acute adverse effects, including death (Lürling and Scheffer, 2007). Impairment of chemosensation can affect vital life processes and has the potential to result in widespread ecological consequences. Among different contaminants, metals have received the most attention and the effects of various concentrations of several single metals have been investigated (Tierny et al, 2010). However, many aspects of olfactory toxicity of metal contaminants remain unknown.

1.3.   Field studies versus laboratory studies

To date, most ecotoxicology studies, including olfactory toxicity studies, have been conducted under controlled laboratory conditions, where a model species of fish is typically exposed to a single contaminant for a relatively short period of time. Exposure water is typically laboratory water with low levels of

DOC, neutral pH and very low concentrations of other elements. This setting is ideal to keep all variables constant to be able to study the effect of the variable of interest. On the other hand, in their natural habitat, fish usually face a mixture of contaminants for a relatively long period of time or even over their entire lifespan. In addition, natural waters normally have high DOC, and pH levels often vary from acidic to basic. Taken together, while laboratory-based studies can be very informative about the mechanism of toxicity, field-based studies are more environmentally realistic (Couture and Pyle, 2011).

1.3.1. Sudbury

The lakes around Sudbury ON have been contaminated by metals for over 100 years because of hard rock mining activities in the region. Many aquatic organisms were extirpated from thousands of Sudbury-area lakes due to high metal concentrations and acidification as the result of mining activities in the "industrial zone of impact" (Keller et al., 1992). Metal emissions from smelters in the Sudbury area peaked in mid-1960 resulting in the metal contamination of over 7000 lakes in a 17,000 km^2 area. In the 1970's remediation activities began in Sudbury and metal concentrations have continually decreased in lakes since then around industrial operations. Although different types of metals have been released to Sudbury lakes, copper and nickel have been traditionally called the "metals of concern" due to their very high concentrations relative to non-contaminated reference lakes. Although the Sudbury region is considered a good example of a successfully recovered region, it seems that concentrations of

contaminants in some lakes are still above the safe concentration for aquatic organisms (Keller et al., 2007). Fish inhabiting Sudbury-area lakes still show elevated tissue metal concentrations relative to fish from reference sites (Pyle et al. 2005). Having been exposed to high levels of metal contamination for generations, these fish provide a unique opportunity to study the effects of long-term exposure to contaminants.

Different aspects of metal toxicity have been studied in Sudbury during the past decades; however, only two studies investigated olfactory toxicity in this area (McPherson et al., 2004; Mirza et al., 2009). McPherson et al. (2004) demonstrated that Iowa darters (*Etheostoma exile*) were unable to avoid traps treated with conspecific skin extract (alarm cue) in metal contaminated lakes, but were able to successfully avoid alarm cue scented traps in clean lakes. Mirza et al. (2009) demonstrated that yellow perch (*Perca flavescens*) from contaminated lakes did not respond to conspecific skin extracts in laboratory water under controlled conditions, whereas those from a clean lake did. However, many aspects of olfactory toxicity in this area, including mechanisms of long-term olfactory toxicity, olfactory recovery potential and olfactory adaptation in metal contaminated habitats remain unstudied.

1.4.   Yellow perch

To date, most olfactory toxicity research has been done on model species, namely rainbow trout or fathead minnows. However, these species are uncommon in Canadian mining areas. Despite their importance as model

species, their environmental relevance is questionable. Yellow perch are nearly ubiquitous throughout Canadian lakes (Scott and Crossman 1973) and occupy various trophic positions in aquatic ecosystems during different stages of their life (i.e., planktivore, benthivore and carnivore; Sherwood et al., 2002). Tolerance of yellow perch to the toxic effects of dissolved metals is considerable (Taylor et al., 2003); in fact, yellow perch is one of the only fish species commonly found in many of the most metal-contaminated lakes around the mining district of Sudbury, ON (Couture and Pyle, 2008). Yellow perch has been heavily studied with respect to the effects of metal uptake, accumulation and subsequent toxicity despite its considerable metal tolerance (Eastwood and Couture, 2002; Couture and Rajotte, 2003; Kraemer et al., 2006; Couture and Pyle, 2008; Pyle et al., 2008). Nevertheless, studies investigating the effect of contaminants (i.e. metals) on olfaction of this ecologically relevant species are rare. In the only other study conducted to date to investigate effects of metal contamination on olfaction of yellow perch, Mirza et al. (2009) demonstrated that yellow perch from contaminated lakes responded to chemical cues electrophysiologically but not behaviourally. Nonetheless, the mechanism of olfactory toxicity of this important species still remains unknown. It is valuable to provide knowledge of how the olfactory system in this species responds to toxicological pressures at multiple levels of biological organization (i.e., behavioural, physiological, cellular, and molecular levels). In addition, it is important to understand how this species is able to handle the excess metal load released to natural receiving waters, and if

any adaptation has happened in the olfactory system of this species after decades of chronic exposure to metal contamination. Revealing the mechanism of this potential adaptation could also answer many questions about different aspects of olfactory toxicity.

1.5. Olfactory toxicity investigation methods

During the past decades several techniques were employed to study the olfactory toxicity of metals in fish from lower levels of biological organization (e.g. molecular) to higher levels (the whole organism). While molecular and cellular studies can provide insight into mechanisms of olfactory toxicity, investigating higher biological levels has greater potential to demonstrate the large-scale consequences of impaired olfaction (i.e., behaviour and survival of the organisms). Although many data are available regarding olfactory toxicity, studies investigating the relationship between the effects of contaminants at different levels of biological organization are rare. Linking different levels of organization will aid in predicting olfactory-based toxicity as well as large-scale consequences on the animals' behaviour and ecological function.

1.5.1. Ecotoxicogenomics

A great deal of research has focused on the effects of metals at the individual, population, and ecosystem level. Metal-induced stress may induce subtle effects at the gene transcription and molecular levels that are not necessarily visible using higher-level physiological measurements. However, transcriptional effects may still evoke adverse metabolic costs to the animal.

Ecotoxicogenomics study the toxicant effects that occur at the molecular level (Neumann and Galvez, 2002). Gene transcription is a sensitive indicator of contaminant exposure and thus contaminant-induced gene expression patterns are evaluated to determine what effect exposure to toxicants will have on the organism (Lettieri, 2006). These patterns are then used to extrapolate the effect to the population and, if possible, to the ecosystem level (Neumann and Galvez, 2002). The use of ecotoxicogenomics to investigate ecosystem effects has numerous benefits, including reduced monitoring costs in terms of both time and expense. In addition, ecotoxicogenomics has been found to be reliable for providing knowledge regarding mechanisms of toxicity, developing "signatures" of toxicity to chemicals, and to identify biomarkers to be used for field and mixture studies (Neumann and Galvez, 2002; Lettieri, 2006; Denslow et al., 2007; Pina and Barata, 2011). During the past few decades, microarrays and real-time polymerase chain reaction have been used in ecotoxicogenomics. While microarrays provide an opportunity to develop molecular signatures for classes of toxicants by studying responses of large number of genes, real-time PCR provides the opportunity to study single genes for mechanism-based toxicology. Although toxicogenomic methods have been a fruitful addition to the field of ecotoxicology, their benefit to olfactory-based toxicology has been limited. Tilton et al. (2008, 2011) used a commercial zebrafish microarray containing 14,900 transcripts to study the effects of exposure to copper and/or chlorpyrifos on the

differential gene expression in olfactory tissue and were able to measure changes at the transcriptional level.

1.5.1.1.   Microarrays in aquatic toxicity studies

Microarray analyses allow rapid assessment of the impacts of stressors at the level of mRNA transcription (Snape et al., 2004; Lettieri, 2006). Microarrays consist of thousands of ordered spots of DNA probes fixed on slides with the potential of each spot to represent a unique gene or DNA sequence and allow for the analysis of the expression of multiple genes simultaneously. In some cases microarrays contain the entire genome of an organism and can theoretically be used to study changes in transcription over the entire genome (Valasek and Repa, 2005). Thus, microarrays are less costly and time-consuming and produce significantly more data than conventional molecular methods (Neumann and Galvez, 2002). Although gene transcription analysis has been used in mammalian toxicology, it has received less attention in aquatic toxicology mainly because of insufficient information in the genome sequence of ecologically relevant aquatic species (Denslow et al., 2007). However, it is possible to obtain gene sequences and construct species-specific arrays for fish of ecological relevance (Pina and Barata, 2011).

1.5.1.1.1.   The novel microarray

The yellow perch genome has not been completely sequenced. Thus, there is no complete-genome microarray available for this environmentally relevant species. Recently a novel custom yellow perch microarray has been

designed and developed by our colleagues, Bougas et al. (2013), for the detection of metal-induced stress and to identify the different mechanisms of sublethal metal toxicity. There are six replications of 1,000 probes on this microarray. The genes selected for this perch mircroarray have been shown to respond to metal exposure and/or represent important physiological processes, such as basal metabolism and chemosensation. This genomic tool has been able to reveal different mechanisms of the sublethal nickel and/or cadmium effects in livers of yellow perch after 45 days of exposure to environmentally relevant concentrations (Bougas et al., 2013). It also was able to detect gene transcription changes in response to non-metal stressors such as hypothermia, hyperthermia, hypoxia and starvation (Bougas et al., 2013). The use of microarray analysis in this dissertation was to test the ability of this microarray to detect gene expression changes of olfactory tissues in exposure to metal contamination.

1.5.1.2.   Real-time polymerase chain reaction

While microarrays provide an opportunity to study a large number of genes simultaneously, RT-PCR experiments allow detailed study of specific genes. Real-time PCR has become a strong and extensively applied method for toxicological studies due to its ability to give a rapid and accurate assessment of very small changes in the transcription of specific genes (Valasek and Repa, 2005). Because of the precision and sensitivity of RT-PCR, even subtle changes in gene expression can be detected. In addition, when it comes to studying a small number of genes, RT-PCR experiments are easier to conduct, require less

time to carry out, and are more cost-effective compared to microarray experiments. In addition, a smaller amount of RNA is needed to conduct a RT-PCR experiment compared to a microarray experiment, making RT-PCR favourable when the amount of tissue is limited. Due to the reliability, RT-PCR is also used as a common validation tool for confirming gene transcription results obtained from microarray analysis (Walker, N. J., 2001; Valasek and Repa, 2005; Morey et al., 2006). Microarrays purport to produce information about a large number of genes. Therefore, getting adequate statistical power could be problematic with present array technology. Thus, researchers choose RT-PCR as a supporting technique to validate and better quantify the most interesting candidate genes from their arrays (Valasek and Repa, 2005). As a common methodology, many toxicology studies have used RT-PCR to validate microarray results (Koskinen et al., 2004; Tilton et al., 2005; Moens et al., 2006; Reynders et al., 2006; Gunnarsson et al., 2007; Moens et al., 2007; Lie et al., 2009).

1.5.1.3. Homeostasis and ecotoxicogenomics

Homeostasis is the ability of cells to regulate their biological activities in order to maintain a stable equilibrium despite the changes in the external environment. Metals are known to disturb the stable conditions of the cells from various mechanisms of action (Wood, 2012). In case of exposure to metals, cells use various mechanisms to maintain their stable conditions and protect themselves from toxic effects of the contaminant as a natural homeostatic response (Wood, 2012). Within hours of exposure to metals gene transcription

changes begin to occur either to reduce the effect of metals in the cells (i.e., induction of detoxifiers) (adaptive response) or to compensate for the loss of essential pathways as a result of exposure to metals (compensatory response) (Denslow et al., 2007). However, at higher concentrations of contaminants or longer exposure duration cells may not be able to adopt or compensate and more severe adverse effects may happen (Denslow et al., 2007). Comparing the gene transcription of cells exposed to contaminants with cells in normal conditions can produce valuable information regarding the homeostatic responses of cells in presence of contaminants to decrease the adverse effects of contaminants. Analysing the homeostatic responses of the cells may lead to understanding the mechanism of toxicity of contaminants.

1.5.2. Neurophysiological response

The acuity of the olfactory system is measurable using a technique called electro-olfactography (EOG). An electro-olfactogram measures an odour-evoked change in the extracellular field potential of the olfactory epithelium, which is a measure of cations (Ca^{2+} and Na^{+}) moving into OSNs and anions (Cl^{-}) moving out of the OSNs in response to a chemosensory stimulus (Scott and Scott-Johnson, 2002). This technique determines if an odour that is presented to the olfactory epithelium of a fish causes the ion channel proteins to open, thereby facilitating the cellular depolarization of olfactory sensory neurons requisite for action potential propagation. The EOG is represented as the signal amplitude between a baseline (no odour present) and the maximum extracellular cation

influx (odour present). The magnitude of this difference (i.e., the amplitude) in an EOG recording measures the olfactory acuity of the animal. The electro-olfactogram has been used as a standard technique in many olfactory toxicity studies and a variety of odourants such as L-alanine (Bilberg et al 2011), L-argenine (Dew, et al., 2012), skin extract (Mirza et al., 2009), taurocholic acid (TCA) (Laframboise and Zielinski, 2011) and sex pheromones (Laframboise et al., 2011) have been used as standard cues in measuring olfactory acuity using EOG. These different cues represent natural cues that fish may detect in natural conditions. Electro-olfactography has been able to reveal many aspects of olfactory toxicity and still is a very useful method in toxicology studies.

1.5.3. Behavioural response

Animal behaviour reflects the relationship between an animal's internal and external environments. It integrates local environmental conditions with the underlying physiology and condition of the behaving animal. Consequently, deviations in behaviours that are critical to the success of an animal, such as finding food, avoiding predation, or reproducing, can have serious ecological consequences—especially if impaired behaviours occur over an entire population. Abnormal behaviour has been used as an indicator in ecotoxicology studies in fish and has provided information of high ecological relevance in ecotoxicology studies (Atchison et al., 1987; Ezeonyejiaku et al., 2011; Hellou, 2011; Powers et al., 2011). For example, swim performance has been used to study the energetics of fish in exposure to contaminants in many studies

(Waiwood and Beamish, 1978; Beaumont et al., 1995; Rajotte and Couture, 2002; Mager and Grosell, 2011; Thomas et al., 2013). In addition, intactness of olfactory mediated behaviours has been used to study olfactory acuity. Since olfaction has been shown to mediate behaviours associated with finding food, avoiding predators, and reproduction, impaired behaviour in these processes could be an indicator of olfactory impairment.

Fish can recognize the presence of a predator by perceiving several chemical cues. As an example, chemical cues released from the injured skin can be perceived by conspecifics as an alarm cue (Wisenden, 2000; Mirza et al ., 2003). Various behavioural strategies (e.g., hiding, reduction in movement and avoidance of high-risk zones) have been observed in response to the presence of predators (Wisenden, 2000; Carreau and Pyle, 2005; Mirza et al. 2009). Thus, a reduced capacity to detect predators impairs the olfactory mediated anti-predator behaviours and consequently increases the probability of being predated upon.

Several studies have used predator alarm cue (i.e., conspecific skin extracts) to induce a natural fright response to study olfactory acuity in aquatic organisms (Carreau and Pyle, 2005; Mirza et al. 2009; Williams and Gallagher, 2013). The attraction of fish to food cue has also been used commonly to study olfactory acuity (Saglio et al., 1996; Kuz'mina, 2011), and a few studies have investigated the effect of contaminants on olfactory mediated reproductive behaviours (Baker and Montgomery, 2001; Moore and Waring, 2001). In general, the behavioural

experiments can still help researchers to understand the ecological effects of impairment of olfaction in fish.

1.6. Copper

Copper's natural concentration in surface freshwater ranges from 0.2 to 30 µg/L (Grosell, 2011). Anthropogenic activities can raise the concentration of copper up to 200 µg/L in water bodies close to mining areas (Grosell, 2011). Despite the fact that copper is necessary for many biological processes, it is also the second most frequent metal reported to impair water quality in the USA (Reiley, 2007). Consequently, much effort has been directed toward revealing the different aspects of both acute and chronic effects of copper on aquatic animals (Grosell, 2011). Copper has also been the center of attention in olfactory toxicity studies. Studying the adverse effects of copper on olfaction in fish began in 1964 when Sprague et al. (1964) showed that Atlantic salmon (*Salmo salar*) avoided high concentrations of copper. Hara et al. (1976) demonstrated that exposure of rainbow trout (*Oncorhyncus mykiss*) to 20 µg/L of copper for two hours reduces the electro-olfactogram (EOG) response to 55 % of the original response. Complete recovery was not observed even after two hours of rinsing with dechlorinated water (Hara et al., 1976). In 1996, Julliard et al. conducted a study to investigate the morphological changes of cells in the olfactory tissues of rainbow trout after exposure to 20 µg/L of copper for 1, 5, 10 and 15 days. They observed that after one day of exposure the number of apoptotic cells increased. After five days of exposure the number of apoptotic cells peaked. While after 10

days of exposure they observed that the number of cell deaths significantly decreased, but a second wave of apoptosis was observed after 15 days of exposure (Julliard et al. 1996). Baldwin et al. (2003) exposed coho salmon (*Oncorhynchus kisutch*) to different concentrations of copper (1, 2, 5, 10 and 20 µg/L) for 30 minutes. They demonstrated that the impairment of EOG responses due to copper exposure is dependent on the concentration of copper and at higher concentrations of copper the impairment is more severe (Baldwin et al., 2003). Bettini et al. (2006) exposed *Tilapia mariae* to 20 µg/L of copper for four days and investigated the process of recovery in olfactory epithelium using light microscopy. They observed that after a significant cell death due to copper exposure, the regeneration of the neurons started within 3 days of being exposed to clean water (Bettini et al., 2006). The neurons were completely regenerated after 10 days being in clean water (Bettini et al., 2006). Sandahl et al. (2006) also demonstrated the olfactory recovery after exposure to copper using EOG (Sandahl et al., 2006). In a recent study Dew et al. (2012) demonstrated that at low concentrations of copper, the impaired olfactory acuity of fathead minnows (*Pimephales promelas*) recover after 96 hours even in the presence of copper (Dew et al., 2012).

The above-cited studies showed copper induced impairment of olfaction at different concentrations on different species of fish. These studies (and others) answered many questions regarding olfactory toxicity of copper, although many more questions remained unanswered. The recent development of molecular

techniques (i.e. gene transcription) has given toxicologists the ability to look at the mechanism of toxicity of many contaminants at the molecular level. In the only two studies that have investigated the effect of copper on olfactory tissues at the molecular level, Tilton et al. (2008, 2011) demonstrated significant differences of gene transcription in pooled olfactory tissues of zebrafish (*Danio rerio*) exposed to copper and/or chlorpyrifos for < 24 h. Tilton et al. (2008) showed that copper caused an under-transcription of genes associated with olfactory signal transduction pathways such as calcium channels, G-proteins, and olfactory receptors. However, these results have yet to be confirmed with traditional techniques measuring higher order physiological effects (Denslow et al 2007).

1.7.   Protection

Many toxicology researchers have explored different factors that affect the adverse effects of contaminants on aquatic organisms. As an example, the toxicity of copper is reduced in water having elevated pH, hardness, sodium, dissolved organic matter, and suspended solids (Laurén and McDonald, 1986; Erickson et al., 1996; Chen et al 2012;). Increased calcium has also shown to decrease the toxic effect of cadmium (Song et al 2013). Pyle et al. (2003) showed that increased dietary sodium decreases the uptake of waterborne copper in gill tissues of rainbow trout. It was also demonstrated that increased dietary sodium is also able to protect gill tissue against some adverse effects of copper such as impairment of Na/K-ATPase (Pyle et al., 2003). There is no

available evidence demonstrating that water quality ameliorates the effects of copper on olfactory function (notwithstanding the ability of DOC to reduce the bioavailability and subsequent olfactory toxicity of copper (McIntyre et al., 2008; Kennedy et al., 2012). In fact, three studies testing the protective effect of aqueous calcium (known to reduce Cu toxicity to the gill) reported that increased aqueous calcium is not protective against copper induced olfactory impairment (McIntyre et al., 2008; Green et al., 2010; Dew et al. 2012).

1.8.   Objectives

This research had four main objectives:

1) To investigate the recovery of olfactory acuity in yellow perch following transplant from metal contaminated lakes to water from a clean reference lake.

2) To understand the relationship between chemosensory mediated behaviours and gene transcription patterns in wild yellow perch from clean and metal contaminated lakes;

3) To determine how copper affects chemosensory pathways at the transcriptional level after different exposure times in yellow perch; and

4) To determine the influence of increased dietary sodium on copper induced olfactory impairment of rainbow trout;

2. CHAPTER 2: Olfactory recovery of wild yellow perch from metal contaminated lakes

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Abstract

Fish depend on their sense of smell for a wide range of vital life processes including finding food, avoiding predators and reproduction. Various contaminants, including metals, can disrupt recognition of chemical information in fish at very low concentrations. Numerous studies have investigated metal effects on fish olfaction under controlled laboratory conditions. However, few have measured olfactory acuity using wild fish in source water. In this study, we used electro-olfactography (EOG) to measure the olfactory acuity of wild yellow perch (*Perca flavescens*) from a clean lake (Geneva Lake) and two metal contaminated lakes (Ramsey and Hannah lakes) from Sudbury, ON, in their own lake water or in water from the other lakes. The results showed that fish from the clean lake had a greater olfactory acuity than those from metal contaminated lakes when fish were tested in their own lake water. However, when fish from the clean lake were held for 24 hours in water from each of the two contaminated lakes their olfactory acuity was diminished. On the other hand, fish from the contaminated lakes held for 24 hours in clean lake water showed a significant olfactory recovery relative to that measured in their native lake water. These results show that although fish from a clean lake demonstrated impaired olfaction after only 24 hours in metal-contaminated water, fish from metal contaminated lakes showed a rapid olfactory recovery when exposed to clean water for only hours.

Keywords: olfactory toxicity, metals, recovery, electro-olfactography, yellow perch

2.1. Introduction

Aquatic animals rely on chemical information in order to mediate vital life processes, such as finding food, avoiding predation, and reproduction (Pyle and Mirza, 2007). Recent work has demonstrated that contamination of aquatic environments can lead to impaired chemosensory function, a process recently coined as 'info-disruption' (Lürling and Scheffer, 2007). However, most studies that have demonstrated contaminant effects on chemosensation in fishes have typically relied on tightly controlled laboratory exposures on model fish species. Under natural conditions, fish are typically exposed to more than one contaminant simultaneously, exposures occur over the fish's lifetime as opposed to the short-term exposures used in most laboratory studies, and responses to contaminants are often affected by site-specific exposure conditions (i.e., water quality). Moreover, model species, such as rainbow trout (*Oncorhynchus mykiss*) or fathead minnows (*Pimephales promelas*) may not inhabit the contaminated system of interest. Consequently, there is a need to investigate info-disruption in a wild fish to determine if metal induced chemosensory deficit observed under controlled laboratory conditions extrapolate to natural populations.

In order to develop a better understanding of the importance of info-disruption in natural fish populations, fish chemosensory function can be tested *in situ* or wild fish can be sampled from their natural habitats and tested under laboratory conditions. McPherson et al. (2004) demonstrated that Iowa darters (*Etheostoma exile*) in contaminated lakes did not avoid traps treated with conspecific alarm

cues whereas those from a clean lake could. This result suggested that wild fish in a contaminated lake are unable to detect an important antipredator cue and may be more susceptible to predation than fish from clean lakes where chemosensory function is intact. Mirza et al. (2009) demonstrated that wild yellow perch (*Perca flavescens*) collected from metal contaminated lakes were unable to respond to standard chemosensory cues in behavioural assays relative to those from clean lakes. However, those same chemosensory-impaired fish showed a significantly greater neurophysiological response to the same standard cues to which they yielded no behavioural response. Although this result might suggest a decoupling between epithelial neurophysiology at the site where the chemosensory cues are detected and higher-order information processing centres related to mounting a behavioural response, it may actually reflect differences in the quality and ionic composition of the water to which the fish was adapted; e.g., elevated Mg concentrations, as proposed by Mirza et.al. (2009).

The objective of this study was to compare olfactory acuity in wild yellow perch from clean and metal contaminated lakes and to determine whether or not observed differences in olfactory acuity are due to permanent olfactory dysfunction or to exposure water quality. A second objective was to determine if olfactory function could recover after only a short exposure to clean lake water if olfactory dysfunction resulted from fish being exposed to contaminated lake water. To address these questions, we conducted a reciprocal cross-exposure study using natural lake water and wild yellow perch from one clean lake, one

moderately metal-contaminated, and one metal-contaminated lake. Olfactory acuity was measured using a common neurophysiological technique, electro-olfactography (EOG).

2.2.   Materials and methods

2.2.1. Study lakes

Three lakes in the industrial region of Sudbury, Ontario, Canada were selected based on previous studies (Figure 2.1, Table 2.1; Unit, C. F. E., 2004; Pyle et al., 2005; Couture et al., 2008). Geneva Lake is 70 km northwest of Sudbury and is considered a clean lake given that it is situated outside of the Sudbury industrial zone of influence and has relatively low dissolved metal concentrations (Table 2.2.). Hannah and Ramsey lakes are located within the metal-contaminated Sudbury zone of influence, and are considered metal contaminated and moderately metal contaminated, respectively (Table 2.2.).

2.2.2. Water sampling and analysis

On-site measurements of pH and temperature were taken using a YSI 6600 V2 multiparameter sonde (YSI Inc, Yellow Springs, Ohio). Water samples were collected from each lake where fish were collected. For each sample one 15 mL container was rinsed three times, containers were submerged and capped under water. Half of the water samples were acidified using 50 μL of trace metals grade high purity nitric acid (Fisher Scientific, Nepean, ON). The acidified samples were passed through a 0.45 μm syringe filter and stored at 4ºC until analysed for
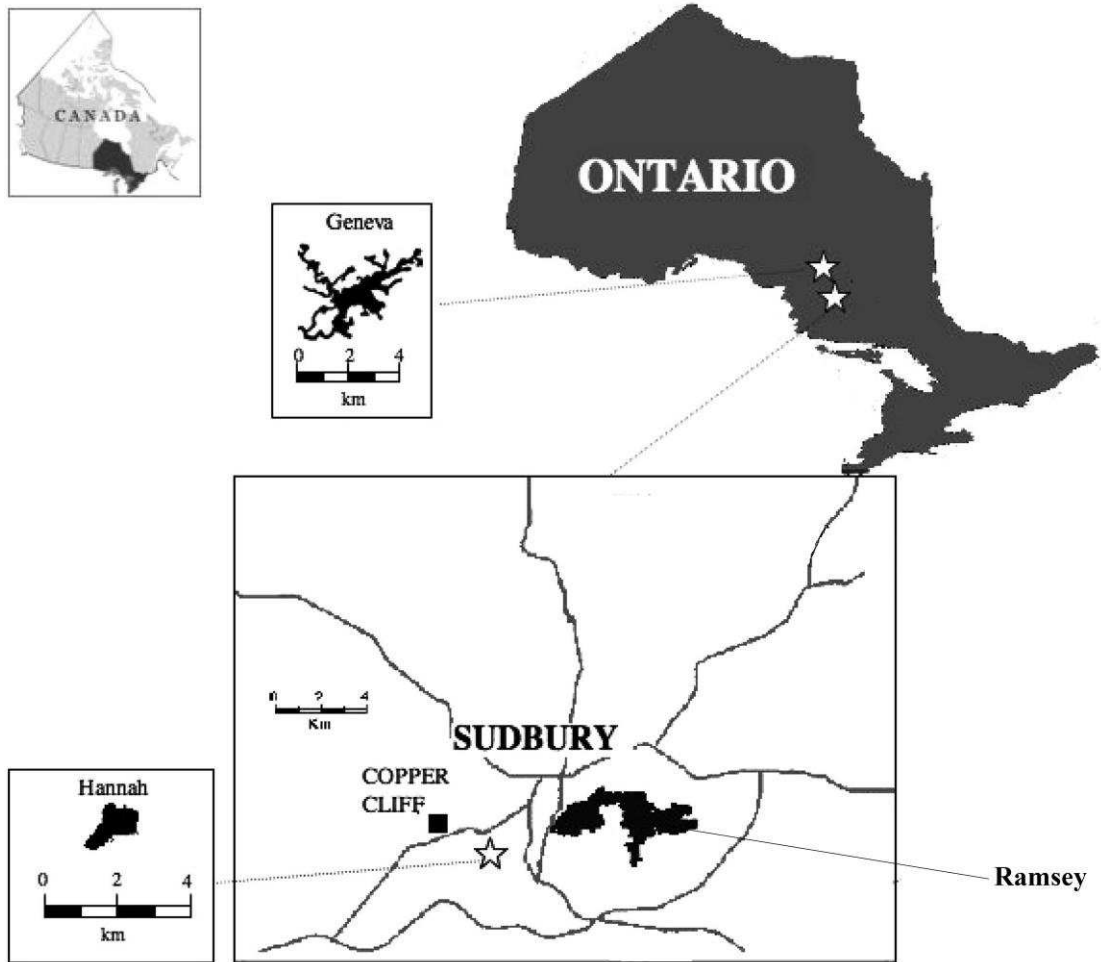
Figure 2.1. Map of study lakes in the Sudbury region (redrawn and modified from Couture et al., 2008).

Table 2.1. Location and surface area of Hannah Lake, Ramsey Lake, and Geneva Lake.

Lake	Decimal Latitude	Decimal Longitude	Lake Area (ha)
Geneva	46.7552	-81.5561	356.4
Ramsey	46.4712	-80.9704	792.2
Hannah	46.4458	-81.0367	27.7

metals. The other half of the water sample was passed through a 0.45 μm syringe filter and stored at 4^0C until analysed for dissolved organic carbon (DOC) concentration. Metal concentrations were measured using inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Varian, Mississauga, ON). The DOC concentration was measured with a San^{++} Automated Wet Chemistry Analyzer (SKALAR, Breda, The Netherlands). All analytical work was 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 LUCAS, including analysis of National Institute of Standards and Technology (NIST) traceable reference material standards. Alkalinity and hardness were determined as previously described (Pyle et al., 2005). All measurements of metal concentrations are summarised in Table 2.2 with measurements of water quality in Table 2.3.

2.2.3. Collecting and maintenance of fish

Fish were collected in early August 2011 using seine nets and angling. Animals were transported back to the lab in aerated lake water and maintained in the lab in static aerated native lake water under a 16:8 light:dark photoperiod and ambient temperature (22 ± 1°C). All animals were held for 24 hours prior to exposures. Animals were not fed during the acclimation or exposure periods.

Table 2.2. Dissolved metal and cation concentrations in water samples (n=3 per lake) from Hannah Lake, Ramsey Lake, and Geneva Lake.

Lake	Cu	Fe	Mn	Ni	Zn	Ca	K	Mg	Na
			(μg l^{-1})				(mg l^{-1})		
Geneva Mean	1.6	21.6	16.9	4.3	2.5	2.7	0.8	0.7	1.8
SEM	0.3	4.0	0.9	0.8	0.5	0.1	0.1	0.1	0.1
Ramsey Mean	8.6	38.4	14.0	32.5	1.7	16.8	1.7	5.3	55.9
SEM	1.5	3.0	0.5	2.0	0.1	0.3	0.2	0.3	2.0
Hannah Mean	13.7	84.3	12.4	48.2	4.2	10.0	1.5	3.6	50.8
SEM	2.3	3.0	0.6	2.0	1.2	0.1	0.1	0.1	0.1

Table 2.3. Ranges of water quality variables in Hannah Lake, Ramsey Lake, and Geneva Lake (n=3 per lake).

Lake	Temp (°C)	pH	Alkalinity	Hardness	DOC
			(mg l^{-1} as $CaCO_3$)	(mg l^{-1} as $CaCO_3$)	(mg l^{-1})
Geneva	25-26	6.85 – 6.98	20 – 22	9.28 – 9.61	4.2 – 5.7
Ramsey	25-26	7.74 – 7.87	35 – 40	62.54 – 65.28	3.9 – 4.7
Hannah	26-27	7.14 – 7.23	25 – 27	39.68 – 40.18	3.7 – 4.1

2.2.4. Experimental design

Yellow perch from Geneva Lake (clean lake), Ramsey Lake, and Hannah Lake (contaminated lakes) were held in their native lake water for 24 hours to acclimate to lab conditions. Baseline olfactory acuity was measured in fish held for 24 hours in their native lake water after the acclimation period. To determine the effect of contaminated lake water on olfactory acuity of fish from a clean lake, fish from Geneva Lake were held in water from Ramsey and Hannah lakes for 24 hours prior to measuring olfactory acuity using exposure water. To determine if recovery of olfactory acuity could be seen by holding fish from contaminated lakes in clean water, fish from Ramsey and Hannah lakes were held in Geneva Lake water for 24 hours and olfactory acuity was measured using Geneva Lake water. In all cases, olfactory acuity was measured using EOG (see below).

2.2.5. Electrophysiological responses

The methods used for EOG analysis were modified from those described by Mirza et al. (2009). Water used to irrigate the olfactory epithelium of each fish was the same as its exposure water. For this study, two different cues, L-alanine and taurocholic acid (TCA), were used. Evidence has demonstrated that amino acids (like L-alanine) and bile salts (like TCA) differently engage the subtypes of olfactory neurons, inducing specific responses in distinct areas of the bulbs (Hamdani and Døving 2007). By using both stimuli we can determine how each type of olfactory neuron responds. Solutions of L-alanine (10^{-3} M) and TCA (10^{-4} M) were made fresh each day in water from the same sources as the exposure

water used for each fish. Each stimulus was delivered at least three times to each fish, with a minimum of 2 minutes between deliveries of any given cue in order to mitigate potential olfactory attenuation. The response to the appropriate blank (i.e., the lake water used to dissolve each stimulus) was also measured. The order of the stimulus delivery (L-alanine, TCA, or a blank) was randomized to ensure there was no bias due to order of delivery.

The raw EOG amplitude for each stimulus delivery was determined by measuring the difference between the baseline EOG response and the maximum response to the stimulus. The raw EOG amplitudes measured to each of the stimuli for an individual fish were then averaged and corrected by subtracting the response elicited by the blank. These corrected values were then averaged across all fish of a specific exposure, such that all data represent mean blank-corrected EOG amplitudes.

2.2.6. Statistical analysis

All statistical analyses were performed using R, version 2.13.0 (R Development Core Team, 2011), with graphics made using the sciplot package (Morales et al., 2010). A fixed-effects ANOVA was used to determine if there were differences among the responses to each cue in fish from each lake exposed to their own lake water. A Dunnett's test was then performed to compare the responses of fish from the two metal contaminated lakes to the responses of fish from Geneva Lake. The same analysis (a fixed-effects ANOVA followed by a Dunnett's test) was performed to determine if there was a

significant difference between the responses of Geneva Lake fish exposed to water from all three lakes, with exposure to native water as the control. In addition, this analysis was used for a comparison between the response of fish from Hannah and Ramsey Lake held in Geneva Lake water with the response of Geneva Lake fish in their native water as the control. For experiments where fish from contaminated lakes (e.g., Hannah and Ramsey) were exposed to either their own water or Geneva Lake water, independent-samples t-tests were used to determine if there was a significant difference. For all analyses, significance was set at $\alpha = 0.05$. Parametric testing was performed prior to each analysis, with a $\log_{10}$ transformation being used to regain parametric assumptions, if needed.

2.3.   Results

2.3.1. Baseline response of fish from clean and metal contaminated lakes

Yellow perch from Ramsey Lake and Hannah Lake showed a lower EOG response to both cues when held in their own lake water relative to those from Geneva Lake held in their own lake water (L-alanine $F_{(2,7)}$ = 8.967, p < 0.02, Figure 2.2.A; TCA $F_{(2, 7)}$ = 8.037, p < 0.02, Figure 2.2.B). The response to 10^{-3} M L-alanine by fish from Ramsey Lake was 17% of the response from Geneva Lake fish, and the response of Hannah Lake fish was 28% of Geneva Lake fish. When 10^{-4} M TCA was used as the stimulus, the response of fish from Ramsey Lake
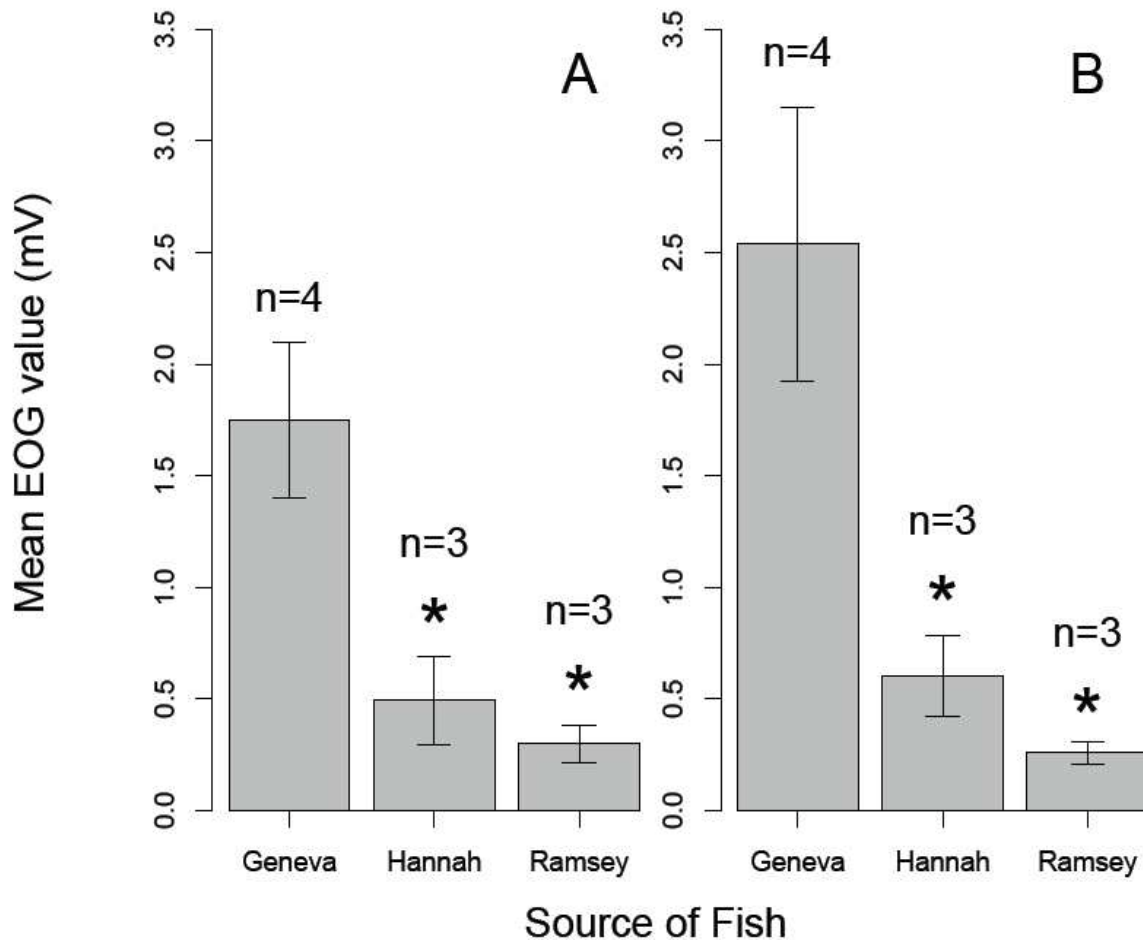
Figure 2.2. Comparison of the mean baseline EOG response (± SEM) to 10-3 M L-alanine (A), and 10-4 M TCA (B) of wild yellow perch from Geneva Lake (clean), Ramsey Lake (moderately contaminated), and Hannah Lake (contaminated). All fish were tested in their native lake water. An asterisk denotes a significant difference from Geneva Lake fish p ≤ 0.05.

was 10% of the response of Geneva Lake fish, and the response of Hannah Lake fish was 24% of the response of fish from Geneva Lake.

2.3.2. Decreased response of fish from clean lake in metal contaminated lake water

The EOG response of Geneva Lake fish to either cue was reduced when they were held in water from Ramsey Lake or Hannah Lake as compared to the response when the same fish were held in their native water (L-alanine $F_{(2,9)}$ = 8.857, $p < 0.01$, Fig. 2.3A; TCA $F_{(2,9)}$ = 6.235, $p < 0.02$, Fig. 2.3B). There was a reduction in EOG response to 10-3 M L-alanine of 75% when Geneva Lake fish were held in Ramsey Lake water, and a 59% decrease when they were held in Hannah Lake water. In response to 10^{-4} M TCA, fish from Geneva Lake held in Ramsey Lake water exhibited a 75% reduction in EOG response relative to those from Geneva Lake held in their own lake water. There was no significant difference detected between the response of Geneva Lake fish held in their source water and Hannah Lake water to 10^{-4} M TCA, even though there was an apparent 58% decrease in response due to the exposure with Hannah Lake water.

2.3.3. Increased response of fish from metal contaminated lakes in clean lake water

Yellow perch from Ramsey Lake held in Geneva Lake water for 24 hours had significantly increased EOG response to both cues, a 3.4 fold increase in response to 10^{-3} M L-alanine (t = -5.25, df = 3.68, $p < 0.008$, Figure 2.4A) and a
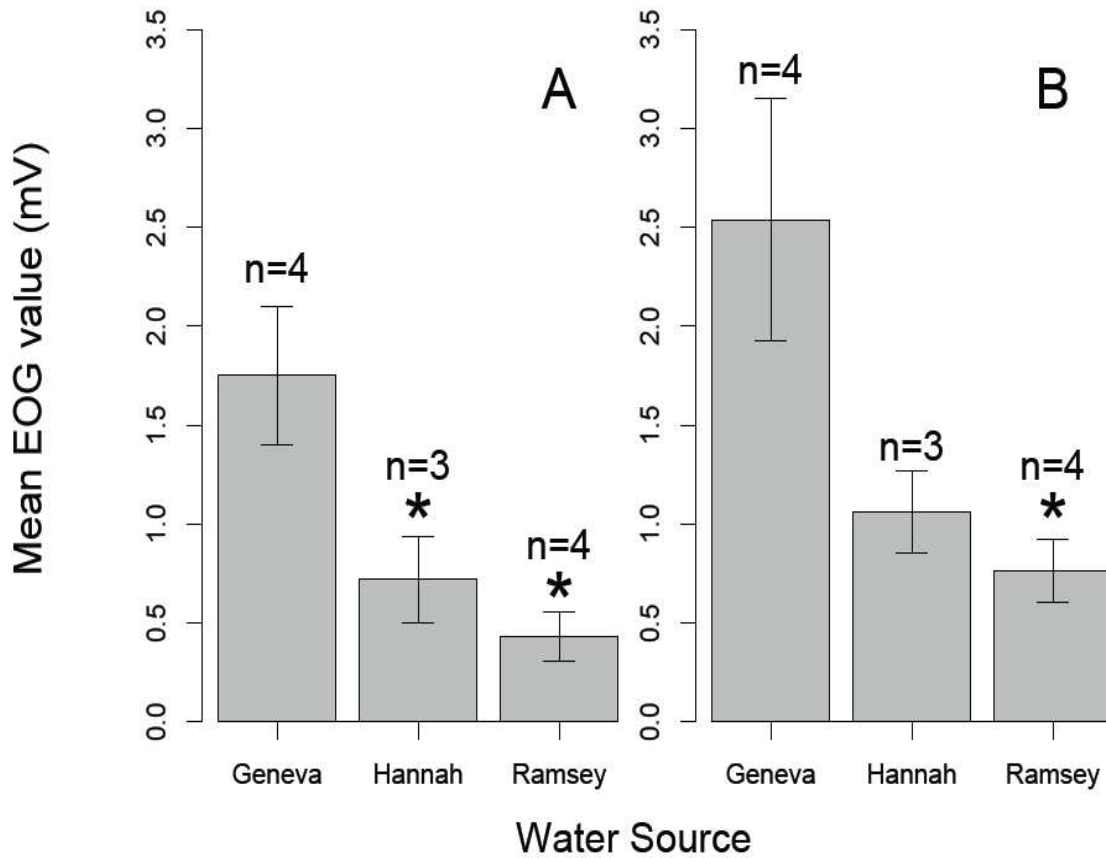
Figure 2.3. Mean EOG response (± SEM) of wild yellow perch from Geneva Lake to 10-3 M L-alanine (A) and 10-4 M TCA (B) after a 24 hours exposure to Geneva Lake (reference), Ramsey Lake (moderately contaminated) and Hannah Lake (contaminated) water. An asterisk denotes a significant difference from the response of Geneva Lake fish held in their own water, p ≤ 0.05.
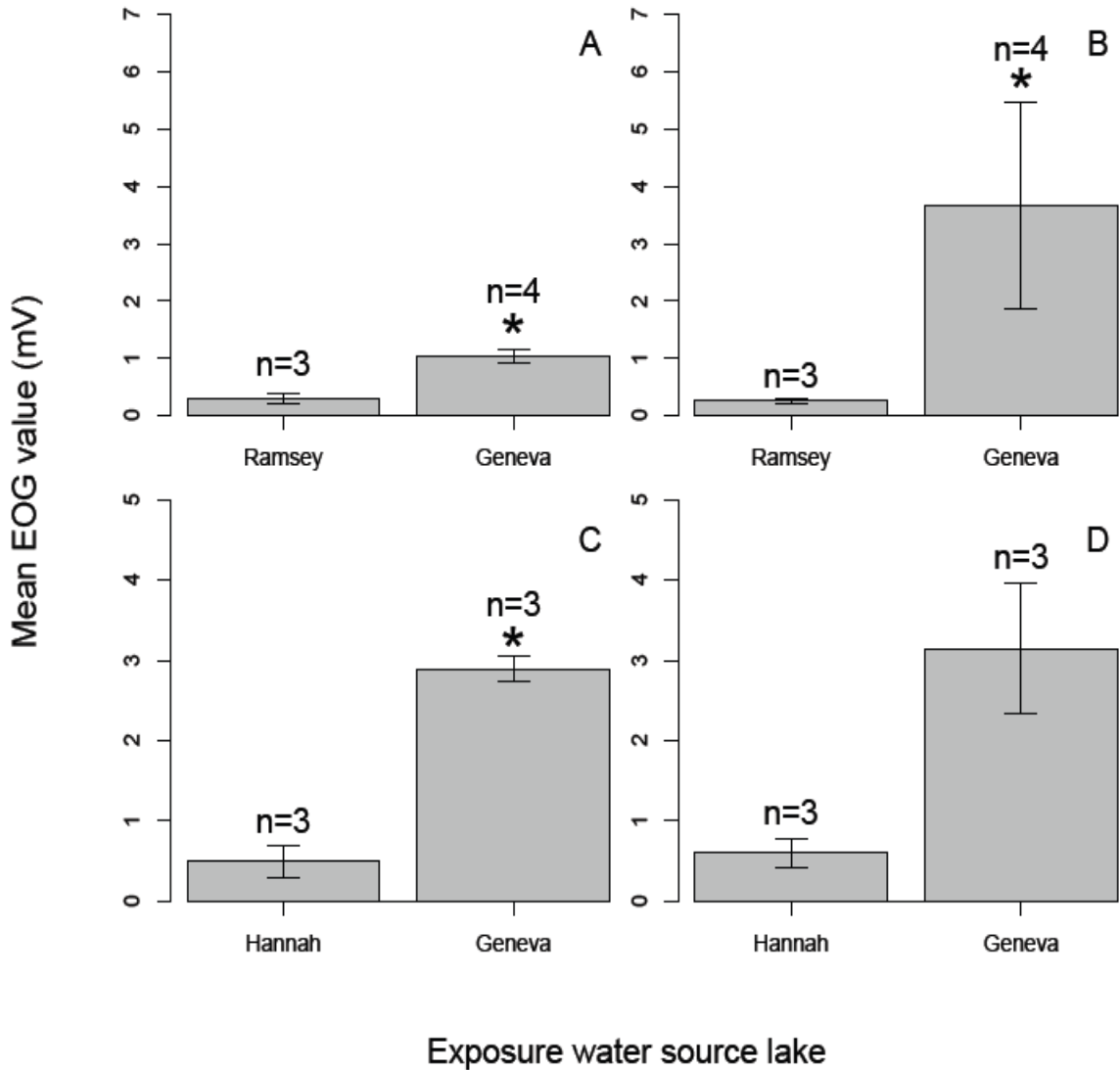
Figure 2.4. Mean EOG response (± SEM) of wild yellow perch from Ramsey Lake to 10-3 M L-alanine (A) and 10-4 M TCA (B) held in their source water or water from Geneva Lake, and response of perch from Hannah Lake to L-alanine (C) and TCA (D) held in their source water or water from Geneva Lake. An asterisk denotes a significant difference between the responses when they were exposed to their source lake water versus Geneva Lake water, $p \leq 0.05$.

14.2 fold increase in response to 10-4 M TCA (t = -4.25, df=2.74, p < 0.01, Figure 2.4B) compared to the response of the same fish held in their own lake water. Fish from Hannah Lake, however, only showed a significantly increased response by 5.8 fold to 10-3 M L-alanine (t = -9.45, df= 3.80, p < 0.001, Figure 2.4C) when held in Geneva lake water as compared to their source lake water. Even though the response to 10-4 M TCA was not statistically different (t = -3.06, df = 2.19, p = 0.082, Figure 2.4D), there was an apparent 5.2 fold increase in EOG response when Hannah Lake fish are held in Geneva Lake water as compared to water from their source lake.

A comparison of the response of yellow perch from all three lakes held in Geneva Lake water for 24 hours demonstrated that fish from Hannah Lake had a 2.4 fold higher response to L-alanine as did fish from Geneva Lake ($F_{(2, 7)}$ = 5.275, p<0.01 Figure 4.5A). There was no difference between the response to L-alanine with fish from Geneva Lake and Ramsey Lake. There was no significant difference detected in the response of fish from all three lakes held in Geneva Lake water to TCA ($F_{(2, 7)}$ = 2.247, p = 0.763 Figure 2.5B).

2.4.   Discussion

In contrast to previously reported results (Mirza et al., 2009), this study demonstrates that yellow perch from metal contaminated lakes show impaired EOG response relative to yellow perch from a clean lake. Water quality parameters of all three lakes are comparable (Table 2.3). Few studies have
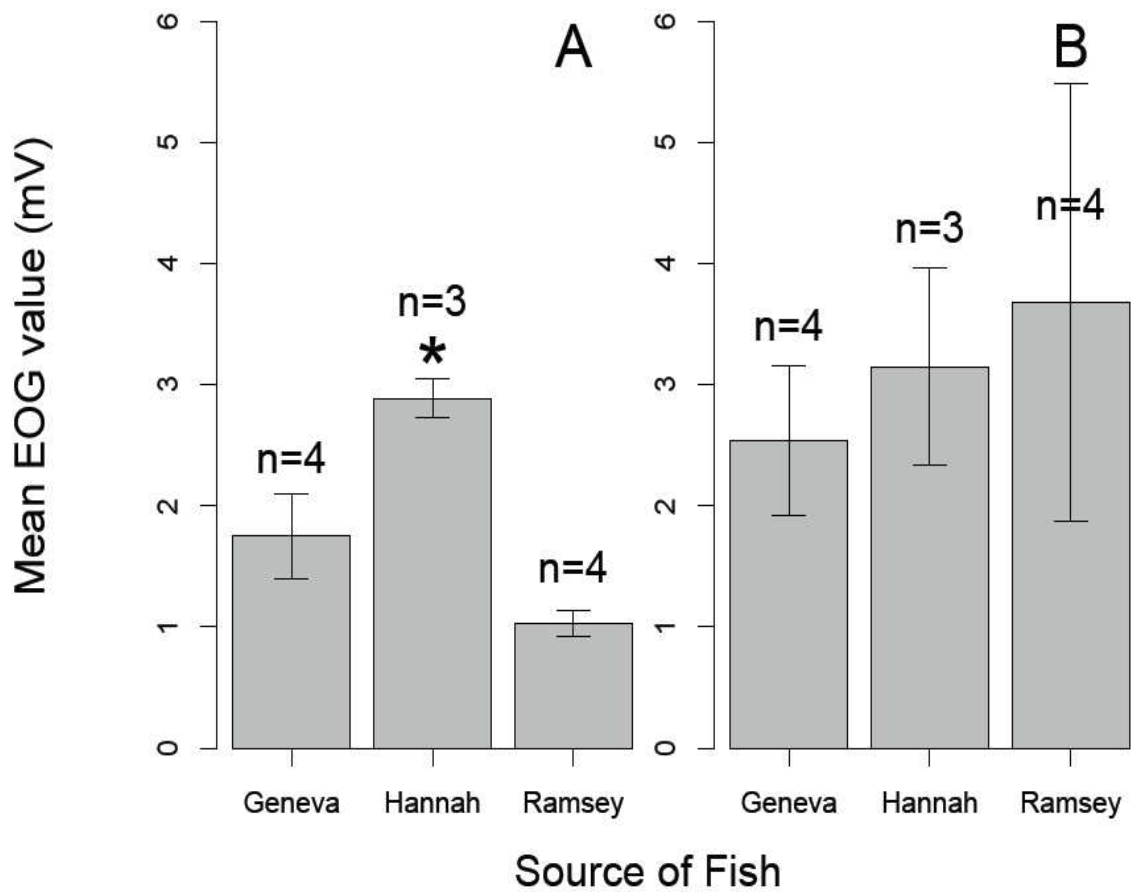
Figure 2.5. Mean EOG response (± SEM) of wild yellow perch from Geneva Lake (clean), Ramsey Lake (moderately contaminated), and Hannah Lake (contaminated)Ramsey Lake to 10-3 M L-alanine (A) and 10-4 M TCA (B) held in Geneva lake water, p ≤ 0.05.

compared olfactory acuity of wild fish from clean and metal contaminated lakes. In an in situ trap experiment, Iowa darters avoided antipredator cue in a clean lake but not in a metal-contaminated lake (McPherson et al., 2004). Behavioural responses of wild perch from clean and contaminated lakes showed a similar trend to that shown by Iowa darters, in that perch from a clean lake avoided an antipredator cue, while perch from contaminated lakes did not (Mirza et al., 2009).

In this study, exposing fish from a clean lake to water from contaminated lakes resulted in a significant reduction in olfactory acuity as measured by EOG. These exposures represent complex mixtures of contaminants, as the concentrations of numerous metals (Table 2.2) are elevated in the contaminated lakes relative to the clean lake. Several studies investigating single metal (such as copper) exposures show impairment of olfaction in a wide variety of fish species such as the pike minnow (*Ptychocheilus lucius*) (Beyers and Farmer 2001), coho salmon (*Oncorhynchus kisutch*) (Baldwin et al., 2003), chum salmon (*Oncorhynchus keta*) (Sandahl et al., 2006), goldfish (*Carassius auratus*) (Kolmakov et al., 2009) and fathead minnow (*Pimephales promelas*) (Green et al., 2010; Dew et al., 2012). To our knowledge, no studies have been performed to investigate the effect of a metal contaminated lake's water on the olfactory function of wild fish. Further work is required to elucidate how complex mixtures in metal contaminated lakes affect olfactory acuity and behavioural responses of fish.

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When fish from metal contaminated lakes were exposed to water from a clean lake for 24 hours, there was a dramatic increase in response to chemosensory cues as compared to fish from the same lake tested in native lake water. This increased response means that olfactory-impaired fish from metal contaminated lakes can quickly recover their ability to respond to cues once the contamination is removed. This recovery was seen for both the moderately contaminated (Ramsey) and contaminated (Hannah) lakes. Recovery of olfactory acuity after exposure to low, ecologically relevant concentrations of copper has been observed after the exposure has been removed in fathead minnows (Green et al., 2010), coho salmon (Baldwin et al., 2003), and chum salmon (Sandahl et al., 2006). Recovery from short-term exposure to higher concentrations of copper has been shown to require longer recovery periods before olfactory responses are fully restored in goldfish (Kolmakov et al., 2009). The need for a longer recovery period is most likely due to copper inducing damage in olfactory sensory neurons (OSNs) in the olfactory epithelium. Recovery after being exposed to high copper concentrations has been shown to occur in *Tilapia mariae* (Bettini et al., 2006), which recovered 10 days after copper was removed. Copper-induced cell death is most likely through an apoptotic mechanism owing to the demonstration of copper-induced apoptosis in rainbow trout OSNs (Julliard et al., 1996). Since recovery of the EOG response of yellow perch was within 24 hours in our study, it is likely that OSNs were not damaged by the mixture of metals in their environment, but instead impaired through a similar mechanism to the studies

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using ecologically-relevant copper concentrations detailed above (Baldwin et al., 2003; Sandahl et al., 2006; Green et al., 2010). An alternate explanation could be that only mature OSNs were affected by the metal exposure such that an intact stem cell layer could regenerate and replace the damaged OSNs.

Contaminated fish tested in clean water responded to both stimuli similarly to clean fish tested in clean water after a 24 h acclimation period, with one exception; Hannah Lake fish yielded a stronger response to L-alanine when tested in clean water than clean fish. This elevated response beyond that of the control fish reflects similar results reported by Mirza et al. (2009), where contaminated fish were tested in clean lab water and showed a stronger response to standard chemosensory stimuli than controls. Although the mechanism for this response is unknown, it may be an adaptive compensatory response by OSNs to maintain chemosensory function in the presence of neurotoxic environmental contaminants.

In all conditions, when either L-alanine or TCA were used, similar trends were observed. As L-alanine and TCA were used to measure different OSN subtypes, the similar trends demonstrate that the toxicity and recovery measured during the experiments was due to a general effect on the olfactory system, and not due to specific OSN subtypes being impaired. In addition, regardless of the source of the fish used, whenever fish were exposed to Geneva Lake water and the EOG response to TCA was measured, there was a high variability to the data. More work is needed to understand why this phenomenon occurred.

Olfactory recovery after chronic exposure to low, environmentally-relevant metal concentrations has not been investigated before this study. This chemosensory recovery after chronic metal exposure may account for the observed differences in the baseline responses in fish from clean and metal-contaminated lakes observed in this study, and those reported by Mirza et al. (2009). Their study used wild yellow perch, as did ours; however, their fish were acclimated to laboratory water and all experiments were performed using laboratory water (i.e., clean water). It is likely that their study showed a higher EOG response to different cues from fish from contaminated lakes because yellow perch from metal contaminated lakes were acclimated to clean water. This acclimation to clean water could have led to a recovery of olfactory response to cues in fish from metal contaminated lakes and may explain their results. Any future experiments involving olfaction of wild fish should be performed using water from their source habitats. Conducting experiments in native lake water also improves the ecological relevance of the results since lake water is their natural environment and reflects the water condition that fish deal with in their habitat. Furthermore, this observation demonstrates that olfactory impairment of fish from metal contaminated lakes can be reversed if the contamination is removed. This observation is important for lake remediation because it demonstrates that olfactory impairment of fish from metal contaminated lakes have the ability to recover once the lake recovers.

2.5. Conclusions

This study demonstrates that yellow perch inhabiting metal contaminated lakes have an impaired olfactory response to standard chemosensory cues compared to fish from a clean lake. In addition, exposure of fish from clean lake to a mixture of different contaminants in metal contaminated lake water for 24 hours water inhibits their olfactory function. Furthermore, fish that have spent their lives in metal contaminated lakes recover quickly (i.e., 24 hours) after being transferred to clean water. Taken together, there are two major implications from this study. First, when performing research on the olfactory response of wild fish it is essential that the experiments be conducted in source water. Second, fish in metal contaminated lakes have impaired olfactory acuity, however, this impairment can be reversed when the contaminants are removed. These outcomes could improve our ability to evaluate the ecological risk of low-level metal release to freshwater, which can eventually lead to improved environmental policies and guidelines that can effectively protect sensitive freshwater ecosystems.

3. CHAPTER 3: Recovery of olfactory mediated behaviours of fish from metal contaminated lakes

Abstract

Fish mediate many biological processes using olfaction (e.g., avoiding predators), and exposure to contaminants can impair these olfactory-mediated behaviours in fish. The recovery potential of olfaction in fish after exposure to contaminants has been investigated in a few studies. While the olfactory recovery of fish from metal contaminated lakes after exposure to clean water has been shown at the neurophysiological level, the recovery potential of olfactory mediated behaviours remains unknown. To study behavioural recovery of fish from metal contaminated lakes, wild yellow perch (*Perca flavescens*) were collected from two metal-contaminated lakes (Ramsey and Hannah lakes) in the metal-mining district of Sudbury, ON. Yellow perch collected from metal contaminated lakes were exposed to clean water from a reference lake (Geneva Lake) for 24 hours before conducting predator avoidance assays. The recovery of predator avoidance behaviour was tested using avoidance responses to conspecific skin extract (i.e., a common anti-predator cue). While olfactory mediated behaviours of fish from Ramsey Lake recovered after 24 hours of exposure to clean water, fish from Hannah Lake did not show recovery within 24 hours. These results demonstrate that although behavioural deficits of fish from metal contaminated lakes are recoverable, the recovery may not occur when fish are from habitats with high concentrations of metals.

Keywords: olfactory toxicity; behavioural deficits; olfactory recovery; yellow perch.

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3.1.   Introduction

Fish live in a world of scents and they use olfaction to mediate important activities, such as finding food, avoiding predators and reproducing. Therefore, olfactory impairment can have severe ecological consequences for fish. The olfactory system in fish is known to be sensitive to low concentrations of contaminants. Most of the olfactory toxicity studies to date have been conducted with short-term exposures to single contaminants under tightly controlled laboratory conditions, while in reality, fish may be exposed to a mixture of contaminants for their entire lives (Couture and Pyle, 2012). Thus, it is necessary to study fish from metal contaminated sources to understand the ecological implications of olfactory impairment.

The recovery potential of impaired olfaction after removing fish from a contaminated environment has been demonstrated in a few studies under laboratory settings (Baldwin et al., 2003; Bettini et al., 2006; Sandahl et al., 2006; Kolmakov et al., 2009; Green et al., 2010). Recently, our group investigated the recovery potential of fish from metal contaminated lakes around Sudbury, ON using electro-olfactography (EOG; Azizishirazi et al., 2013). We demonstrated that impaired olfaction in fish collected from metal contaminated lakes recovers at the neurophysiological level after 24 hours in clean water. However, it is still unknown if rapid neurophysiological recovery translates into rapid behavioural recovery.

To test if metal-exposed yellow perch (*Perca flavescens*) recover their ability to detect and respond to predators via olfaction, yellow perch were collected from two metal contaminated lakes in the Sudbury region and kept in either their own lake water or water from a clean lake for 24 hours. The intactness of olfactory mediated predator avoidance was tested using the natural avoidance response of yellow perch to conspecific skin extract.

3.2. Materials and Methods

3.2.1. Fish collection, maintenance, and exposure

All experiments were conducted in accordance with the guidelines of the Canadian Council of Animal Care. In May 2013, fish were collected from Ramsey Lake (46°28'48"N 80°56'32"W) and Hannah Lake (46°26'37"N 81°02'17"W) using a seine net (Azizishirazi et al., 2013). Both Ramsey and Hannah lakes are situated in Sudbury, ON. and known to be metal contaminated for years (Unit, C. F. E., 2004; Azizishirazi et al., 2013). After seining, 30 fish were randomly selected and the rest were released. Collected fish were transported back to Cooperative Freshwater Ecology Unit, Sudbury, and acclimated to laboratory conditions in their native lake water for 24 hours. Water was changed every 12 hours using fresh water from each lake. After the acclimation period, fish were kept for another 24 hours in their native lake water or Geneva Lake water (Azizishirazi et al., 2013) based on the experimental design (exposure time). The temperature of the holding water was 18 ± 1°C and the photoperiod was 16:8 h light:dark. Fish were not fed during the acclimation, exposure or experiment.

3.2.2. Water sampling

On site temperature and pH was measured with a YSI 6600 V2 multiparameter sonde (YSI Inc, Yellow Springs, Ohio). Water samples were collected at the same locations as fish collection sites (Azizishirazi et al., 2013). For dissolved metal concentrations, water samples were filtered using a 0.45 µm syringe filter and stored in 50 mL tubes. At the laboratory, samples were acidified with 200 µL of trace metals grade high purity nitric acid (Fisher Scientific, Nepean, ON) and stored at 4°C until analysed. Dissolved metal concentrations were measured via inductively coupled plasma atomic emission spectrometry (ICP-AES) by the Lakehead University Instrumentation Laboratory, (LUIL) Thunder Bay, ON, Canada (Table 3.1). To measure dissolved organic carbon (DOC), samples taken from the lakes were refrigerated at 4°C for five days until DOC was measured by the Lakehead University Centre for Analytical Services (LUCAS), Thunder Bay, ON. Alkalinity and hardness were measured as previously described (Pyle et al., 2005) (Table 3.1). The Canadian Association for Laboratory Accreditation (CALA) accredited both LUIL and LUCAS and all QA/QC procedures followed internal standard operating procedures of the LUCAS and LUIL. Alkalinity and hardness was measured as previously described (Pyle et al., 2005).

Table 3.1. Concentrations of dissolved metals and cations as well as water quality parameters in water samples (n=3) from lakes of study.

Source		Cu	Fe	Mn	Ni	Zn	Ca	K	Mg	Na
		$(\mu g\ L^{-1})$					$(mg\ L^{-1})$			
Geneva Lake	Mean	0.9	24.1	12.9	1.3	1.4	2.7	0.3	0.6	0.8
	SEM	0.2	2.2	0.2	0.2	0.3	0.1	0.1	0.1	0.1
Ramsey Lake	Mean	8.1	12.9	5.3	29.5	2.3	15.2	1.4	4.6	49.3
	SEM	0.1	0.8	0.3	0.3	0.3	0.1	0.2	0.5	2.7
Hannah Lake	Mean	14.4	22.2	5.1	45.4	1.8	8.9	1.4	3.2	46.5
	SEM	0.2	1.3	0.2	2.1	0.2	0.2	0.1	0.2	1.9

Source	Temp	pH	Alkalinity	Hardness	DOC
	(°C)			$(mg\ L^{-1}$ as CaCO3)	
Geneva Lake	18-20	6.82 - 6.97	4.5 – 4.7	8.5 – 9.2	3.7 – 3.8
Ramsey Lake	19-21	6.65 – 6.73	18.2–19.3	54.5 – 59.2	4.0 – 4.2
Hannah Lake	17-19	6.89 – 7.10	14.7– 14.9	34.1– 36.7	3.5 – 3.6

3.2.3. Behavioural experiments

Behavioural experiments were conducted as previously described by Azizishirazi et al. (2014) using a behavioural choice assay with two arms, one containing a blank cue, the other containing a conspecific skin extract cue which serves as an alarm cue. Exposure water was used for the behavioural trials and for making both the control and experimental alarm cues used in the behavioural assays.

3.2.4. Statistical analysis

Behavioural data were analyzed using R, version 3.0.3 (R Development Core Team, 2012). To determine if there was a difference between time spent in the stimulus arm versus time spent in the blank arm in each experiment, a paired t-test was used. The significance level, alpha, was set *a priori* to 0.05.

3.3.   Results

Yellow perch from Ramsey Lake and Hannah Lake did not show any preference for either arm of the maze containing blank or alarm cue (Ramsey Lake fish: $t_8 = 0.994$, p=0.34; Hannah Lake fish: $t_8 = 1.314$, p=0.22; Fig. 3.1). When given the choice, fish from Ramsey Lake held in Geneva Lake water avoided the alarm cue and spent 2.7 fold more time in the blank arm ($t_{10} = 3.243$, p<0.01; Fig. 3.1). Hannah Lake fish held in Geneva Lake water did not show any preference between the blank and alarm cue ($t_{10} = 0.698$, p= 0.50; Fig. 3.1).
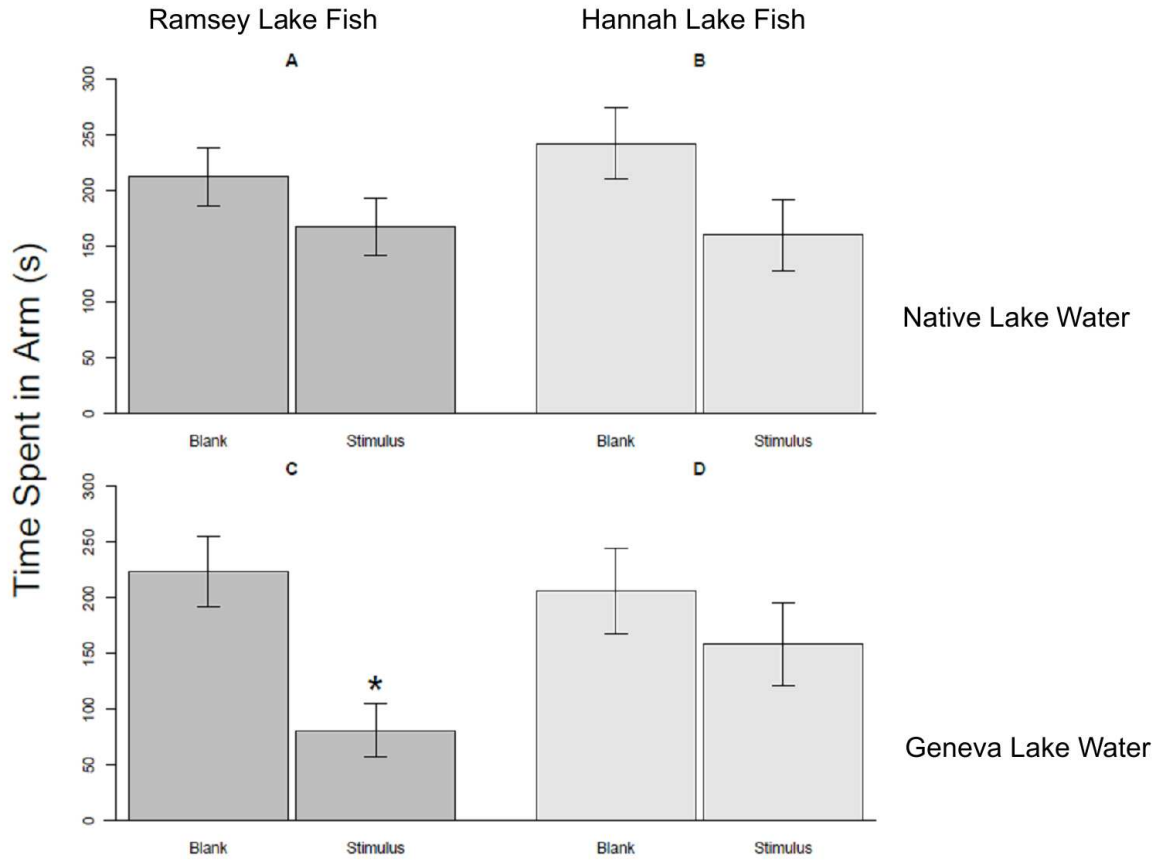
Figure 3.1. Mean time spent by fish from Ramsey (A; n=9) and Hannah (B; n=9) lakes, held in their own native lake water during the exposure time, and time spent by fish from Ramsey (C; n=11) and Hannah (D; n=11) lakes, held in Geneva Lake water during the exposure in either stimulus or blank arms (± SEM). An asterisk denotes a significant difference between the time spent in each arm at α = 0.05.

3.4.   Discussion

The current study demonstrates that impaired olfactory-mediated behaviours of fish from a moderately metal contaminated lake (Ramsey Lake) recovers after 24 hours of being kept in clean water. However, olfactory mediated behaviours of fish from a metal contaminated lake (Hannah Lake) do not recover after being kept for 24 hours in clean water. It is very likely that because the copper concentration is relatively low in Ramsey Lake water, the olfactory impairment recovers in a short period of time (i.e., 24 hours; Table 3.1). However, because the copper concentration is relatively higher in Hannah Lake water compared to Ramsey Lake (Table 3.1), impaired olfactory related predator avoidance behaviour did not recover following the transplantation of fish into clean lake water. In a recent study we investigated the olfactory recovery potential of fish from the same metal contaminated lakes (Ramsey and Hannah lakes) at the neurophysiological level using EOG responses to two different cues (Azizishirazi et al., 2013). When taurocholic acid (TCA) was used as the chemosensory stimulus, fish from Ramsey Lake showed recovery, whereas fish from Hannah Lake did not recover (Azizishirazi et al., 2013). Taking the current study and our previous 2013 study, we demonstrated that both EOG response to TCA and behavioural anti-predator responses recovered in fish from Ramsey Lake, whereas neither EOG nor behavioural responses recovered in fish from Hannah Lake. It has been previously demonstrated that TCA evokes an EOG response in a subtype of olfactory sensory neurons that are known to be

responsible for anti-predator behaviours (Dew et al., 2014). Copper specifically damages ciliated cells, which are responsible for both the EOG response to TCA and the behavioural anti-predator response (Dew et al., 2014). Thus, it can be concluded that because of the higher concentration of copper in Hannah Lake water compared to Ramsey Lake water, the damage to ciliated cells is more serious in Hannah Lake than in Ramsey Lake. It has been previously demonstrated that when the concentration of contaminants increased the damage to olfactory tissues were more severe (Bettini et al., 2006; Sandahl et al., 2006; Dew et al., 2012). It is possible that because of greater damage in olfactory tissues of fish from Hannah Lake compared top fish from Ramsey Lake their olfactory acuity needs a longer time to recover.

Mirza et al. (2009) did not observe any behavioural recovery in yellow perch from either Ramsey or Hannah lakes; however, the results from this study still corroborate their findings. The reported concentrations of copper were higher in both Ramsey Lake and Hannah Lake during the time of Mirza et al.'s sampling in 2005 compared to our copper measurements in 2013 (i.e., Ramsey Lake: 14 μg/L versus 8.1 μg/L; Hannah Lake: 39 μg/L versus 14.4 μg/L). Nonetheless, the concentration of copper in Ramsey Lake in 2005 was very close to the concentration of copper at Hannah Lake at 2013. Therefore, it is very likely that the behavioural olfactory recovery did not happen in Ramsey Lake fish because the high concentration of copper in Ramsey Lake water in 2005.

3.5. Conclusions

In conclusion, fish from moderately metal contaminated lakes with impaired olfactory mediated behaviours can recover their olfactory function after being held in clean water for 24 hours. However, the recovery of olfactory mediated behaviours may not occur when the concentrations of metals to which they have been exposed in their habitat is higher.

4. CHAPTER 4: Chemosensory mediated behaviours and gene transcription profiles in wild yellow perch (*Perca flavescens*) from metal contaminated lakes

Abstract

The olfactory system of fish is sensitive to the toxic effects of low concentrations of contaminants. To investigate the effects of long-term metal exposure on olfaction in wild yellow perch (*Perca flavescens*), fish from one clean (Geneva Lake) and two metal-contaminated lakes (Ramsey and Hannah lakes) were collected in and around the metal-mining district of Sudbury, ON. Two different techniques were used to measure the effects of exposure to environmental contamination: (i) behavioural responses were recorded in response to conspecific skin extract and (ii) gene transcription differences in olfactory rosettes were characterized using a novel, 1000-candidate gene yellow perch microarray. Behavioural assays performed on fish from the clean lake demonstrated avoidance of a conspecific skin extract, while fish from metal contaminated lakes showed no avoidance response. A total of 109 out of the 1000 genes were differentially transcribed among the lakes. Most of the differentially transcribed genes were between the two metal contaminated lakes relative to either of the contaminated lakes and the reference lake. No genes were differentially expressed between Geneva Lake (clean) and Hannah Lake (metal contaminated). These results demonstrated that even though the different populations of fish from both Hannah and Ramey lakes were affected at the behavioural level, the impairment of olfaction was not measurable using gene transcriptional changes in olfactory rosettes.

Keywords: olfactory toxicity; behavioural deficits; gene transcription; microarray; yellow perch.

4.1.   Introduction

Traditional studies designed to characterize metal toxicity in fish have relied on single-metal exposures to model species under the tightly controlled conditions of the laboratory (Wood et al., 2012a, 2012b). These studies have provided a wealth of information about the basic biology of metal exposures, including important routes of uptake, preferential tissue accumulation, and modes of toxicity. Moreover, many of the effects revealed by these studies have been induced only after animals have been exposed to relatively high contaminant concentrations, usually in exposure water having comparatively simple water chemistry relative to natural waters. Much less work has been done to characterize the subtle effects of low, environmentally relevant metal concentrations in wild fish adapted to either clean or contaminated waters (Couture and Pyle, 2012).

Recently, considerable research attention has been directed towards understanding how dissolved contaminants affect chemosensation and chemical communication in fish (Carreau and Pyle, 2005; Bettini et al., 2006; Sandahl et al., 2006; Blechinger et al., 2007; Kolmakov et al., 2009; Tierney et al., 2010; Dew et al., 2012). Aquatic animals rely on important chemicals in the water that inform about the location of food, the risk of predation, or the reproductive status of potential mates (among others) (Hamdani and Døving, 2007). In other words, information conveyed through chemical communication is essential for maintaining healthy populations. Anything that could disrupt the perception of

these important chemical cues, such as environmental contamination, has the potential to cause a significant ecological perturbation by interfering with an animal's ability to find food, avoid predators, or reproduce (Lürling and Scheffer, 2007). Detecting the presence of a predator is vital for any prey and aquatic organisms are known to use olfaction to evaluate the local risk of predation (Kats and Dill, 1998). Several studies have investigated the fright response in aquatic organisms and fishes can recognize the presence of a predator through various cues including conspecific skin extract (Brown, 2003). Impaired anti-predator responses could reduce the ability of fish to detect predators and decrease their chance of survival, which could in turn change predator–prey dynamics to benefit predators, and at a population scale would likely cause changes to the whole ecosystem.

Gene transcription is a sensitive indicator of contaminant exposure, and contaminant-induced gene transcription changes have been measured to determine what effect contaminant exposure will have on an organism (Hogstrand et al., 2002; Snape et al., 2004; Lettieri, 2006; Reynders et al., 2006; Sheader et al., 2006; Moens et al., 2007; Craig et al., 2010). Using microarrays to study sublethal toxicity allows for the analysis of several physiological pathways simultaneously to highlight those pathways that are most sensitive to site-specific contamination. In addition, physiological pathways that may not have been considered to be at risk from environmental contamination could also be affected by the environmental contamination (Denslow et al., 2007). In the only two

studies using microarray technology to characterize the effects of a contaminant (in this case copper and or/chlorpyrifos) on gene transcription in olfactory tissues of fish, Tilton et al. (2008, 2011) demonstrated that zebrafish (*Danio rerio*) exposed to copper and/or chlorpyrifos for < 24 h showed significant differences of gene transcription in pooled olfactory tissues. For these studies, olfactory rosettes, telencephalon, and the underlying olfactory bulb were pooled and a commercial zebrafish microarray containing 14,900 transcripts was used to measure gene transcription patterns associated with contaminant exposure. Tilton et al. (2008) showed that copper caused an under-transcription of key genes associated with the olfactory signal transduction pathway such as calcium channels, G-proteins, and olfactory receptors. In 2011 they exposed fish to copper, chlorpyrifos and mixtures of both and found that copper and chlorpyrifos cause their own transcriptional signatures (Tilton et al., 2011). However, the transcriptional signature of the contaminant mixtures was more similar to that in zebrafish exposed to copper (Tilton et al., 2011). One question that remains from that work is whether or not those same gene transcription patterns can be observed in wild fish populations where long-term metal exposure has led to impaired chemosensory function.

The industrial region of Sudbury, ON, Canada provides an excellent opportunity to study metal-impaired chemical communication in wild yellow perch (*Perca flavescens*) populations (Pyle et al., 2005). Sudbury is a top nickel-producing region in the world (Chau and Kulikovsky-Cordeiro 1995). Mining has

taken place in the region since the late 1800s which has resulted in acidification and metal contamination in over 7,000 lakes in a 17,000 ha industrial 'zone of impact' (Keller et al., 1992). The dominant fish species is yellow perch, mainly because of its acid tolerance (Freda and McDonald, 1988) and well-documented ability to tolerate dissolved metals at concentrations elevated significantly above background concentrations (Taylor et al., 2003). Recent studies have demonstrated that yellow perch from metal-contaminated lakes in the Sudbury area have impaired chemosensory function when presented with pure olfactory chemicals (such as amino acids) or natural chemosensory cues (such as conspecific skin extracts containing chemical alarm cues) (Mirza et al., 2009; Azizishirazi et al., 2013). Recently, a novel 1000 candidate-gene yellow perch microarray was developed as a tool for the detection of metal-induced stress and to identify the different mechanisms of sublethal metal toxicity in yellow perch (Bougas et al., 2013). The microarray contains genes associated with a wide variety of cellular process including genes associated with olfaction. The 1000 candidate-gene microarray revealed different mechanisms of the sublethal effects of nickel and/or cadmium in livers of yellow perch after 45 days of exposure to environmentally relevant concentrations (Bougas et al., 2013).

The objective of this study was to determine if behavioural deficits induced by metal-impaired chemosensory function in wild yellow perch from metal contaminated lakes are linked to gene transcription patterns in olfactory tissues. Such a link could provide insights into the mechanism(s) of toxic action related to

metal-impaired olfaction in wild fish populations. To investigate this question, we collected wild yellow perch from a clean lake and two metal-contaminated lakes in the Sudbury region. The natural avoidance response of yellow perch to conspecific skin extract was used to test the olfactory acuity of fish from all lakes. The endpoints used in this experiment were fleeing and avoiding an olfactory-labeled "high risk zone" of a choice maze. Gene transcription patterns of the most exposed olfactory tissue, the olfactory rosette, were examined using the yellow perch microarray, and these expression patterns were analysed relative to behavioural responses.

4.2.   Materials and Methods

4.2.1. Water sampling

Temperature and pH were measured on site using a YSI 6600 V2 multiparameter sonde (YSI Inc, Yellow Springs, Ohio). Water samples were collected from Hannah Lake, Ramsey Lake, and Geneva Lake (Azizishirazi et al., 2013) in close proximity to where fish were collected. Samples were stored in 50 mL tubes and capped under water to decrease the headspace, and were split into three groups for subsequent analysis. Total dissolved metal concentrations were determined in 50 mL water samples acidified with 200 µL of trace metals grade high purity nitric acid (Fisher Scientific, Nepean, ON) passed through a 0.45 µm syringe filter. After acidification and filtration, samples were stored at 4°C until analyzed via inductively coupled plasma atomic emission spectroscopy (ICP-AES) by the Lakehead University Instrumentation Laboratory, Thunder Bay,

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ON, Canada for metal concentrations (Table 4.1). Dissolved organic carbon (DOC) concentration was measured by the Lakehead University Centre for Analytical Services using a San^{++} Automated Wet Chemistry Analyzer (SKALAR, Breda, the Netherlands). Alkalinity and hardness were measured as previously described (Pyle et al., 2005).

4.2.2. Fish Collection

All experiments were conducted in accordance with the guidelines of the Canadian Council of Animal Care. Fish were collected from Geneva Lake, Ramsey Lake, and Hannah Lake using seine nets and angling in June 2011 for use in gene transcription experiments, and in June 2012 for behavioural experiments. At each collection site, 20 fish for behavioural experiments and 12 fish for the gene expression experiment were randomly selected. Randomly-selected fish were transported to Laurentian University, Sudbury, ON, in aerated native lake water for gene expression and behavioural experiments.

4.2.3. Behavioural assessment

4.2.3.1.   Maintenance of the fish

Fish collected from each of the three lakes were kept in their native lake water in 30 L plastic tanks for 24 hours to acclimate to laboratory conditions. Each tank was aerated and water was changed every 12 hours using fresh water

Table 4.1. Concentrations of dissolved metals and cations as well as water quality parameters in water samples (n=3 per lake) from Hannah, Ramsey, and Geneva lakes in June 2011 and June 2012.

Lake		Cu	Fe	Mn	Ni	Zn	Ca	K	Mg	Na
		(μg L^{-1})					
Geneva	Mean	0.9	24.8	10.0	4.0	2.5	2.7	0.4	0.7	0.9
(2011)	SEM	0.2	2.0	0.5	0.9	0.5	0.2	0.1	0.1	0.1
Ramsey	Mean	12.6	124.4	33.0	44.5	5.1	16.5	1.5	4.9	52.8
(2011)	SEM	0.5	10.0	2.5	3.0	1.9	0.7	0.1	0.2	3.0
Hannah	Mean	17.7	81.3	19.5	70.1	4.2	11.5	1.6	3.7	54.9
(2011)	SEM	1.5	4.0	2.3	3.5	0.8	0.7	0.2	0.4	3.5
Geneva	Mean	0.7	21.6	16.9	4.1	2.5	2.5	0.4	0.6	0.8
(2012)	SEM	0.1	4.0	0.9	0.5	0.5	0.3	0.1	0.1	0.1
Ramsey	Mean	7.9	11.9	12.0	30.1	7.8	14.8	1.4	4.4	47.5
(2012)	SEM	0.8	3.0	0.6	1.9	0.9	0.8	0.1	0.3	2.0
Hannah	Mean	11.5	16.3	7.4	44.2	4.2	8.8	1.4	4.4	47.5
(2012)	SEM	0.6	2.0	0.3	5.0	0.9	0.7	0.1	0.3	2.0

Lake	Temp (°C)	pH	Alkalinity (mg L^{-1} as CaCO$_3$)	Hardness (mg L^{-1} as CaCO$_3$)	DOC (mg L^{-1})
Geneva (2011)	22-23	6.8 – 6.9	10 – 12	8.7 – 10.5	3.8 – 3.9
Ramsey (2011)	22-24	7.8 – 7.9	25 – 28	58.8 – 63.9	3.9 – 4.2
Hannah (2011)	23-25	7.2 – 7.4	16 –18	40.5 – 47.3	3.5 – 3.7
Geneva (2012)	22-24	6.7 – 6.8	8 –10	7.5 – 9.9	3.7 – 3.9
Ramsey (2012)	24-25	7.9 – 8.1	17 –19	51.8 – 58.3	4.0 – 4.1
Hannah (2012)	25-26	6.7 – 6.8	14 –15	32.1 –37.7	3.6 – 3.8

from each lake. The temperature of the holding water was 25 ± 1°C and the photoperiod was 16:8 light:dark.

4.2.3.2. Experimental design

Conspecific skin extract was made fresh prior to each behaviour trial. Donor fish were sacrificed with a sharp blow to the head. Skin was removed from both sides of two yellow perch from Geneva, Hannah, and Ramsey lakes. In three separate watch glasses (one per source of fish), 10 ± 0.5 cm^2 of skin was chopped using fine dissecting scissors and a scalpel. Native lake water (1 L) was used to dilute the skin extract to a concentration of 10 cm^2/L. The solution was mixed for 5 minutes and allowed to settle for 5 minutes, after which the top 800 mL was poured in 50 mL plastic tubes. Troughs measuring 70 cm x 20 cm x 15 cm (L x W x H) were used as behaviour mazes for this experiment. Lines were drawn on the edge of the trough (visible from the top) to divide the maze into three zones. Each distal segment of the maze was 27 cm long with a middle zone of 15 cm. Mazes were filled using 8 L of native lake water for the fish to be tested. Fish were randomly assigned to the mazes and allowed to acclimate to the trough for 20 minutes prior to delivering the stimulus and blank. After the acclimation period, 50 mL of stimulus (conspecific skin extract) or blank (native lake water) was delivered to either end of the maze using syringes and aeration tubing from behind a curtain. The end of the trough maze receiving either the stimulus or blank was randomized for each behavioural trial. After allowing 2 minutes for the cue to diffuse throughout the maze, fish movement was recorded

for 8 minutes using a Logitech C615 high definition web camera (Logitech, Mississauga, ON). All videos were scored by an observer for fish position in the maze in 10 s intervals and the number of entries into each end of the maze. To reduce bias the observer was blind to the position of the stimulus or blank.

4.2.3.3.   Statistical analysis

Behavioural data were analyzed using R, version 2.15.2 (R Development Core Team, 2012). Two separate analyses were performed on the behavioural data, the time spent in the stimulus zone versus the time spent in the blank zone, and the number of entries into the stimulus zone. A paired t-test was used to determine if there was a difference between time spent in the stimulus versus time spent in the blank zone for fish from each lake. The number of entries into the stimulus zone was compared between fish from Geneva Lake and either of the other two lakes using two Student's t-tests. Prior to the Student's t-tests an outlier was identified in the data for Geneva Lake using a Grubb's test and removed. The significance level, alpha, was set a priori to 0.05. However, p-values were adjusted using a Holm's correction to compensate for an increased probability of committing a Type 1 statistical error owing to serial hypothesis testing.

4.2.4. Gene transcription

4.2.4.1. Total RNA Extraction

Randomly selected fish were euthanized in a 2 L bath of 200 mg/L MS-222, (Syndel Laboratories Ltd, Nanaimo, BC) buffered with sodium bicarbonate (Fisher Scientific, Nepean, ON) within 10 minutes of arriving at the laboratory. Sacrificed fish from each lake were divided into 4 groups, and olfactory rosettes of all three fish in each group were collected and pooled together. Skin covering both nares of each perch (located dorsally between the eyes and snout of the fish) were removed using a scalpel and forceps (Fig. 4.1.A). The olfactory rosette was located in each olfactory chamber, grasped with forceps, and the underlying connective tissue and olfactory nerve were cut with a scalpel. The olfactory rosettes were removed and stored in *RNAlater®* Solution (QIAGEN Inc, Toronto, ON) in 1.5 mL tubes, refrigerated overnight, and then stored at -80°C until RNA was extracted. Prior to RNA extraction, all six rosettes were thawed, disrupted and homogenized together using a motorized homogenizer (Silentcrusher M, Heidolph, Elk Grove Village, IL). Total RNA was extracted using the PureLink® RNA Mini Kit (Life Technologies Inc, Burlington, ON) according to the manufacturer's instructions. Extracted RNA was stored at -80°C until used.

4.2.4.2. Labeling and cDNA hybridization

The quality and integrity of the total RNA was checked using an Experion Automated Electrophoresis Station and RNA HighSens Chips (Bio-Rad, Hercules, CA). For each sample, 1.5 µg total RNA was retro-transcribed and the
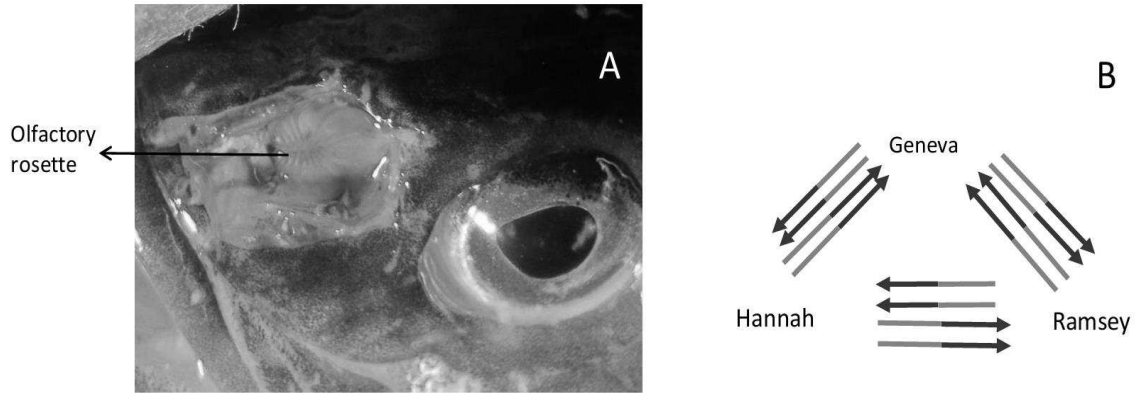
Figure 4.1.A. Dissection of an olfactory rosette from yellow perch. B. Experimental design of the microarray experiment. Each arrow represents a microarray, the black end of which represents red fluorescent dye, with the gray end representing green fluorescent dye.

cDNA samples were labeled using the Genisphere 3DNA Array 350 Kit, Invitrogen's Superscript II retro-transcriptase, and *Cyanine3* and *Cyanine5* fluorescent dyes (Genisphere), following the procedures described at http://genisphere.com/products/3dna-array-detection/support (Genisphere Array 350 Protocol). Four samples from each lake were compared and analyzed through a loop design (Fig. 4.1.B) for a total of 12 microarrays. The loop design included pair-wise direct comparisons among samples of the different lakes. Each pooled sample was technically replicated on two bi-coloured microarrays and dye-swapped.

4.2.4.3. Data acquisition, preparation, and statistical analysis

Scan, localization and quantification of the spots, and data analysis were conducted as described by Bougas et al. (2013). Using a mixed model ANOVA, we tested for the presence of significant lake effects (Geneva, Hannah, and Ramsey) with the "Array" term included as a random effect, and "Dye" and "Lake" included as fixed effects (Fs, with 1000 sample ID permutations). A False Discovery Rate correction (FDR = 0.1) was applied within the R / MAANOVA package to reduce the Type I error rate associated with multiple comparisons and the corrected p-values were used to determine the significance of differential gene transcription levels.

A posteriori tests were conducted with 1000 permutations for the three possible comparisons to generate a list of genes with significant differential expression among the different lakes (FDR = 0.1).

4.2.4.4. Functional classification

Gene ontology (GO) and assessment of significant differential representation of functional classes was performed in the Blast2Go software as described by Bougas et al. (2013).

4.3. Results

4.3.1. Behavioural experiment

When given the choice between a zone of a maze containing a blank cue or an alarm cue, yellow perch collected from Geneva Lake actively avoided the alarm cue by spending 6.8 fold more time in the zone containing the blank relative to the zone containing the alarm cue (t_7=4.56, p<0.01; Fig. 4.2), while yellow perch from Ramsey Lake and Hannah Lake showed no preference for either zone (t_{10}=1.24, p=0.36 and t_7=-0.31, p=0.76, respectively; Fig. 4.2).

Fish from Ramsey Lake entered the zone containing alarm cue 3-fold more frequently than fish from Geneva Lake (t_{16}=-3.14, p<0.01; Fig. 4.3), while fish from Hannah Lake entered the alarm cue containing zone 4 times more frequently than fish from Geneva Lake ($t_{9.7}$=-3.02, p<0.02; Fig. 4.3).
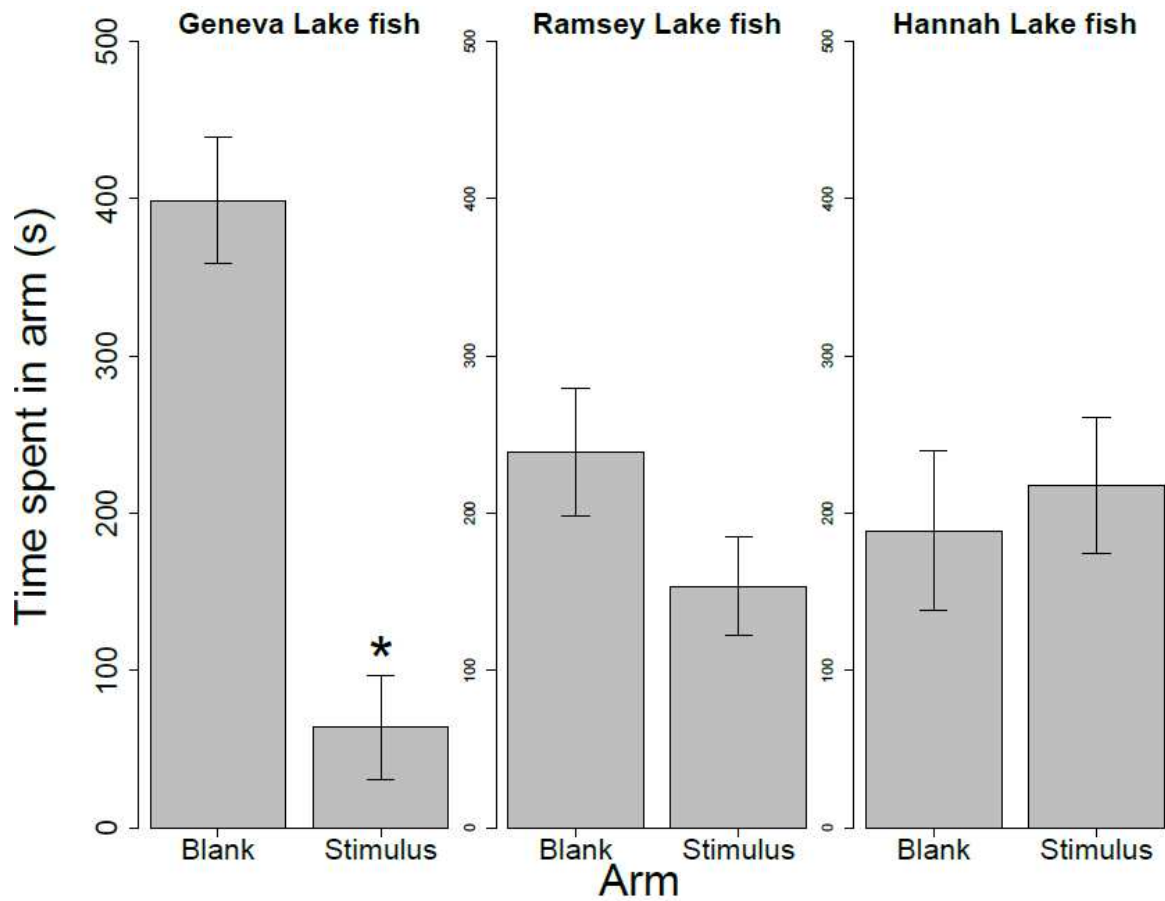
Figure 4.2. Mean time spent by fish from Geneva (n=9), Ramsey (n=11) and Hannah (n=9) lakes in either stimulus or blank zone (± SEM). All trials were conducted in native lake water. An asterisk denotes a significant difference between the time spent in zone receiving the stimulus (conspecific alarm cue) relative to the zone receiving the blank (native lake water), p ≤ 0.05.
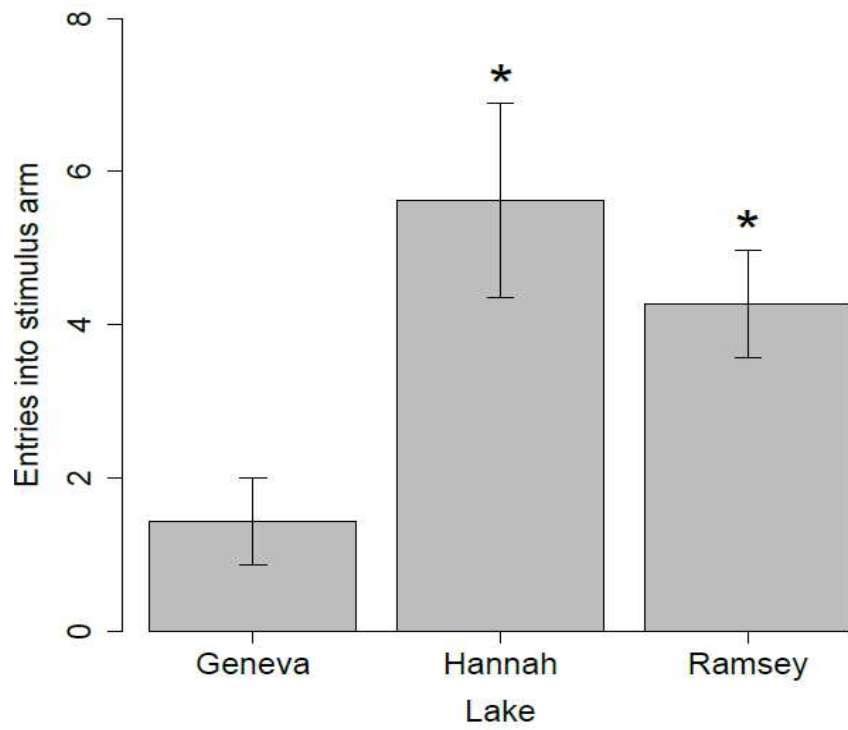
Figure 4.3. Number of times fish from Geneva, Ramsey and Hannah lakes entered into the zone of the behavioural maze receiving a conspecific alarm cue. An asterisk denotes a significant difference from Geneva Lake fish, $p \le 0.05$.

4.3.2. Gene transcription

4.3.2.1. Gene transcription differences

A total of 109 out of the 1000 analyzed genes showed a significant differential transcription between at least one pair of lakes (FDR < 0.1, p < 0.015). The comparison between Ramsey and Hannah lakes showed more differentially transcribed genes (n = 91) compared to 64 and 0 differentially transcribed genes in Ramsey Lake versus Geneva Lake and Geneva Lake versus Hannah Lake, respectively (Fig. 4.4) and most of the differentially transcribed genes between Hannah-Ramsey and Geneva-Ramsey were the same (Fig. 4.4). In the Ramsey Lake and Hannah Lake comparison, 54 genes in Ramsey Lake fish were under-transcribed (average $\log_2$ of fold change: -0.19) and 37 genes were over-transcribed (average $\log_2$ of fold change: 0.41) relative to Hannah. In the Ramsey and Geneva comparison, 35 genes from Ramsey Lake fish were under-transcribed (average $\log_2$ of fold change: -0.2) and 29 genes were over-transcribed (average $\log_2$ of fold change: 0.39) relative to Geneva (Table 4.2).

4.3.2.2. Functional categories of differentially transcribed genes

Gene ontology annotation was used to identify over-represented GO terms (Fisher tests, p < 0.05) in the lists of genes that were significantly differentially transcribed among the Geneva and Ramsey lakes and among Hannah and

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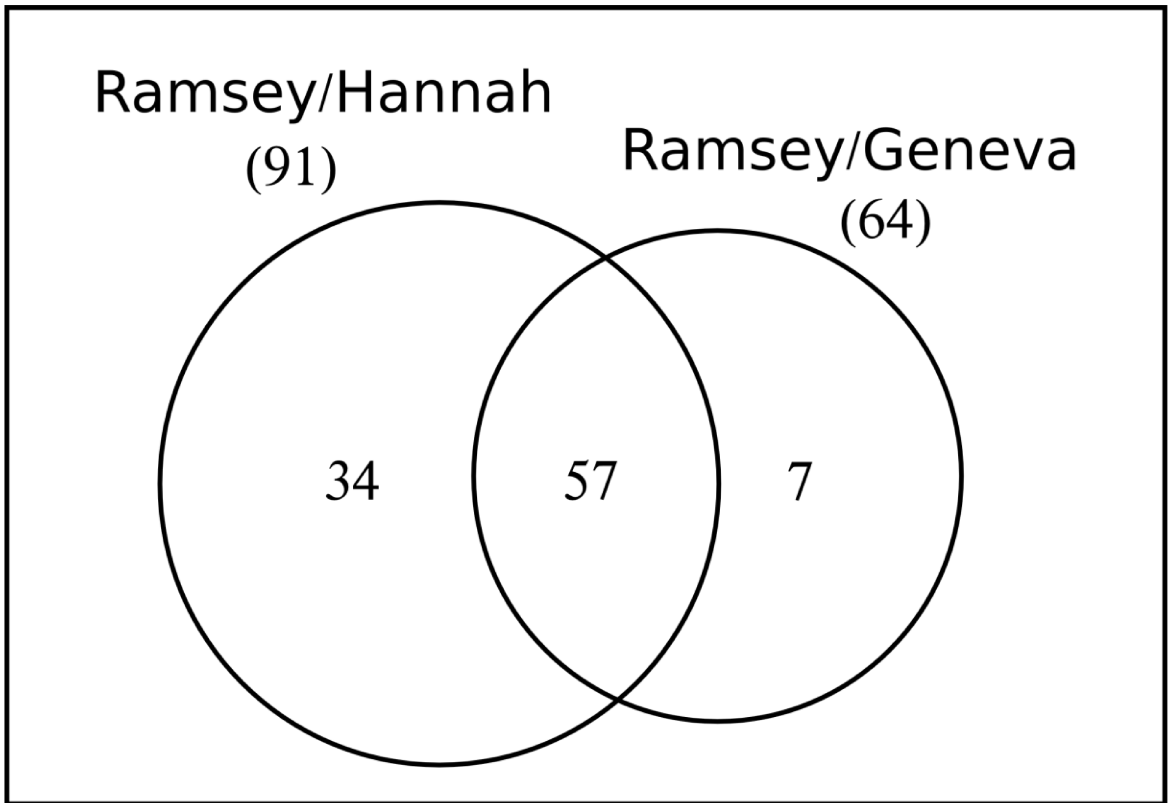
Figure 4.4. Venn diagram representing the number of differentially transcribed genes between Ramsey/Hannah and Ramsey/Geneva comparisons (FDR=0.1).

Table 4.2. List of differentially transcribed genes among Ramsey and Geneva lakes. Fold-changes represent the base 2 logarithm of the absolute fold-change.

Probe name	Gene name	P	Fold-change
		Anova (FDR)	Ramsey/Geneva
Overtranscribed genes			
Pf_probe_0036	5-aminolevulinate synthase, erythroid-specific	0.01	0.451
Pf_probe_0047	Histone H1.0	0.08	0.129
Pf_probe_0104	activating transcription factor 4	0.08	0.133
Pf_probe_0256	UDP-N-acetylglucosamine transporter [*Salmo salar*]	0.04	1.643
Pf_probe_0260	Pyruvate kinase muscle isozyme	0.04	0.698
Pf_probe_0277	Sulfotransferase 1C1	0.09	0.098
Pf_probe_0310	MAGUK p55 subfamily member 5-A	0.03	0.259
Pf_probe_0336	Ceruloplasmin	0.05	0.346
Pf_probe_0340	Hydroxyacid oxidase 1	0.08	0.127
Pf_probe_0344	Probable E3 ubiquitin-protein ligase MYCBP2	0.03	0.244

Probe name	Gene name	P	Fold-change
		Anova (FDR)	Ramsey/Geneva
Pf_probe_0350	Actin-related protein 3	0.04	0.453
Pf_probe_0352	snp_neutre_no_id_006	<0.01	1.506
Pf_probe_0356	2-Cys peroxiredoxin 4	0.04	0.164
Pf_probe_0401	Endoplasmin	0.03	0.182
Pf_probe_0488	ferritin H subunit	0.05	0.273
Pf_probe_0497	Dynein light chain 2, cytoplasmic	0.03	0.212
Pf_probe_0528	glucose-regulated protein 94	0.03	0.285
Pf_probe_0563	putative F-type lectin	0.07	0.172
Pf_probe_0614	snp_neutre_no_id_011	0.04	0.134

Probe name	Gene name	P	Fold-change
		Anova (FDR)	Ramsey/Geneva
Pf_probe_0716	VHSV-induced protein-10	0.06	0.110
Pf_probe_0725	Microsomal glutathione S-transferase 3 [Anoplopoma fimbria] gb|ACQ58963.1| Microsomal glutathione S-transferase 3 [Anoplopoma fimbria	0.04	0.141
Pf_probe_0798	Galectin-9	0.01	0.187
Pf_probe_0815	44.2 kDa connexion	0.03	0.255
Pf_probe_0836	Lysine-specific demethylase 5A	0.05	0.120
Pf_probe_0837	secreted immunoglobulin domain 4	0.05	0.294
Pf_probe_0860	Ras-related protein Ral-B	0.05	0.556
Pf_probe_0908	Glutathione S-transferase 3	0.04	0.083
Pf_probe_0910	heparin cofactor II	0.04	2.025

Probe name	Gene name	P	Fold-change
		Anova (FDR)	Ramsey/Geneva
Undertranscribed genes			
Pf_probe_0016	QM-like protein	0.09	-0.149
Pf_probe_0020	60S acidic ribosomal protein P2	0.06	-0.148
Pf_probe_0058	ADP/ATP translocase	0.04	-0.271
Pf_probe_0066	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	0.06	-0.117
Pf_probe_0073	AP-3 complex subunit delta-1	0.06	-0.165
Pf_probe_0077	Multivesicular body subunit 12B	0.08	-0.093
Pf_probe_0090	Elongation factor 1-alpha	0.04	-0.229
Pf_probe_0110	poly A binding protein, cytoplasmic 1 b	0.07	-0.159
Pf_probe_0188	stress protein HSC70-1	0.07	-0.159
Pf_probe_0220	cytosolic malate dehydrogenase A	0.06	-0.135
Pf_probe_0262	Cystathionine gamma-lyase	0.07	-0.100
Pf_probe_0287	nascent polypeptide-associated complex subunit α	0.04	-0.324

Probe name	Gene name	P	Fold-change
		Anova (FDR)	Ramsey/Geneva
Pf_probe_0375	Eukaryotic translation initiation factor 3 subunit B	0.04	-0.097
Pf_probe_0395	60S ribosomal protein L26	0.08	-0.124
Pf_probe_0440	Glutathione S-transferase Mu 3	0.04	-0.257
Pf_probe_0487	hyperosmotic glycine rich protein	0.04	-0.216
Pf_probe_0509	60S ribosomal protein L8	0.04	-0.191
Pf_probe_0521	Eukaryotic translation initiation factor 3 subunit 3	0.06	-0.153
Pf_probe_0546	rpL14 protein	0.04	-0.114
Pf_probe_0623	Extracellular superoxide dismutase	0.08	-0.097
Pf_probe_0626	serine/threonine-protein kinase RIO1	0.08	-0.124
Pf_probe_0633	Plasminogen activator inhibitor 1 RNA-binding protein	0.03	-0.184
Pf_probe_0676	Guanine nucleotide-binding protein subunit beta-2-like	0.05	-0.244
Pf_probe_0793	Selenoprotein Pa	0.04	-0.110
Pf_probe_0804	Eukaryotic initiation factor 4A-I	0.03	-0.186

Probe name	Gene name	P	Fold-change
		Anova (FDR)	Ramsey/Geneva
Pf_probe_0944	Calmodulin	0.04	-0.156
Pf_probe_0946	60S ribosomal protein L27	0.01	-0.191
Pf_probe_0949	Calmodulin	0.04	-0.234
Pf_probe_0990	Phosphate carrier protein, mitochondrial precursor	0.04	-0.118
Pf_probe_0996	High mobility group protein B1	<0.01	-0.911

Table 4.3. List of differentially transcribed genes among Ramsey and Hannah lakes. Fold-changes represent the base 2 logarithm of the absolute fold-change.

Probe name	Gene name	Pvalue	Fold-change
		Anova (FDR)	Ramsey/Hannah
Overtranscribed genes			
Pf_probe_0013	Actin, alpha sarcomeric/cardiac	0.09	0.127
Pf_probe_0036	5-aminolevulinate synthase	0.01	0.578
Pf_probe_0256	UDP-N-acetylglucosamine transporter	0.04	1.928
Pf_probe_0260	Pyruvate kinase muscle isozyme	0.04	0.789
Pf_probe_0310	MAGUK p55 subfamily member 5-A	0.03	0.375
Pf_probe_0336	Ceruloplasmin	0.05	0.421
Pf_probe_0340	Hydroxyacid oxidase 1	0.08	0.145
Pf_probe_0344	Probable E3 ubiquitin-protein ligase MYCBP2	0.03	0.268
Pf_probe_0350	Actin-related protein 3	0.04	0.513
Pf_probe_0352	snp_neutre_no_id_006	<0.01	1.520

Probe name	Gene name	Pvalue	Fold-change
		Anova (FDR)	Ramsey/Hannah
Pf_probe_0401	Endoplasmin	0.03	0.195
Pf_probe_0433	immunoglobulin IgL light chain precursor	0.02	1.255
Pf_probe_0437	Purine nucleoside phosphorylase	0.07	0.196
Pf_probe_0488	ferritin H subunit	0.05	0.237
Pf_probe_0497	Dynein light chain 2, cytoplasmic	0.03	0.295
Pf_probe_0528	glucose-regulated protein 94	0.03	0.333
Pf_probe_0563	putative F-type lectin	0.07	0.143
Pf_probe_0604	fatty acid synthase	0.08	0.057
Pf_probe_0614	snp_neutre_no_id_011	0.04	0.078
Pf_probe_0621	flavin-containing monooxygenase	0.09	0.088
Pf_probe_0627	C-4 methylsterol oxidase	0.09	0.080
Pf_probe_0704	heme oxygenase 1	0.07	0.107
Pf_probe_0716	VHSV-induced protein-10	0.06	0.067
Pf_probe_0725	Microsomal glutathione S-transferase 3	0.04	0.112

Probe name	Gene name	Pvalue	Fold-change
		Anova (FDR)	Ramsey/Hannah
Pf_probe_0801	ferritin heavy subunit	0.08	0.380
Pf_probe_0815	44.2 kDa connexin	0.03	0.254
Pf_probe_0831	2-amino-3-ketobutyrate coenzyme A ligase, mitochondrial precursor	0.09	0.083
Pf_probe_0836	Lysine-specific demethylase 5A	0.05	0.133
Pf_probe_0837	secreted immunoglobulin domain 4	0.05	0.336
Pf_probe_0860	Ras-related protein Ral-B	0.05	0.653
Pf_probe_0908	Glutathione S-transferase 3	0.04	0.117
Pf_probe_0910	heparin cofactor II	0.04	2.420
Pf_probe_0945	78 kDa glucose-regulated protein	0.04	0.203
Pf_probe_0958	peptidylprolyl isomerase B	0.04	0.147
Pf_probe_1000	fibrinogen alpha chain [Epinephelus coioides	0.05	0.124

Probe name	Gene name	Pvalue	Fold-change
		Anova (FDR)	Ramsey/Hannah
Undertranscribed genes			
Pf_probe_0058	ADP/ATP translocase	0.04	-0.159
Pf_probe_0066	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	0.06	-0.111
Pf_probe_0073	AP-3 complex subunit delta-1	0.06	-0.138
Pf_probe_0075	noggin-1	0.06	-0.110
Pf_probe_0078	Histone H3.3 type 1	0.05	-0.223
Pf_probe_0090	Elongation factor 1-alpha	0.04	-0.283
Pf_probe_0110	poly A binding protein, cytoplasmic 1 b	0.07	-0.118
Pf_probe_0153	mitochondrial ATP synthase F0 complex subunit c isoform 1	0.03	-0.199
Pf_probe_0157	Glyceraldehyde-3-phosphate dehydrogenase	0.08	-0.182
Pf_probe_0170	Heterogeneous nuclear ribonucleoprotein A/B	0.07	-0.145
Pf_probe_0220	cytosolic malate dehydrogenase A	0.06	-0.194
Pf_probe_0240	cyclin G1	0.04	-0.261

Probe name	Gene name	Pvalue	Fold-change
		Anova (FDR)	Ramsey/Hannah
Pf_probe_0319	protein phosphatase-2A catalytic subunit-like	0.09	-0.086
Pf_probe_0374	Hnrpa01 protein	<0.01	-0.579
Pf_probe_0375	Eukaryotic translation initiation factor 3 subunit B	0.04	-0.139
Pf_probe_0387	Troponin I, fast skeletal muscle	<0.1	-0.095
Pf_probe_0395	60S ribosomal protein L26	0.08	-0.163
Pf_probe_0411	cytochrome b	<0.01	-0.252
Pf_probe_0415	carnitine palmitoyltransferase 1B	0.04	-0.188
Pf_probe_0417	retinol-binding protein	0.07	-0.150
Pf_probe_0421	Cytochrome c oxidase subunit 3	<0.01	-0.247
Pf_probe_0440	Glutathione S-transferase Mu 3	0.04	-0.296
Pf_probe_0471	C-type lectin domain family 4 member E	0.05	-0.130
Pf_probe_0506	60S ribosomal protein L28	0.04	-0.201
Pf_probe_0509	60S ribosomal protein L8	0.04	-0.232

Probe name	Gene name	Pvalue	Fold-change
		Anova (FDR)	Ramsey/Hannah
Pf_probe_0545	ependymin	0.07	-0.228
Pf_probe_0546	rpL14 protein	0.04	-0.196
Pf_probe_0615	60S ribosomal protein L24	0.08	-0.193
Pf_probe_0623	Extracellular superoxide dismutase	0.08	-0.091
Pf_probe_0626	serine/threonine-protein kinase RIO1	0.08	-0.134
Pf_probe_0633	Plasminogen activator inhibitor 1 RNA-binding protein	0.03	-0.196
Pf_probe_0648	Junction plakoglobin	0.08	-0.114
Pf_probe_0676	Guanine nucleotide-binding protein subunit beta-2-like 1	0.05	-0.136
Pf_probe_0723	Sodium/potassium-transporting ATPase subunit beta-233	0.08	-0.147
Pf_probe_0748	Cytochrome c oxidase subunit 1	0.01	-0.178
Pf_probe_0781	creatine kinase	0.06	-0.134
Pf_probe_0793	Selenoprotein Pa	0.04	-0.120
Pf_probe_0804	Eukaryotic initiation factor 4A-I	0.03	-0.167

Probe name	Gene name	Pvalue	Fold-change
		Anova (FDR)	Ramsey/Hannah
Pf_probe_0856	40S ribosomal protein S5	0.08	-0.223
Pf_probe_0931	60S ribosomal protein L15	0.08	-0.234
Pf_probe_0944	Calmodulin	0.04	-0.169
Pf_probe_0946	60S ribosomal protein L27	0.01	-0.277
Pf_probe_0949	Calmodulin	0.04	-0.242
Pf_probe_0953	Elongation factor 1-gamma	0.08	-0.193
Pf_probe_0990	Phosphate carrier protein, mitochondrial precursor	0.04	-0.172
Pf_probe_0996	High mobility group protein B1	<0.01	-0.737

Table 4.4. Over-represented processes categories in the Ramsey versus Hannah and Ramsey versus Geneva comparisons (Fisher's exact test, significance threshold: p-value = 0.05, http://www.blast2go.com/b2ghome). Abbreviations: OG: over transcribed gene, UG: under transcribed gene, fold change are $\log_2$ transformed.

Comparison	Processes category	Number of genes	% OG	OG fold change	UG fold change
Ramsey vs. Hannah	tetrapyrrole metabolic process	5	100	0.34	-
	metal ion homeostasis	5	60	0.27	-0.2
	cellular homeostasis	6	67	0.26	-0.2
	ribosome biogenesis	10	0	-	-0.2
	translation	15	0	-	-0.19
	cellular component biogenesis	16	17	0.43	-0.24
	response to chemical stimulus	17	59	0.27	-0.24
	localization	23	39	0.48	-0.23
Ramsey vs. Geneva	cellular homeostasis	5	60	0.2	-0.19
	transmembrane transport	6	0	-	-0.19
	ribosome biogenesis	7	0	-	-0.15
	translation	11	0	-	-0.16
	cellular component biogenesis	11	27	0.38	-0.25
	response to chemical stimulus	12	58	0.27	-0.34
	localization	17	47	0.44	-0.29

In addition, under-transcription of all genes was also observed for transmembrane transport in Ramsey Lake fish compared to Geneva Lake fish and over-transcription of all genes was found for tetrapyrrole metabolic process in Ramsey Lake fish compared to Hannah Lake fish.

4.4.   Discussion

This study demonstrates that chronically metal exposed wild yellow perch from a contaminated environment cannot respond behaviourally to a conspecific alarm cue. This impaired chemosensory function in fish from metal-contaminated lakes mirrors a study by Mirza et al. (2009) in which yellow perch from Hannah Lake and Ramsey Lake had a reduced anti-predator response when compared to fish from a control lake, James Lake. In their study, experiments were performed in laboratory water, while our experiments were performed in native lake water. Previously, it was demonstrated that the source water for exposures was an important consideration for using a neurophysiological technique (electro-olfactography; EOG) to measure olfaction; however, it appears that wild yellow perch from contaminated lakes fail to respond to conspecific alarm cue, despite being tested in native lake water or clean laboratory water, which is not true for neurophysiological (i.e., EOG) endpoints (Azizishirazi et al., 2013).

Previous laboratory studies indicate that fish continuously exposed to metals can recover olfactory function after an initial impairment (Beyers and Farmer, 2001; Dew et al., 2012). The results of the current study indicate that chronically exposed yellow perch from contaminated lakes show an impaired

behavioural response to a conspecific alarm cue relative to fish from a clean lake. One explanation for this discrepancy is that fish from our study were naturally exposed to contaminants for their entire lives, while laboratory fish used in the previous studies were exposed to contaminants for only a short period. Dew et al. (2012) showed that adult fathead minnows (*Pimephales promelas*) recovered olfactory function during continuous exposure to sublethal concentrations of copper. In contrast, when the same species was exposed to almost the same concentration of copper during embryonic development, fish were not able to detect predators, and did not recover from chemosensory dysfunction after 84–96 days in clean water (Carreau and Pyle, 2005). These studies suggest that the ability of fish to recover from contaminant-impaired olfaction is related to the life-stage exposed to chemosensory-impairing contaminants. In the current study, wild yellow perch would have been exposed to contaminants during embryonic development; therefore, recovery of olfactory function in contaminated water is unlikely, which is consistent with our data. In addition, Beyers and Farmer (2001) and Dew et al. (2012) used single-contaminant (i.e., copper), which was added to laboratory water under controlled exposure conditions, whereas wild fish inhabit waters with variable exposure conditions and are exposed to a mixture of contaminants and modifying factors (Table 4.1). It is plausible that differences in water quality, as well as the fact that the wild fish used in the present study were likely exposed to contaminants during embryonic development, in part explain the lack of chemosensory recovery in continuous exposure found in this study.

Microarray analysis using the olfactory rosettes of fish from different lakes demonstrated some differential gene transcription patterns. However, data from the 1000-gene yellow perch microarray used to monitor gene transcription patterns in olfactory rosettes was not able to predict olfactory impairment of yellow perch from metal contaminated habitats. A great number of differentially transcribed genes was expected between Hannah and Geneva lakes due to the dramatic difference in metal concentrations between the two lakes, but this was not seen (Table 4.2). Yellow perch from Hannah Lake showed olfactory impairment using neurophysiological and behavioural endpoints, however, no differences in gene transcription were seen between the fish from these two lakes (Mirza et al., 2009; Azizishirazi et al., 2013; this study). In previous studies, microarray assays showed promising potential for predicting toxicity in fish (Hogstrand et al., 2002; Reynders et al., 2006; Sheader et al., 2006; Moens et al., 2007; Bougas et al., 2013). The 1000 gene yellow perch array used in this study has previously been used and was able to detect the gene transcription changes in liver in a 45-day exposure to sublethal concentrations of nickel and/or cadmium (Bougas et al., 2013). In terms of olfaction, microarray analyses have been used to study the differential gene transcription in the olfactory tissues of zebrafish in response to various contaminants (Tilton et al., 2008; Tilton et al, 2011). In addition to species and microarray dissimilarity, one major difference between our study and research conducted by Tilton et al. (2008, 2011) was that the exposure time in their study was 24 hours, whereas in our study fish were exposed to contaminants for their entire lives. In our study, however, because the

chronic contaminant exposure in Hannah Lake, it is plausible that the transcription of genes in olfactory sensory neurons (OSNs) compensated for the effect and reached a new steady state at the time of sampling. This speculation may explain why no changes have been observed in the transcription of genes in OSNs, although this hypothesis remains to be rigorously tested.

The greatest number of differentially transcribed genes was observed between Hannah and Ramsey lakes, the two metal-contaminated lakes in the study. In addition, most of the differentially transcribed genes between Hannah-Ramsey and Geneva-Ramsey were the same (Fig. 4.4), which suggests that a factor other than contaminant exposure most likely had the strongest effect on gene transcription in olfactory tissues. Considering that no genes were differentially transcribed between Geneva Lake and Hannah Lake fish and the similarity of differentially transcribed genes between Hannah-Ramsey and Geneva–Ramsey leads to the conclusion that one or more factors in Ramsey Lake fish caused this differential transcription.

Higher concentrations of iron in Ramsey Lake water at the time of sampling in 2011 compared to the time of sampling in 2012 could be responsible for changes in gene transcription of fish from Ramsey Lake (Table 4.1). Over transcription of genes like ferritin H subunit, ferritin heavy subunit and ceruloplasmin which all have a role in iron metabolism, support the role of Fe in gene transcription changes of fish from Ramsey Lake compared to fish from Hannah or Geneva lakes In addition, since Ramsey Lake is situated in the city of

Sudbury and receives contamination from storm water, it is possible that some other non-metal contaminant could be responsible for the differential gene transcription in fish from Ramsey Lake (Roberts et al., 2005).

Measuring gene transcription with cDNA microarrays represents a snapshot of gene transcription at a specific time in a specific tissue (Neumann and Galvez, 2002) and transcription of genes can change quickly (Tadiso, et al., 2011; Beggel et al., 2012). On the other hand, the conditions in a lake are variable and many chemical and physical factors, such as temperature and dissolved oxygen, can vary on a time scale ranging from hours to months or even years. Therefore, it is likely that the relatively short-term changes in the conditions of the environment could influence gene transcription and induce "noise" in gene transcription profiles (Van der Meer et al., 2005; Bougas et al., 2013).

4.5. Conclusions

In conclusion, fish from metal contaminated lakes around Sudbury, ON are olfactory-impaired, as demonstrated by behavioural testing. However, no link could be made between the behavioural and transcriptional levels of biological organization. This incongruity leads to the conclusion that the impairment of olfaction of chronically exposed wild fish would not necessarily be measurable using gene transcriptional changes in olfactory rosettes. Fortunately, classical techniques such as measuring behavioural responses can still be used to investigate olfactory impairment of fish from metal contaminated sites.

Acknowledgements

5. CHAPTER 5: Protection of fish olfaction against copper induced impairment

Abstract

Exposure to low concentrations of copper impairs olfaction in fish. To determine the transcriptional changes in the olfactory epithelium induced by copper exposure, wild yellow perch (*Perca flavescens*) were exposed to 20 µg/L of copper for 3 and 24 hours. A novel yellow perch microarray with 1000 candidate genes was used to measure differential gene transcription in the olfactory epithelium. While three hours of exposure to copper changed the transcription of only one gene, the transcriptions of 70 genes were changed after 24 hours of exposure to copper. Real-time PCR was utilized to determine the effect of exposure duration on two specific genes of interest, two sub-units of Na/K-ATPase. At 24 and 48 hours, Na/K-ATPase transcription was down-regulated by copper at olfactory rosettes. As copper-induced impairment of Na/K-ATPase activity in gills can be ameliorated by increased dietary sodium, rainbow trout (*Oncorhynchus mykiss*) were used to determine if elevated dietary sodium was also protective against copper-induced olfactory impairment. Measurement of the olfactory response of rainbow trout using electro-olfactography demonstrated that sodium was protective of copper-induced olfactory dysfunction.

Keywords: olfaction, olfactory toxicity, copper, microarray, real-time PCR, electro-olfactography.

5.1.   Introduction

An intact sense of smell is crucial for many aquatic animals, including fish, as many vital activities such as finding food, avoiding predators, homing, and finding an appropriate mate are mediated by olfaction (Laberge and Hara, 2001). To efficiently detect odour molecules the olfactory system of fish, specifically olfactory sensory neurons (OSNs), is in direct contact with the surrounding water. As a consequence, sensitive neural tissue can easily come into contact with environmental contaminants and impair olfaction even at very low concentrations (Tierney et al. 2010). Impairment of olfaction in fish due to contaminant exposure has received a considerable amount of attention. During the past five decades several studies have investigated the effects of various contaminants on fish olfaction, with metals being the most studied class of contaminant. While the effect of copper on olfaction has received most of the attention (Hara et al., 1976; Julliard et al., 1996; Beyers and Farmer, 2001; Baldwin et al., 2003; Carreau and Pyle, 2005; Bettini et al., 2006; Sandahl et al. 2006; Kolmakov, et al., 2009; Dew et al., 2012; Dew et al., 2014), other metals such as nickel (Brown et al., 1982; Tallkvist et al. 1998; Dew et al., 2014), zinc (Brown et al. 1982; Cancalon, 1982; Kuz'mina, 2011), and cadmium (Baker and Montgomery, 2001; Scott et al., 2003; Blechinger et al., 2007) have also been studied. Regardless of the extensive efforts to explore the effects of copper on olfaction, the underlying mechanism of olfactory toxicity of copper remains unknown. Furthermore, no effort has been directed towards understanding the factors that may protect olfactory function against copper-induced impairment.

Measuring the effects of contaminants on the transcriptome of exposed animals has become an established method in toxicology studies (Walker, 2001; Schirmer et al., 2010). Highly sensitive molecular techniques provide the opportunity to reveal mechanisms of toxicity by investigating rapid and subtle changes at the molecular level (Denslow et al., 2007; Schirmer et al., 2010). Specifically, microarrays allow for the study of large numbers of genes simultaneously. Genes demonstrated to respond to the experimental treatment (e.g., Cu-exposure) on the microarray can be further explored individually using real-time PCR (Snape et al., 2004; Valasek and Repa, 2005; Lettieri, 2006; Schirmer, et al., 2010; Pina and Barata, 2011).

Two microarray studies have investigated the effect of metal contamination on olfactory tissues. Tilton et al. (2008, 2011) exposed zebrafish (*Danio rerio*) to low concentrations of copper and/or chlorpyrifos. Tilton et al. (2008) exposed zebrafish to increasing concentrations of copper (6.3, 16 and 40 µg/L) for 24 hours and found that the transcription of many genes was altered in response to copper exposure, with the largest number of genes having altered transcription at the highest tested concentrations. Tilton et al. (2011) exposed zebrafish to copper and/or chlorpyrifos (an organophosphate pesticide) for 24 hours and both contaminants showed unique transcriptional signatures. However, when they used a mixture of copper and chlorpyrifos, the transcriptional signature was closer to that of copper, even at high concentrations of chlorpyrifos.

Like any toxicological endpoint, differential gene expression is influenced by exposure duration (Denslow et al., 2007; Heckmann et al., 2008; Ankley et al., 2009). However, to date no time series studies have been done at the transcriptional level in regards to olfactory toxicity. At other levels of biological organization, it has been demonstrated that low concentrations of copper will cause different effects on the olfactory system at different exposure durations. For example, fathead minnows (*Pimephales promelas*) exposed to low concentrations of copper for short durations (1, 3, 24, and 96 hours) will initially have a decreased neurophysiological response to odours, which recovers over time (Dew et al., 2012). In terms of behaviour, Colorado pikeminnows (*Ptychocheilus lucius*) have greater copper-induced impairment of behavioural response following 24-hour exposures compared to 96-hour exposures (Beyers and Farmer, 2001).

Parallel to understanding the different aspects of toxicity of any contaminant, many researchers are trying to understand the factors that affect toxicity to make better ecological risk assessments. For example, calcium has been shown to reduce the lethal effects of copper (Chen et al. 2012); however, calcium is not protective against olfactory impairment caused by exposure to low concentrations of copper (Green et al., 2010; Dew et al. 2012). Sodium may also protect against copper-induced olfactory dysfunction. Increasing dietary sodium has been demonstrated to decrease the uptake of waterborne copper in gill tissues of rainbow trout (*Oncorhynchus mykiss*; Pyle et al., 2003). Interestingly, in the same study it was demonstrated that copper-induced impairment of Na/K-

ATPase in gill tissue was reversed by increased dietary sodium. This protective effect of dietary sodium has yet to be studied in olfactory tissue (Pyle et al., 2003).

In the current study, the effects of copper on olfactory tissue at the transcriptional level were investigated using a novel 1000 candidate gene yellow perch microarray (Bougas et al. 2013). Real-time PCR was used to confirm the results of microarray analyses and to measure the transcription of both subunits of Na/K-ATPase in response to different exposure durations of copper. Due to the reason that wild yellow perch do not feed in captivity, we chose another species for testing the effect of increased dietary sodium on copper-induced olfactory dysfunction. Rainbow trout were fed with control and sodium-rich diets and then were exposed to waterborne copper to determine if an increase in dietary sodium in fish could be protective against exposure to copper.

5.2. Materials and methods

5.2.1. Gene transcription

5.2.1.1. Collection and acclimation of fish

Yellow perch were collected from Geneva Lake, near Sudbury, Ontario (46°45'59" N, 81°32'41" W) using seine nets and angling during June 2012 for microarray experiments, and during June 2013 for real-time PCR experiments. Fish were transported in aerated native lake water to the Cooperative Freshwater Ecology Unit, Sudbury, and acclimated to laboratory conditions in water from

Geneva Lake for 24 hours in 12 L plastic tanks. In each container, four fish were held in eight L of water. All tanks were aerated during acclimation and the temperature was kept close to Geneva Lake water temperature (22-23^{0}C).

5.2.1.2. Exposure protocol

To test the effects of copper on olfactory tissue at the transcriptional level (microarray experiments), after 24 hours of acclimation fish were randomly assigned to two treatment groups: control (Geneva Lake water), and copper (Geneva Lake water with 20 µg/L of elevated copper). For each group, four aerated tanks containing three fish were set up for 3 or 24 hours.

To confirm the results of microarray analyses and to measure the transcription of genes of interest (real-time PCR experiments), fish were exposed to either control water (i.e., Geneva Lake water) or control water plus 20 µg/L copper, and held for either 1, 3, 12, 24 or 48 hours. Three fish were randomly assigned to each of 30 experimental replicates (tanks) containing six L of aerated exposure water.

5.2.1.3. Water quality analysis

To measure temperature and pH in the exposure tanks, a YSI 6600 V2 multiparameter sonde (YSI Inc., Yellow Springs, Ohio) was used. Water samples were collected from exposure tanks at the end of the exposure period. From each exposure or control tank, three water samples were analyzed for dissolved metal concentrations, dissolved organic carbon (DOC), and alkalinity. Total dissolved metal concentrations were determined by first passing unfiltered water samples

through a 0.45 μm nylon syringe filter. Filtered water samples were acidified by adding 200 μL of trace metals grade high purity nitric acid (Fisher Scientific, Nepean, ON) to 50 mL of water sample. Samples were stored at 4^0C until analyzed via inductively-coupled plasma atomic emission spectrometry (ICP-AES) by the Lakehead University Instrumentation Laboratory (LUIL), Thunder Bay, Ontario, Canada for metal concentrations (Tables 5.1; 5.2). Dissolved organic carbon was measured by the Lakehead University Centre for Analytical Services (LUCAS) using a San^{++} Automated Wet Chemistry Analyzer (SKALAR, Breda, The Netherlands). Both LUIL and LUCAS are accredited through the Canadian Association for Laboratory Accreditation (CALA). All QA/QC procedures followed internal standard operating procedures of the LUCAS and LUIL. Alkalinity and hardness was measured as previously described (Pyle et al., 2005).

5.2.1.4. Total RNA extraction

Three fish from each exposure tank (for both microarray and real-time PCR experiments) were euthanized by exposure to 200 mg/L MS-222, (Syndel Laboratories Ltd, Nanaimo, BC) buffered to pH 7.4 with sodium bicarbonate (Fisher Scientific, Nepean, ON). Olfactory rosettes were collected from each fish as previously described by Azizishirazi et al. (2014) (chapter 4). Olfactory rosettes were stored in RNAlater® Solution (QIAGEN Inc, Toronto, ON) and used for RNA extraction as previously described by Azizishirazi et al. (2014) (chapter 4). Extracted RNA was stored at -80°C until used for either microarray or real-time PCR experiments.

Table 5.1. Concentrations of dissolved metals and cations as well as water quality parameters in water samples (n=3) from the tanks of control and copper groups for microarray experiment.

Tank		Cu	Fe	Mn	Ni	Zn	Ca	K	Mg	Na
		(μg L^{-1})					(mg L^{-1})			
Control	Mean	0.7	24.8	10.6	4.3	4.1	2.8	0.4	0.7	1.1
	SEM	0.2	1.0	0.2	0.1	0.4	0.4	0.1	0.1	0.1
Copper	Mean	19.8	29.8	9.3	4.2	7.6	3.2	0.6	0.7	1.2
	SEM	0.3	4.0	0.8	0.4	0.4	0.4	0.2	0.1	0.1

Tank	Temp (ºC)	pH	Alkalinity mg/L as CaCO3	Hardness (mg/L as CaCO3)	DOC mg/L
Control	22-23	6.90 - 7.03	20 – 22	8.4 – 10.3	4.2 – 5.7
Copper	22-23	6.83 – 6.99	20 – 22	9.5 – 12.2	4.2 – 5.7

Table 5.2. Concentrations of dissolved metals and cations as well as water quality parameters in water samples (n=3) from the tanks of control and copper groups for real-time PCR experiment.

Tank		Cu	Fe	Mn	Ni	Zn	Ca	K	Mg	Na
		(μg L^{-1})					(mg L^{-1})			
Control	Mean	0.7	21.2	12.9	1.3	3.5	2.3	0.5	0.6	0.9
	SEM	0.1	1.0	0.2	0.2	0.2	0.1	0.1	0.1	0.1
Copper	Mean	18.1	20.4	14.3	0.9	3.3	2.2	0.5	0.6	0.7
	SEM	0.3	0.8	0.3	0.2	0.6	0.1	1.5	0.1	0.1

Tank	Temp (ºC)	pH	Alkalinity mg/L as CaCO3	Hardness (mg/L as CaCO3)	DOC mg/L
Control	20-22	6.80 - 6.95	19 – 23	7.5 – 8.8	4.4 – 5.6
Copper	20-22	6.75 – 6.78	19 – 23	7.3 – 8.6	4.4 – 5.6

5.2.1.5. Microarray experiments

5.2.1.5.1. Labeling and cDNA hybridization

The quality and integrity of the total RNA was verified using an Experion Automated Electrophoresis Station and RNA HighSens Chips (Bio-Rad, Hercules, CA). For each sample, 1.5 µg total RNA was retro-transcribed and the cDNA samples were labeled using the Genisphere 3DNA Array 350 Kit, and Cyanine3 and Cyanine5 fluorescent dyes, following the procedures described in Azizishirazi et al. (2014). Four pooled samples from each condition (control 3 hours, copper 3 hours, control 24 hours, copper 24 hours) were compared and analyzed through a dye-swapped pairwise design for a total of 8 microarrays distributed as follows: 4 microarrays for both copper and control samples after 3 hours of exposure, and 4 microarrays for both copper and control after 24 hours of exposure. The transcription profiles were measured using the yellow perch 1,000 candidate-gene microarray described in Bougas et al. (2013) that was successfully tested and applied using olfactory rosette tissue (Azizishirazi et al. 2014).

5.2.1.5.2. Data acquisition, preparation, and statistical analysis

Scanning, localization, and quantification of the spots and data analysis were conducted as described by Bougas et al. (2013). Using a mixed model ANOVA, we tested for the presence of significant copper effects with the "Array" term included as a random effect, and "dye" and "copper" included as fixed

effects (Fs, with 1000 sample ID permutations). A False Discovery Rate correction (FDR = 0.1) was applied within the R/MAANOVA package to reduce the Type I error rate associated with multiple comparisons and the corrected p-values were used to determine the significance of differential gene transcription levels at α = 0.05. Gene ontology (GO) and assessment of significant differential representation of functional classes was performed in the Blast2Go software (http://www.blast2go.com/b2ghome) as described by Bougas et al. (2013).

5.2.1.6. Quantitative Real-time PCR

Gene specific β-actin primers were used as previously designed by Pierron et al. (2009) and specific primers for both subunits of Na/K-ATPase and GADPH (Table 5.3) were designed using Primer Design Tool (Eurofins MWG Operon). All the primers were ordered from Eurofins MWG Operon (Louisville, KY). The quantity and quality of total RNA was tested using an Epoch Micro-Volume Spectrophotometer (BIOTEK, Winooski, VT). cDNA was synthesized using iScript™ Reverse Transcription Supermix (Bio-Rad, Hercules, CA) according to the manufacturer's instructions and stored in -20°C until analyzed by real-time PCR. Real-time PCR reactions were conducted in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) reagents. Preliminary experiments were conducted to find the optimal concentration of cDNA, optimal annealing temperature of each primer, and the amplification efficiencies of all primers. Based on the preliminary results, the cDNA was diluted 100 fold. Each 20 µL reaction consisted of 10 µL of RT-PCR

Table 5.3. Sequences of primer pairs used in RT- PCR analyses

Genes	Primer	Primer sequence
β-actin	Forward	GCCTCTCTGTCCACCTTCCA
	Reverse	GGGCCGGACTCATCGTACT
GADPH	Forward	ACGCTGGTGTGGACTATGTT
	Reverse	ACCCTCTTAGCTCCACCCTT
Na/K-ATPase subunit α (SPA)	Forward	ATG ATT GAC CCT CCT CGT GC
	Reverse	ATA GCC TTG GCT GTG ATG GG
Na/K-ATPase subunit β (SPB)	Forward	ATT GGG ACC ATT CAG GCG
	Reverse	GAG GGT GGG GTG TGT GAT AG

reagents, the specific primer pairs (final concentration of 500 nM each) and the cDNA as template. Based on the optimal annealing temperature of each primer different protocols have been used for each gene. Two negative controls of non-reverse transcribed total RNA and reaction without template were also amplified to control the genomic DNA contamination and reagents contaminations respectively.

Real-time PCR data were analyzed using R, version 2.15.2 (R Development Core Team, 2012). For each gene (target or housekeeping) and each biological replication ΔCt was calculated by subtracting the Ct number of the housekeeping gene from that of the target gene (Yuan et al., 2006). The ΔCt values for copper exposed and control were compared using Student's t-test for each target gene at each time-point (Yuan et al., 2006). In all comparisons, p-values were adjusted using a Bonferroni's correction to compensate for an increased probability of committing a Type 1 error. All comparisons were considered statistically significant at α = 0.05. The $\Delta\Delta$Ct values were calculated using the following equation (Yuan et al., 2006);

$\Delta\Delta$Ct = ΔCt reference - ΔCt target

The expression ratio was calculated from the below equation:

Ratio = $2^{-\Delta\Delta Ct}$

5.2.2. Neurophysiological response

5.2.2.1. Acclimation and maintenance of the fish

Considering the difficulties of feeding wild fish in captivity, we selected a model species, rainbow trout, which feed more actively in captivity and consequently we were able to feed them with different diets. Due to sensitivity of rainbow trout to copper (Taylor et al., 2003) we decided to decrease the concentration of copper for rainbow trout experiment (10 µg/L). Juvenile rainbow trout were obtained from the Allison Creek Trout Brood hatchery station and held at the University of Lethbridge's Aquatic Research Facility. Fish were acclimated to laboratory conditions (12°C and a 16h:8h light:dark cycle) for a minimum of three weeks in recirculating tanks with supplemental aeration. During the acclimation period fish were fed twice a day (1% body weight each time) with granulated rainbow trout food (EWOS, Surry, BC, Canada). After feeding, the excess food was removed and 80% of water was changed using fresh lab water.

5.2.2.2. Diet preparation

Three different diets were prepared by first grinding granulated rainbow trout food (EWOS, Surry, BC, Canada) into flour. Up to 300 mL of distilled deionised water was added to 500 g of the rainbow trout flour with or without adding NaCl (Fisher, Burlington, ON, Canada) to produce dough for a control, low Na or high Na diet. The dough was then extruded through a potato ricer (President's Choice, Brampton, ON, Canada) and cut with a sharp knife as it was being extruded through the ricer. The new diets were air-dried overnight and

stored in airtight containers at -20°C until used. The measured concentrations of Na in different diets were as follows: control (0.7% w/w Na), low Na (3% w/w Na) or high Na (5% w/w Na) diet.

5.2.2.3. Exposure protocol

Six fish were randomly assigned to each of six 150 L tanks with conditions matched to holding conditions (12°C, 16h:8h light:dark cycle, and supplemental aeration). Three separate feeding regimes were used, control food (0.7% Na), food containing 3.0% Na, and food containing 5.0% Na. Fish were fed 1% of their body weight twice a day with one of the three diets (two tanks of fish per diet) for five days. A minimum of 80% water change was done in each tank 15 minutes following feeding to ensure there was no increase in sodium content in the exposure water. On day 6 fish were exposed to either control water or water containing 10 µg/L of copper. Fish were exposed for 24 hours and then their olfactory acuity was measured using electroolfactography (EOG).

5.2.2.4. Water quality analysis

Water samples were collected from fish tanks after each exposure period, passed through a 0.45 mm syringe filter and stored in 50 mL plastic tubes. Right after the water sampling, 200 µL of trace metals grade high purity nitric acid (Fisher Scientific, Nepean, ON) added to each sample to acidify the samples. The samples were then stored at 4°C until analyzed via ICP-AES by the Lakehead University Instrumentation Laboratory, Thunder Bay, Ontario, Canada for metal concentrations (Table 5.4).

Table 5.4. Concentrations of dissolved metals and cations as well as water quality parameters in water samples (n=3) from the tanks of control and copper groups for the EOG experiment.

Lake		Cu	Fe	Mn	Ni	Zn	Ca	K	Mg	Na
		$(\mu g\ L^{-1})$					$(mg\ L^{-1})$			
Control	Mean	0.1	3.9	0.7	4.2	3.5	44.3	1.4	18.4	20.1
	SEM	0.1	1.0	0.1	0.2	0.4	0.1	0.1	0.5	0.3
Copper	Mean	8.5	2.9	0.2	3.9	3.3	44.7	1.3	18.6	20.2
	SEM	0.8	0.7	0.1	0.2	0.5	1.5	0.1	0.4	0.4

Tank	Temp (ºC)	pH	Alkalinity (mg/L as CaCO3)	Hardness (mg/L as CaCO3)	DOC mg/L
Control	12-13	6.90 - 7.03	20 – 22	186.4-188.2	1.6 – 1.9
Copper	12-13	6.83 – 6.99	20 – 22	184.9-186.6	1.6 – 1.9

5.2.2.5. Electro-olfactography

The electrophysiological responses of the olfactory tissue in rainbow trout were recorded as previously described by Azizishirazi et al. (2013). All solutions were kept at 12°C during the experiment to prevent any heat shock interfering with the neurophysiological olfactory response. Previous research has demonstrated that L-alanine and TCA engage different subtypes of olfactory neurons (Dew et al., 2014). To test both subtypes of olfactory receptor neurons, two different cues, L-alanine (MP Biomedicals, Solon, OH, USA) and taurocholic acid (TCA) (Fisher Scientific, Toronto, ON, Canada), were used. Solutions of 5 x 10^{-3} M L-alanine and 10^{-4} M TCA were made fresh each day.

5.2.2.6. Blood sodium concentration

Blood samples were collected from the caudal peduncle of anaesthetised rainbow trout immediately after EOG experiments. Fish were sacrificed in a 2 L bath of 200 mg/L MS-222 (Syndel Laboratories Ltd, Nanaimo, BC) buffered with sodium bicarbonate (Fisher Scientific, Nepean, ON). Immediately after death, 100 µL of blood was collected from the perforated caudal peduncle and stored at 4°C until analyzed for sodium by LUIL.

5.2.2.7. Statistical analysis

Electro-olfactography data and blood sodium concentration data were analyzed using R, version 2.15.2 (R Development Core Team, 2012). Independent sample t-tests were used to compare the corrected EOG response to L-alanine or TCA between control and copper exposed fish for each of the

three feeding regimes. A two-way analysis of variance (ANOVA) was used to examine the single or combined influences of copper exposure and different diets on the blood sodium concentrations of rainbow trout. To account for unbalanced data a Type II sum-of-squares was used for ANOVA. A Dunnett's test was then used to compare the blood sodium concentrations of fish fed with low or high sodium diet and the blood sodium concentration of control fed fish.

5.3.   Results

5.3.1. Microarray experiment

Only one gene showed significantly different transcription after 3 hours of exposure to copper compared to 70 differentially transcribed genes after 24 hours of exposure to copper (FDR<0.1, p <0.008, Tables 5.5 and 5.6). Among the 70 differentially transcribed genes, 39 genes were over-transcribed (average $\log_2$ of fold change: 0.18) and 31 genes were under-transcribed (average $\log_2$ of fold change: 0.21) in the 24-hour copper-exposed group relative to the control group. Gene ontology (GO) annotation was used to identify the over-represented GO terms in the list of the significant genes relative to the list of genes represented in the microarray. The analysis showed that 70 of the significant genes were included in eight over-represented functional categories (Fisher tests, p<0.05). Within the structural constituents of ribosomes, mRNA binding, and peptidyl-prolyl cis-trans isomerase activity functional categories, all genes were over-transcribed in the copper-exposed group relative to the control group.

Table 5.5. List of differentially transcribed genes among the control and 3 hours of copper exposure. Fold-changes represent the base 2 logarithm of the absolute fold-change. p (FDR) corresponds to the FDR-corrected p-value of the pairwise tests. Abbreviations: Cu = copper, Co = control.

Probe name	Gene name	P ANOVA (FDR)	Fold-change
			Cu/Co
Pf_probe_0252	Hemoglobin subunit alpha-1	<0.01	0.6028

Table 5.6. List of differentially transcribed genes among the control and 24 hours of copper exposure. Table convention follow those for table 2.5.

Probe name	Gene name	P	Fold-change
		ANOVA (FDR)	Cu/Co
Overtranscribed genes			
Pf_probe_0180	14-3-3 protein beta/alpha-1 [*Salmo salar*]	0.04	0.1776
Pf_probe_0526	40S ribosomal protein S13	0.03	0.2078
Pf_probe_0694	40S ribosomal protein S17	0.07	0.1190
Pf_probe_0761	40S ribosomal protein S2 [*Salmo salar*]	0.04	0.1540
Pf_probe_0575	40S ribosomal protein S3-A	0.07	0.0714
Pf_probe_0914	40S ribosomal protein SA	0.07	0.1083
Pf_probe_0564	60S ribosomal protein L10a [*Anoplopoma fimbria*]	0.08	0.1057
Pf_probe_0931	60S ribosomal protein L15	0.05	0.1335
Pf_probe_0294	60S ribosomal protein L18a	0.07	0.1378
Pf_probe_0643	60S ribosomal protein L19	0.03	0.1228
Pf_probe_0004	60S ribosomal protein L22	0.04	0.1188
Pf_probe_0275	60S ribosomal protein L23a [*Anoplopoma fimbria*]	0.04	0.1443
Pf_probe_0615	60S ribosomal protein L24	<0.01	0.1861
Pf_probe_0946	60S ribosomal protein L27	0.08	0.1160

Probe name	Gene name	P	Fold-change
		ANOVA (FDR)	Cu/Co
Overtranscribed genes			
Pf_probe_0950	60S ribosomal protein L28 [*Platichthys flesus*]	0.05	0.1224
Pf_probe_0213	60S ribosomal protein L34	0.08	0.1261
Pf_probe_0125	ADP/ATP translocase 2 [*Osmerus mordax*]	0.05	0.1536
Pf_probe_0587	AF450502_1 ribosomal protein L32 [*Epinephelus coioides*]	0.04	0.1568
Pf_probe_0998	C1q-like-1 [*Siniperca chuatsi*]	0.03	0.2677
Pf_probe_0465	C1q-like-2 [*Siniperca chuatsi*]	0.03	0.2105
Pf_probe_0249	calreticulin [*Paralichthys olivaceus*]	0.07	0.1075
Pf_probe_0479	CCAAT/enhancer binding protein beta [*Rachycentron canadum*]	0.03	0.1929
Pf_probe_0131	CCAAT/enhancer binding protein delta2 [*Oncorhynchus mykiss*]	0.06	0.1645
Pf_probe_0542	Claudin-4 [*Osmerus mordax*]	0.05	0.1386
Pf_probe_0834	Cold-inducible RNA-binding protein [*Salmo salar*]	0.03	0.1778
Pf_probe_0321	complement component 1 q subcomponent gamma polypeptide [*Paralichthys olivaceus*]	<0.01	0.4026
Pf_probe_0804	cyclophilin type peptidyl-prolyl cis-trans isomerase [*Glossina morsitans morsitans*]	0.03	0.2159

Probe name	Gene name	P	Fold-change
		ANOVA (FDR)	Cu/Co
Overtranscribed genes			
Pf_probe_0324	gluthathione S-transferase omega [*Takifugu obscurus*]	0.03	0.1375
Pf_probe_0528	Eukaryotic initiation factor 4A-I	0.07	0.1119
Pf_probe_0428	glucose-regulated protein 94 [*Paralichthys olivaceus*]	0.03	0.1713
Pf_probe_0153	mitochondrial ATP synthase F0 complex subunit c isoform 1 [*Takifugu rubripes*]	0.05	0.1276
Pf_probe_0714	peptidyl-prolyl cis-trans isomerase FKBP5 [*Danio rerio*]	0.09	0.1121
Pf_probe_0958	peptidylprolyl isomerase B [*Oncorhynchus masou formosanus*]	0.03	0.1836
Pf_probe_0563	putative F-type lectin [*Perca flavescens*]	<0.01	0.4930
Pf_probe_0054	ribosomal protein L11 [*Solea senegalensis*]	0.06	0.1445
Pf_probe_0644	ribosomal protein large P0-like protein [*Perca flavescens*]	<0.01	0.3840
Pf_probe_0982	ribosomal protein S25 [*Solea senegalensis*]	0.03	0.1951
Pf_probe_0637	Transaldolase [*Osmerus mordax*]	0.08	0.1416

Probe name	Gene name	P	Fold-change
		ANOVA (FDR)	Cu/Co
Undertranscribed genes			
Pf_probe_0036	5-aminolevulinate synthase, erythroid-specific, mitochondrial	0.05	-0.1103
Pf_probe_0835	B Chain B, Met-Perch Hemoglobin	<0.01	-0.7499
Pf_probe_0151	B-cell translocation protein 1 [*Epinephelus coioides*]	0.09	-0.1067
Pf_probe_0627	C-4 methylsterol oxidase [*Osmerus mordax*]	0.04	-0.1298
Pf_probe_0949	Calmodulin; Short=CaM	0.03	-0.1823
Pf_probe_0944	Calmodulin; Short=CaM	0.03	-0.1514
Pf_probe_0671	CDGSH iron sulfur domain-containing protein 1 [*Anoplopoma fimbria*]	0.03	-0.1389
Pf_probe_0303	Cell cycle progression protein 1 [*Salmo salar*]	0.07	-0.0735
Pf_probe_0420	Cu/Zn superoxide dismutase [*Oplegnathus fasciatus*]	0.04	-0.1515
Pf_probe_0425	Cytochrome P450 24A1, mitochondrial precursor [*Salmo salar*]	0.09	-0.1333
Pf_probe_0618	Death-associated protein-like 1-A [*Anoplopoma fimbria*]	0.03	-0.2809
Pf_probe_0823	ferritin M subunit [*Larimichthys crocea*]	0.09	-0.1373
Pf_probe_0252	Hemoglobin subunit alpha-1	<0.01	-0.7212
Pf_probe_0910	heparin cofactor II [*Paralichthys olivaceus*]	0.03	-0.2307
Pf_probe_0996	High mobility group protein [*Anoplopoma fimbria*]	0.03	-0.3184

Probe name	Gene name	P	Fold-change
		ANOVA (FDR)	Cu/Co
Undertranscribed genes			
Pf_probe_0047	Histone H1.0 [*Caligus rogercresseyi*]	0.03	-0.2810
Pf_probe_0078	Histone H3.3 type 1	0.03	-0.1562
Pf_probe_0374	Hnrpa01 protein [*Epinephelus coioides*]	0.03	-0.1557
Pf_probe_0433	immunoglobulin IgL light chain precursor [*Larimichthys crocea*]	0.07	-0.1680
Pf_probe_0520	L-threonine 3-dehydrogenase, mitochondrial [*Danio rerio*]	0.05	-0.1159
Pf_probe_0310	MAGUK p55 subfamily member 5-A	0.04	-0.1333
Pf_probe_0168	malate dehydrogenase, [*Ixodes scapularis*]	0.03	-0.2373
Pf_probe_0976	Membrane-associated progesterone receptor component 1 [*Anoplopoma fimbria*]	0.06	-0.0862
Pf_probe_0209	MHC II invariant chain [*Siniperca chuatsi*]	0.09	-0.1432
Pf_probe_0941	snp_neutre_no_id_004	0.03	-0.2528
Pf_probe_0352	snp_neutre_no_id_006	<0.01	-0.4341
Pf_probe_0933	Sodium/potassium-transporting ATPase subunit alpha-1	0.03	-0.1791
Pf_probe_0723	Sodium/potassium-transporting ATPase subunit beta-233 [*Salmo salar*]	0.04	-0.1449
Pf_probe_0839	Tubulin beta-4 chain	0.09	-0.1311
Pf_probe_0279	Tumor-associated calcium signal transducer 2 [*Salmo salar*]	0.03	-0.1459

Probe name	Gene name	P	Fold-change
		ANOVA (FDR)	Cu/Co
Pf_probe_0297	ubiquilin-4 [*Danio rerio*]	0.08	-0.0904

Table 5.7. Functions and the corresponding genes. Fold-changes represent the base 2 logarithm of the absolute fold-change. p corresponds to the Fisher's exact test in Blast2go.

GO number	Functions	P	Probe name	Gene name	Fold-change
					Cu/Co
GO:0003735	structural constituent of ribosome	<0.0001	Pf_probe_0004	60S ribosomal protein L22	0.1188
			Pf_probe_0054	ribosomal protein L11 [*Solea senegalensis*]	0.1445
			Pf_probe_0125	AF450502_1 ribosomal protein L32	0.1568
			Pf_probe_0275	60S ribosomal protein L23a [*Anoplopoma fimbria*]	0.1443
			Pf_probe_0294	60S ribosomal protein L18a	0.1378
			Pf_probe_0506	60S ribosomal protein L28 [*Platichthys flesus*]	0.1224
			Pf_probe_0526	40S ribosomal protein S13	0.2078
			Pf_probe_0564	60S ribosomal protein L10a [*Anoplopoma fimbria*]	0.1057
			Pf_probe_0575	40S ribosomal protein S3-A	0.0714
			Pf_probe_0615	60S ribosomal protein L24	0.1861
			Pf_probe_0643	60S ribosomal protein L19	0.1228

GO number	Functions	P	Probe name	Gene name	Fold-change
					Cu/Co
			Pf_probe_0644	ribosomal protein large P0-like protein	0.3840
			Pf_probe_0761	40S ribosomal protein S2 [*Salmo salar*]	0.1540
			Pf_probe_0914	40S ribosomal protein SA	0.1083
			Pf_probe_0931	60S ribosomal protein L15	0.1335
			Pf_probe_0946	60S ribosomal protein L27	0.1160
			Pf_probe_0950	60S ribosomal protein L34	0.1261
			Pf_probe_0982	ribosomal protein S25 [*Solea senegalensis*]	0.1951
GO:0003755	peptidyl-prolyl cis-trans isomerase activity	0.01	Pf_probe_0321	cyclophilin type peptidyl-prolyl cis-trans isomerase [Glossina morsitans morsitans]	0.2159
			Pf_probe_0958	peptidylprolyl isomerase B *[Oncorhynchus masou formosanus]*	0.1836
GO:0003684	damaged DNA binding	0.01	Pf_probe_0575	40S ribosomal protein S3-A; AltName: S1A	0.0714
			Pf_probe_0996	High mobility group protein B1 [*Anoplopoma fimbria*]	-0.3184

GO number	Functions	P	Probe name	Gene name	Fold-change Cu/Co
GO:0016709	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, NAD(P)H as one donor	0.01	Pf_probe_0425	Cytochrome P450 24A1, mitochondrial precursor [*Salmo salar*]	-0.1333
			Pf_probe_0627	C-4 methylsterol oxidase [*Osmerus mordax*]	-0.1298
GO:0005391	sodium:potassium-exchanging ATPase activity	0.02	Pf_probe_0723	Sodium/potassium-transporting ATPase subunit beta-233 [*Salmo salar*]	-0.1449
			Pf_probe_0933	Sodium/potassium-transporting ATPase subunit alpha-1	-0.1791
GO:0020037	heme binding	0.04	Pf_probe_0252	Hemoglobin subunit alpha-1	-0.7212
			Pf_probe_0425	Cytochrome P450 24A1, mitochondrial precursor [*Salmo salar*]	-0.1333
			Pf_probe_0835	B Chain B, Met-Perch Hemoglobin	-0.7499
			Pf_probe_0976	Membrane-associated progesterone receptor component 1 [*Anoplopoma fimbria*]	-0.0862

GO number	Functions	P	Probe name	Gene name	Fold-change
					Cu/Co
			Pf_probe_0804	Eukaryotic initiation factor 4A-I	0.1119
			Pf_probe_0575	40S ribosomal protein S3-A; AltName: S1A	0.0714
GO:0019904	protein domain specific binding	0.03	Pf_probe_0180	14-3-3 protein beta/alpha-1 [*Salmo salar*]	0.1776
			Pf_probe_0944	Calmodulin; Short=CaM	-0.1514
			Pf_probe_0949	Calmodulin; Short=CaM	-0.1823

In the other three functional categories, heme binding, oxidoreductase activity, and Na/K-ATPase activity, all genes were under-transcribed in the copper-exposed group relative to the control group. Details about the functions and the genes represented in each of them are presented in Table 5.7.

5.3.2. Quantitative Real-Time PCR

When yellow perch were exposed to 20 µg/L of copper for 1, 3, or 48 hours, the transcription of both subunits of Na/K-ATPase did not show differential expression compared to control (Fig. 5.1). When yellow perch were exposed for 12 hours, the expression of Na/K-ATPase sub-unit β was down-regulated by 32% compared to control (t_9=3.22, p<0.05; Fig. 5.1) while the expression of the Na/K-ATPase α sub-unit did not show any difference from the control. When yellow perch were exposed to copper for 24 hours both α and β subunits of Na/K-ATPase were down regulated 38% and 37% respectively (t_8=3.71, p<0.01 and t_9=2.69, p<0.05; respectively; Fig. 5.1).

5.3.3. Electro-olfactography

Rainbow trout fed the control diet and exposed to copper showed no impairment of EOG response to L-alanine (t_8=0.29, p=0.76; Fig. 5.2A), but did show a 42% decrease in EOG response to TCA (t_8=0.61 p<0.01; Fig. 5.2B) in copper exposed fish as compared to control animals. There was no significant difference between EOG responses for control and copper exposed fish in response to L-alanine (t_8=-0.73, p=0.46; Fig. 5.2A) or TCA (t_8=0.19, p=0.80; Fig.

5.2B) in fish fed a low sodium diet. Fish fed a high sodium diet did not show any impairment of response to L-alanine (t_8=-0.58, p=0.57; Fig. 5.2A) or TCA (t_8=0.33, p=0.70; Fig. 5.2B) due to copper exposure.

5.3.4. Blood sodium concentration

There was no statistical interaction between the diet used and exposure in the blood sodium concentration ($F_{(2, 20)}$ = 0.60, *p*=0.55). While exposure did not have any effect on the blood sodium concentrations of test animals ($F_{(1, 24)}$ = 3.22, *p*=0.08) diet showed to have an effect on the blood sodium concentration ($F_{(2, 23)}$ = 6.90, *p*<0.01, Fig. 5.3). When Dunnett's test was used, although no difference was detected between fish fed a low sodium diet and fish fed with normal food fish fed with high sodium diet showed a 27% increase in their blood sodium concentration compared to control group (*p*= 0.04, Fig. 5.3).

5.4.   Discussion

While 24 hours exposure to copper caused changes in transcription of 70 genes, 3 hours of exposure to copper altered the transcription of only one gene (Table 2.5). This disparity in the number of genes differentially transcribed at various time points confirms that the effect of copper on the transcription of genes in olfactory rosettes varies over time. It is clear that exposure to copper for a short duration (i.e., 3 hours) did not allow sufficient time for the contaminant to alter the expression of genes. Previously, it was discussed that the first changes at the transcriptional level normally occur within a few hours (Denslow et al.

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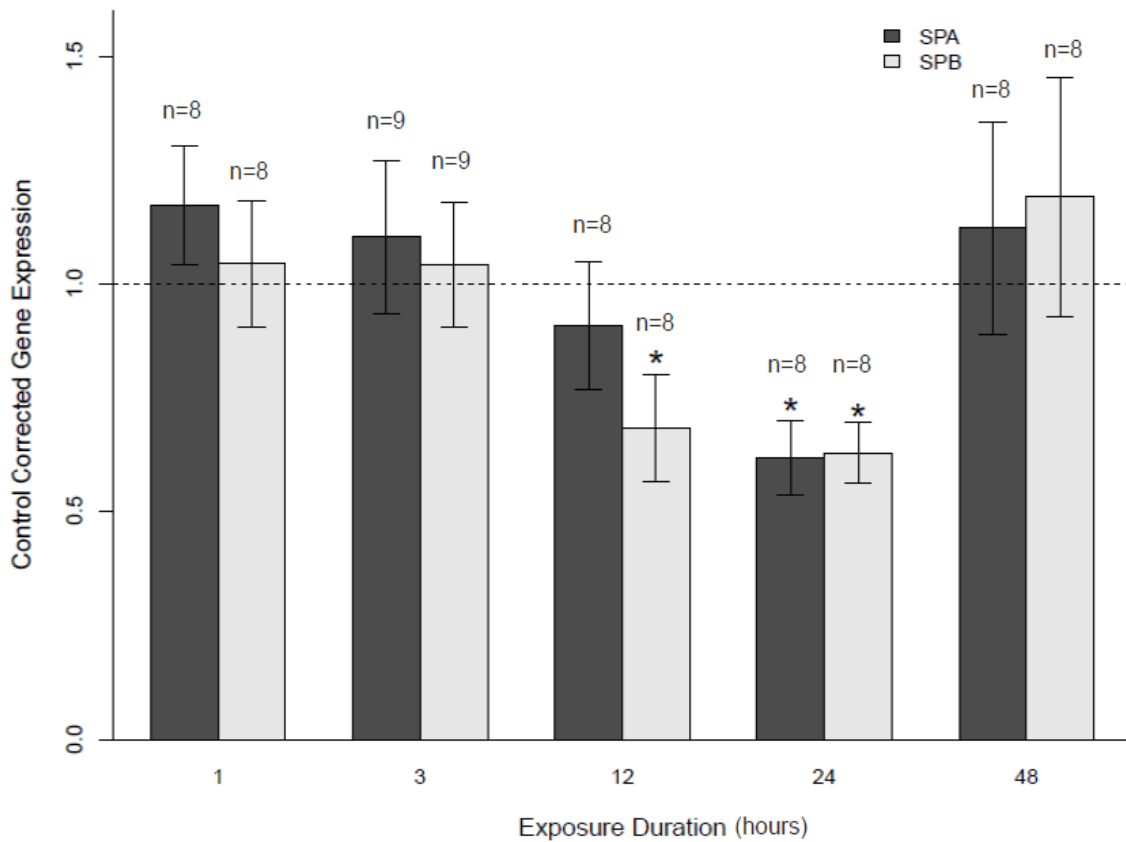
Figure 5.1. Means (+/-SEM) of Na/K-ATPase subunit α (SPA) and Na/K-ATPase subunit β (SPB) expression in olfactory rosettes of yellow perch after different exposure durations. The gene expression is corrected for the reference gene and then expressed relative to control. Asterisks denote difference from control (non-exposed fish).
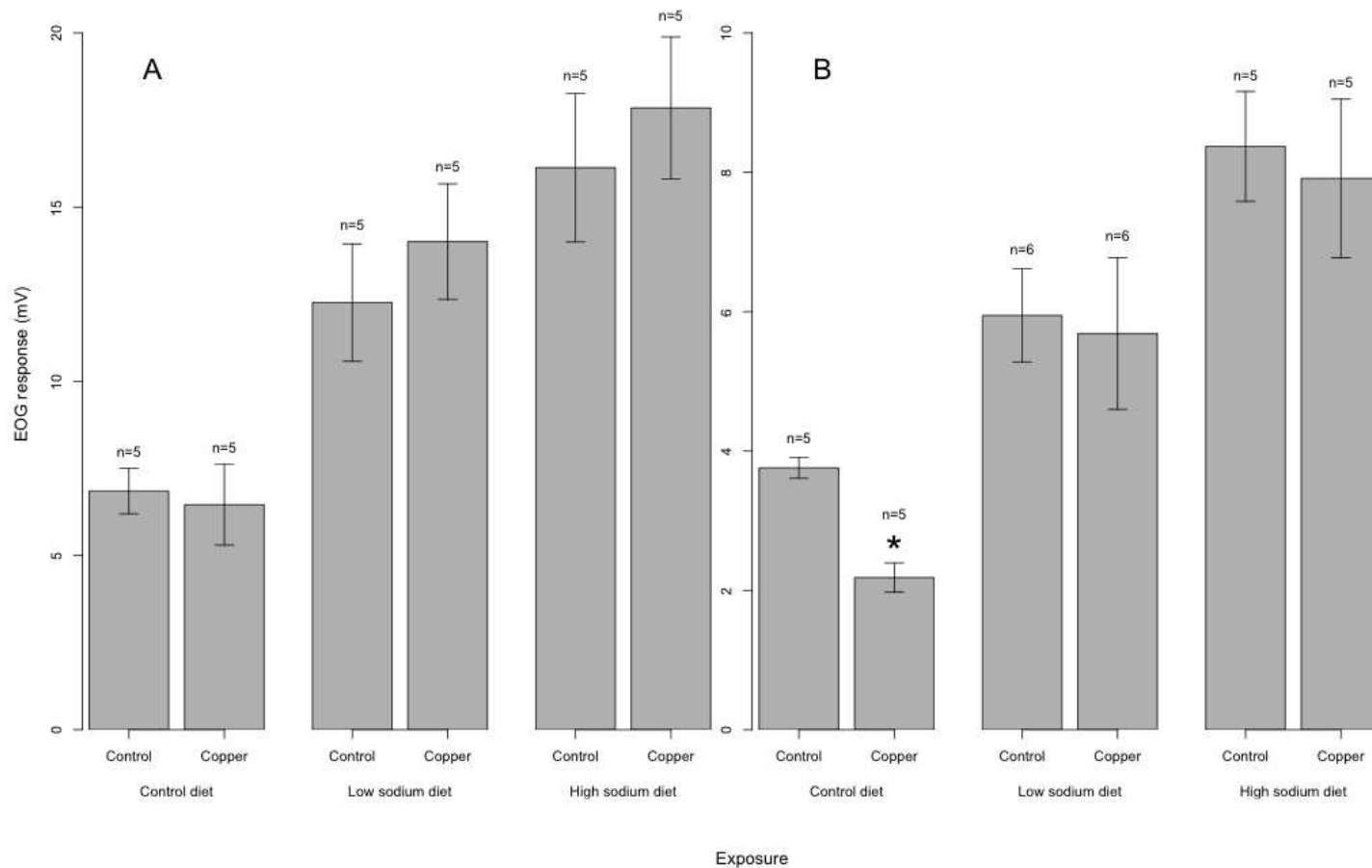
Figure 5.2. Mean EOG response (+/-SEM) of rainbow trout fed with control, low sodium or high sodium diets to 10^{-3} M L-alanine (A) and 10^{-4} M TCA (B) exposed to control or 10 μg/L of copper. An asterisk denotes a significant difference between the TCA response of copper-exposed and control fish fed with control food, $p \leq 0.05$.
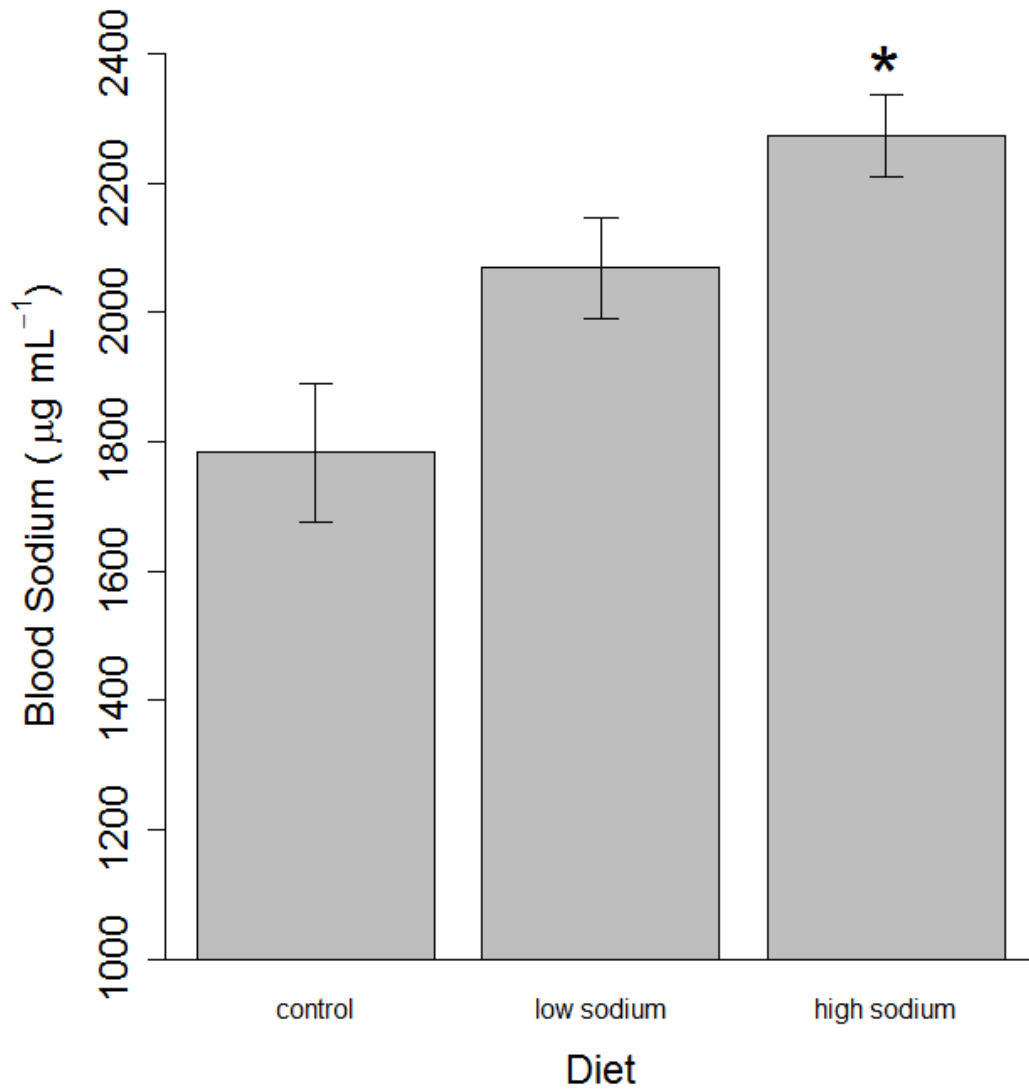
Figure 5.3. Mean blood sodium concentrations (+/-SEM) of rainbow trout fed with different diets; normal, low sodium and high sodium. The asterisk denotes a significant difference between the blood sodium concentrations of fish fed with high sodium diet from the blood sodium concentration of fish fed with control food, $p \leq 0.05$.

2007). However, it is possible that high concentrations of contaminants may affect the transcription of genes more quickly (Denslow et al 2007). While a few studies showed that various contaminant-exposure durations can lead to different effects on gene transcription specific tissues, including brain, pituitary, testes, ovaries, as well as whole body tissue (Van der Ven et al., 2005; Jönsson et al., 2007; Ankley et al., 2009; Beggel et al., 2012), no studies have investigated the effect of contaminants on the transcription of genes in olfactory tissues at various time points. However, the effect of exposure duration on olfactory acuity (e.g., EOG measurements) has been demonstrated (Beyers and Farmer, 2001; Dew et al., 2012).

After 24 hours of exposure to copper, Na/K-ATPase subunit α, Na/K-ATPase subunit β, hemoglobin subunit alpha-1, heparin cofactor II and Cu/Zn superoxide dismutase were down regulated, whereas glutathione S-transferase theta-1 and gluthathione S-transferase omega were over transcribed (Table 5.6). Considering the function of these genes (Table 5.7), it is likely that while copper altered the expression of many genes, detoxifiers like glutathione were up-regulated to protect the cells against the effects of copper exposure (e.g., oxidative stress).

The results from this study do not completely agree with the findings of Tilton et al. (2008) who reported on the effect of copper at the transcriptional level. Tilton et al. reported a substantial difference in the transcription of genes in pooled olfactory tissues of zebrafish, including, olfactory rosettes, telencephalon,

and the underlying olfactory bulb. Among all the copper concentrations they used (6, 16 and 40 µg/L); their 16 µg/L treatment of copper most closely matched the findings of the current study. Tilton et al. demonstrated that exposure to 16 µg/L of copper changed the transcription of 390 genes over/under 1.5 fold. Nevertheless, in the current study just two genes were changed over 1.5 fold (hemoglobin subunit alpha-1 and perch methemoglobin) (Table 2.6). In addition, most of the genes differentially transcribed in our study were dissimilar to their study. One explanation is the difference in the number of studied genes. In their study they used the Affymetrix GeneChip Zebrafish Genome Arrays containing 14,900 transcripts whereas in our study used 1,000 genes. The other reason could be that they pooled the olfactory rosettes, olfactory nerves, telecephalon and the olfactory bulbs whereas in our study we used just olfactory rosettes. Changes at transcriptional level could be different at different tissues (Wang et al., 2008). In addition in the current study we used yellow perch, a metal tolerant fish (Taylor et al., 2003; Couture and Pyle, 2008). It is plausible that zebrafish are more sensitive to exposure to copper compared to yellow perch, and exposure to copper affected the transcription of genes in zebrafish more severely. Despite the difference in differentially transcribed genes, a few genes followed the same pattern in both studies. In both studies two genes encoding Na/K-ATPase, showed down regulation as a result of exposure to copper. Exposure to copper decreases the branchial activity of Na/K-ATPase in the gills of Mozambique tilapia (*Oreochromis mossambicus*; Li et al., 1998), red belly tilapia (*Tilapia zillii*;

Ay et al., 1999), and rainbow trout (Pyle et al., 2003). It has also been demonstrated that Na/K-ATPase is involved in restoring the resting potential of recently-fired neurons by actively exporting sodium ions and importing potassium ions to and from the extracellular environment (Skou, 1965; Thomas,1969; Klimmeck et al., 2008; Kleene, 2009). The sodium pump (Na/K-ATPase) is also known to be active in olfactory sensory neurons of different fish species like garfish (*Lepisosteus osseus*) (Kracke et al., 1981) and Atlantic salmon (*Salmo salar*) (Lo et al., 1991). In fact, it is demonstrated that the concentration of Na/K-ATPase is three times higher in the cilia of olfactory sensory neurons of Atlantic salmon compared to the decilliated olfactory rosettes (Lo et al., 1991). It has been previously shown in many studies that the primary mechanism of toxicity of copper is a general sodium efflux, leading to reduced whole body sodium, resulting in increased blood viscosity, tachycardia, eventually leading to death (Laurén, and McDonald, 1986; Grosell and Wood, 2002; Taylor et al., 2003). It is likely that the general copper-induced whole body sodium efflux also led to reduced sodium concentrations in olfactory tissues. In response to low sodium concentrations in OSNs, Na/K-ATPase is down-regulated to efflux less amount of sodium from intracellular as homeostasis response. The down regulation of Na/K-ATPase allows the cell to restore the sufficient amount of sodium in the cell in the lack of sodium due to exposure to copper. Down regulation of Na/K-ATPase can impair the ability of neurons in maintaining the electrochemical gradients which can lead to impairment of response in olfactory sensory neurons.

The result from real-time PCR experiments investigating the transcription of two subunits of Na/K-ATPase after 3 and 24 hours of exposure to copper confirmed the microarray results (Fig. 5.1). Traditionally, real-time PCR is used to confirm and validate the results from microarray analysis (Walker, 2001; Morey et al., 2006). The results of real-time PCR for the two subunits of Na/K-ATPase validate the microarray results. Both techniques showed down-regulation of both subunits of Na/K-ATPase following 24 hours of exposure to copper. However, when fish were exposed to copper for 3 hours, no changes were detected in the transcription of the genes of interest. These results confirm the robustness of the results from the microarray in detecting the changes at the molecular level in olfactory rosettes of yellow perch exposed to copper. The real-time PCR results also demonstrated that the transcription of Na/K-ATPase at relatively short exposure durations (1 and 3 hours) did not change. The results of the current study indicate that copper requires more than 3 hours to alter the transcription of Na/K-ATPase when present at 20 µg/L. Nonetheless, it has been demonstrated that copper can impair olfactory function (i.e., EOG response) after only a very short exposure duration (i.e., 30 minutes) (Green et al., 2010; Dew et al., 2012). Thus, it is likely that the impairment of Na/K-ATPase is not responsible for the observed olfactory impairment from short copper exposures, and that an alternative mechanism of toxicity exists. However, it is also possible that because the species involved in the current study and the studies conducted by Green et

al. (2010) and Dew et al. (2012) were different, they have different sensitivity to copper.

At relatively long exposure times (e.g., 48 hours) the transcription of both subunits of Na/K-ATPase returned to control levels. Thus, there is a U-shaped pattern in the copper-induced transcriptional modulation of Na/K-ATPase, where between 12 and 24 hours of exposure, gene transcription is modulated, but before and after this time period, gene transcription is unaffected. It is plausible that between 24 and 48 hours of exposure copper is being detoxified, effectively reducing the effect of copper (i.e., the down regulation of Na/K-ATPase, and impaired olfactory acuity). This result corroborates other studies that demonstrated recovery of impaired olfaction in continuous exposure to low concentration of copper (Beyers and Farmer, 2001; Dew et al., 2012).

Considering the impairment of Na/K-ATPase (down regulation of Na/K-ATPase genes) in olfactory tissues after exposure to copper and the role of Na/K-ATPase in restoring the resting potential of neurons a new study was designed. We hypothesised that it is possible that by protecting Na/K-ATPase against copper in olfactory tissues, the olfactory acuity of the organism can be protected against copper exposure. Pyle et al. (2003) demonstrated that by increasing the dietary sodium, the function of Na/K-ATPase could be protected in gills of rainbow trout (Pyle et al., 2003). To test if increased dietary sodium can protect Na/K-ATPase in olfactory tissues, and consequently the olfactory acuity, we

conducted an experiment in which we fed fish and checked their olfactory acuity after exposure to copper.

This study is the first to demonstrate the ability of increased dietary sodium to protect copper-induced olfactory impairment. Rainbow trout fed a normal diet and exposed to relatively low concentration of copper showed a reduction in EOG response to TCA but not L-alanine (Fig. 5.2). The specific impairment of EOG response has been previously demonstrated where copper impaired the EOG response to TCA but did not have any effect on the response to L-alanine in two species of fish; yellow perch and fathead minnows (Dew et al., 2014). The present study coincides with their findings and shows a contaminant-specific copper induced impairment of olfactory sensory neurons in another species, rainbow trout.

In the current study, rainbow trout were fed with food having elevated levels of sodium. Exposure to copper showed to have no effect on the blood sodium concentrations of fish fed with different levels of sodium. It is likely that the effect was not observed because of the small sample size (p=0.08). Although the blood sodium concentrations of fish fed with low sodium diet did not show any difference with control fed fish, the blood sodium concentrations of fish fed with high sodium diet for 6 days was higher compared to fish fed with control food (Fig. 5.3).

When the olfactory acuity of the experimental fish was tested, exposure to 10 µg/L of copper for 24 hours impaired the EOG response of normal-fed

rainbow trout to TCA. However, copper exposed rainbow trout fed with either low or high concentrations of supplementary sodium (either 2.3% or 4.3%; Fig 5.2) showed an intact response to both cues (L-alanine and TCA). One explanation could be that the increased sodium decreased the copper uptake in the olfactory tissues. Pyle et al. (2003) examined the effect of increased dietary sodium on copper induced changes in gills and other tissues of rainbow trout. They demonstrated that increased dietary sodium decreased copper uptake to gills (Pyle et al., 2003). It is possible that increased dietary sodium inhibits the uptake of copper into olfactory tissues as well. Pyle et al. (2003) discussed that the protection of branchial Na/K-ATPase activity is a result of reduced copper uptake caused by increased dietary sodium. It is plausible that increased dietary sodium protects the activity of Na/K-ATPase in olfactory tissues through the same mechanism. However, the ability of increased sodium to decrease copper uptake and to protect the activity of Na/K-ATPase at the protein level against copper in olfactory tissues has yet to be investigated. It is also possible that elevated dietary sodium led to increased sodium efflux from the olfactory epithelium leading to higher sodium concentrations in the extracellular environment of the olfactory epithelium. Therefore, there was a larger transepithelial electrochemical gradient that allowed for a greater sensitivity to odours.

5.5.   Conclusion

This study demonstrates that 24 hours of exposure to copper alters the transcription of many genes in olfactory rosettes of yellow perch. In addition, the

alteration of genes (i.e., Na/K-ATPase) in response to copper exposure is dependent on the exposure duration. Furthermore, increased dietary sodium can protect the olfactory function of rainbow trout against copper-induced olfactory impairment.

Acknowledgements

6. CHAPTER 6: Conclusion

Contaminants exert their sublethal effects at low concentrations and those effects can impair normal behaviours in fish. Effects on individual fish can translate to entire populations, which can ultimately affect ecosystems. Olfaction is sensitive to environmental contamination, and is important for mediating vital biological activities that can have large biological costs if perturbed. Knowledge about how contaminants interact with the olfactory system and how it responds (including recovery) can lead to understand how contaminants affect individuals, populations, and ecosystems which can in turn lead to proper ecological risk evaluation.

The studies reported in this thesis improve our knowledge about the effects of contaminants in natural conditions of metal contaminated lakes. The work presented here is the first to investigate olfactory impairment of fish from metal contaminated lakes at three different levels of biological organisation. The results from neurophysiological and behavioural testing demonstrated that fish from metal contaminated lakes around Sudbury, ON are olfactory-impaired (Chapters 2 and 4). No link was observed between gene transcription and neurophysiological or behavioural responses. Since neurophysiological and behavioural effects have been shown to be promising in producing reliable results in many studies, it is concluded that the impairment of olfaction of chronically exposed wild fish would not necessarily be measurable using gene

transcriptional changes in olfactory rosettes. It is also revealed in this study that exposing fish for a relatively short period of time (24 hours) from a clean lake to water from metal contaminated lakes that have a mixture of different contaminants inhibits their olfactory function. Furthermore, this work reported that fish from metal contaminated lakes with impaired olfaction have the potential to recover. The recovery has been measured and shown at both neurophysiological and behavioural levels.

The work presented in this dissertation reveals that copper alters the transcription of some genes that have important roles in essential biological pathways. Knowledge about the impaired pathways could reveal the mechanism of olfactory toxicity of copper in fish. As an example, this study demonstrated that the Na/K-ATPase genes were down-regulated as a homeostatic response in order to be able to compensate for sodium efflux (as a well-known mechanism of toxicity of copper). The down regulation of these genes helps the cells in olfactory tissues to be able to compensate for copper induced sodium loss and restore enough amount of sodium. Therefore, by having increased amount of sodium in olfactory tissues the cells in olfactory tissues may be protected against copper (discussed below). This research also reveals that the alteration the transcription of genes at the olfactory tissues is time sensitive. While on one hand too short exposure times may not be enough to alter the transcription of genes, on the other hand, at long exposures the transcription of genes may come back to normal. This research showed that there is a small window of time (i.e. 24 hours)

to study genes transcription changes in olfactory tissues due to exposure to copper. Considering the down regulation of Na/K-ATPase genes (discussed above) a method was proposed to protect olfaction in fish against copper, which was the first method that successfully protected fish olfaction against exposure to contaminants. This work revealed that increased dietary sodium protects olfaction against copper induced impairment. This protection could be used to protect olfaction against copper in fish populations which feed on modifiable food sources (e.g. hatcheries). It can also be first step to for finding other methods to protect against metal impaired olfaction in fish.

Taken together, there are three major implications from this dissertation. First, various methods for future olfactory toxicity studies (e.g. testing the yellow perch microarray for both short-term and long-term exposures in olfactory toxicity studies as well as modifying the methods of olfactory toxicity studies of fish from natural habitats) were tested, validated and modified. Second, the recovery of olfaction in fish from metal contaminated lakes both neurophysiologically and behaviourally were demonstrated. Third, one of the factors that reduce the toxic effect of copper on olfaction of fish was characterized. Fish with impaired olfaction are not able to properly execute their basic biological processes. The impairment of basic biological processes can threaten fish populations and consequently impair the whole ecosystem. Understanding that olfactory toxicity is recoverable reveals that fish from moderately metal contaminated sites can recover from impaired olfaction when contaminants are removed. These

outcomes improved our ability to evaluate the ecological risk of low-level metal release to freshwater that could eventually lead to improved environmental policies. The data are also helpful in protecting fish against metal exposure, and these findings can be used in metal exposed fish recovery programs.

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