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Dietary sodium protects fish against copper-induced olfactory impairment

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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 h. 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 h 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 h, 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. This work demonstrates that the transcriptions of both subunits of Na/K-ATPase in the olfactory epithelium of fish are affected by Cu exposure, and that dietary Na protects against Cu-induced olfactory dysfunction.

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 odor 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, 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, studies discovering potential ways to protect olfaction against copper-induced impairment are rare.

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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 exposure to copper 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 h 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 h 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 h) will initially have a decreased neurophysiological response to odors, which recovers over time (Dew et al., 2012). In terms of behavior, Colorado pikeminnows (*Ptychocheilus lucius*) have greater copper-induced impairment of behavioral response following 24-h exposures to copper 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 on 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.

2. Materials and methods

2.1. Experimental animals

Yellow perch is indigenous to North American freshwater lakes and enjoys a nearly ubiquitous distribution east of the Rocky Mountains in Canada and the northern United States (Scott and Crossman, 1973). Because of its known tolerance to metals, it is often one of the first species to recolonize metal-contaminated habitats or the last to be extirpated (Couture and Pyle, 2008). Consequently, yellow perch was studied here for its relevance to Canadian freshwater ecosystems. Regarding the feeding experiment, it has been our experience that yellow perch are not amenable to long-term holding in an aquatic facility. Therefore, we were required to use another species for the feeding experiments as we were required to hold fish for a minimum of four weeks (three weeks for acclimation, one week for the feeding experiments). 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.

2.2. Gene transcription experiment

2.2.1. 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 h in 12 L plastic tanks. In each container, four fish were held in 8 L of water. All tanks were aerated during acclimation and the temperature was kept close to Geneva Lake water temperature (22–23 °C).

2.2.2. Exposure protocol

To test the effects of copper on olfactory tissue at the transcriptional level (microarray experiments), after 24 h of acclimation fish were randomly assigned to two treatment groups: control (Geneva Lake water), and copper (Geneva Lake water with 20 μg/L (0.32 μM) of elevated copper). For each group, four aerated tanks containing three fish were set up for 3 or 24 h.

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 (0.32 μM) copper, and held for either 1, 3, 12, 24, or 48 h. Three fish were randomly assigned to each of 30 experimental replicates (tanks) containing 6 L of aerated exposure water.

2.2.3. 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 °C until

Table 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.

Lake		Cu	Fe	Mn	Ni	Zn	Ca	K	Mg	Na
		($\mu g\,L^{-1}$)					($mg\,L^{-1}$)			
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 (°C)	pH	Alkalinity	Hardness (mg/L as $CaCO_3$)	DOC
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

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 1 and 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).

2.2.4. 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). Olfactory rosettes were stored in RNAlater® Solution (QIAGEN Inc., Toronto, ON) and used for RNA extraction as previously described by Azizishirazi et al. (2014). Extracted RNA was stored at −80 °C until used for either microarray or real-time PCR experiments.

2.2.5. Microarray experiments

2.2.5.1. 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 h, copper 3 h, control 24 h, copper 24 h) 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 h of exposure, and 4 microarrays for both copper and control after 24 h of exposure. The transcription profiles were measured using the yellow perch 1000 candidate-gene microarray described in Bougas et al. (2013) that was successfully tested and applied using olfactory rosette tissue (Azizishirazi et al., 2014).

Table 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 RT-PCR experiment.

Tank		Cu	Fe	Mn	Ni	Zn	Ca	K	Mg	Na
		($\mu g\,L^{-1}$)					($mg\,L^{-1}$)			
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 (°C)	pH	Alkalinity	Hardness (mg/L as $CaCO_3$)	DOC
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

Table 3
Sequences of primer pairs used in RT- PCR analyses.

Genes	Function	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

2.2.5.2. 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 $\alpha = 0.05$. To measure the fold change the level of transcription in fish from the copper exposed group was divided by the level of transcription in fish from the control group. The result is then transformed with a base 2 logarithm.

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).

2.2.6. 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 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 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 I error. All comparisons were considered statistically significant at $\alpha = 0.05$. The ΔΔCt values were calculated using the following equation (Yuan et al., 2006);

$$\Delta\Delta Ct = \Delta Ctreference - \Delta Cttarget$$

The expression ratio was calculated from the below equation:

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

2.3. Diet modification experiment

2.3.1. Acclimation and maintenance of the fish

Due to sensitivity of rainbow trout to copper (Taylor et al., 2003) we decided to decrease the concentration of copper for rainbow trout experiment down to 10 μg/L (0.16 μM). 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 16 h:8 h light:dark cycle) for 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.

2.3.2. 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.

2.3.3. Exposure protocol

For EOG experiments 36 fish were randomly assigned to six 150 L tanks (six fish in each group) with conditions matched to holding conditions (12 °C, 16 h:8 h 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 min 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 (0.15 μM) of copper. Fish were exposed for 24 h and then their olfactory acuity was measured using electroolfactography (EOG).

2.3.4. Water quality analysis

Water samples were collected from fish tanks after each exposure period, passed through a 0.45 μm 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 4).

Table 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 EOG experiment.

Lake		Cu ($\mu g L^{-1}$)	Fe	Mn	Ni	Zn	Ca ($mg L^{-1}$)	K	Mg	Na
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 (°C)	pH	Alkalinity	Hardness (mg/L as $CaCO_3$)	DOC
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

2.3.5. 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 5×10^{-3} M L-alanine and 10^{-4} M TCA were made fresh each day.

2.3.6. 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.

2.3.7. 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 concentrations of control fed fish.

3. Results

3.1. Microarray experiment

Only one gene showed significantly different transcription after 3 h of exposure to copper compared to 70 differentially transcribed genes after 24 h of exposure to copper (FDR < 0.1, $p < 0.008$, Table S1). Among the 70 differentially transcribed genes, 39 genes

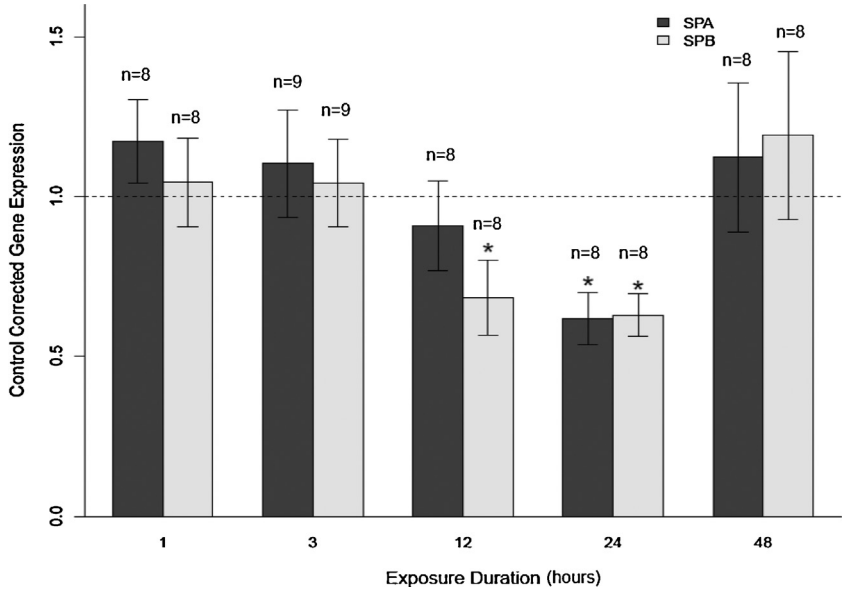

Fig. 1. Means (+/−SEM) of Na/K-ATPase subunit α (SPA) and Na/K-ATPase subunit β (SPB) expression in olfactory rosettes of yellow perch after different exposure durations. The gene expression is corrected for the reference gene and then expressed relative to control. Asterisks denote difference from control (non-exposed fish).

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. 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 S2.

3.2. Quantitative Real-Time PCR

When yellow perch were exposed to 20 μg/L of copper for 1, 3, or 48 h, the transcription of both subunits of Na/K-ATPase did not show differential expression compared to control (Fig. 1). When yellow perch were exposed for 12 h, 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. 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 h, 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. 1).

3.3. Electro-olfactogram

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. 2A), but did show a 42% decrease in EOG response to TCA ($t_8 = 6.16$ $p < 0.01$; Fig. 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 ($t_9 = -0.74$, $p = 0.48$; Fig. 2A) or TCA ($t_9 = 0.19$, $p = 0.85$; Fig. 2B) in fish fed a low sodium diet. Fish fed a high sodium diet did not show any impairment of response to L-alanine ($t_8 = 0.58$, $p = 0.58$; Fig. 2A) or TCA ($t_8 = 0.33$, $p = 0.75$; Fig. 2B) due to copper exposure.

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. 3).

4. Discussion

While 24 h exposure to copper caused changes in transcription of 70 genes, 3 h of exposure to copper altered the transcription of only one gene (Table S1). 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 h) 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., 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 three hours of exposure to copper, hemoglobin subunit alpha-1 was up regulated (52% as compared to the control group).

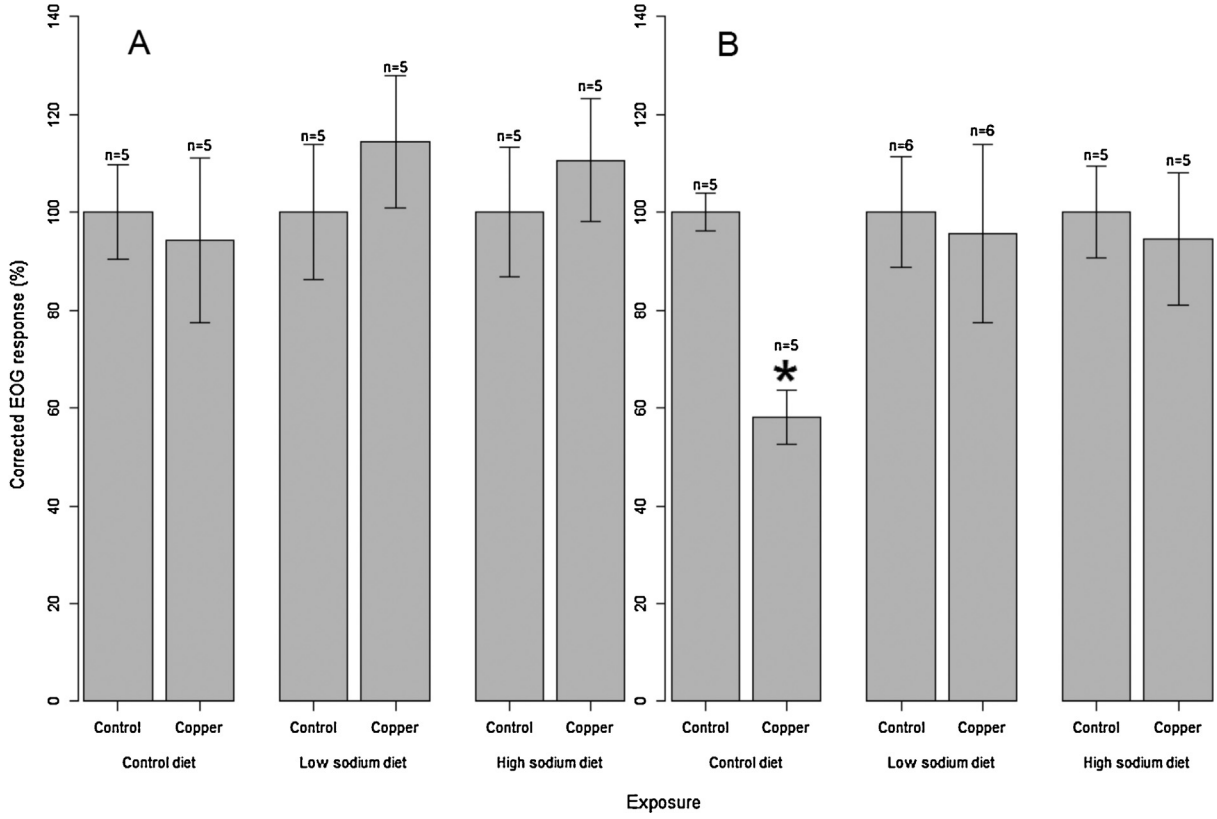

Fig. 2. Mean relative EOG response (+/−SEM) of rainbow trout fed with control, low sodium or high sodium diets to 10–3 M L-alanine (A) and 10-4 M TCA (B) exposed to control or 10 μg/L of copper. An asterisk denotes a significant difference between the TCA response of copper-exposed and control fish fed with control food, $p \leq 0.05$.

However, the transcription of hemoglobin subunit alpha-1 did not show any difference with the control group after 24 h of exposure to copper. Although increase of hemoglobin in olfactory tissues in response of copper has not been studied, copper exposure has been demonstrated to increase hemoglobin in the blood of different fish species (Cerqueira and Fernandes, 2002; Mazon et al., 2002; Carvalho and Fernandes, 2006; Abdel-Tawwab et al., 2007). The increase of hemoglobin is a compensatory response to increase the oxygen transfer in fish, which is affected by copper exposure (Cerqueira and Fernandes, 2002; Mazon et al., 2002). It is likely that in the current study, genes encoding hemoglobin (i.e., hemoglobin subunit alpha-1) were over transcribed as an early response of olfactory tissues to loss of oxygen transfer due to copper exposure. It is also plausible that after 24 h required amount of hemoglobin was provided and the transcription of the genes encoding hemoglobin (i.e., hemoglobin subunit alpha-1) was back to normal.

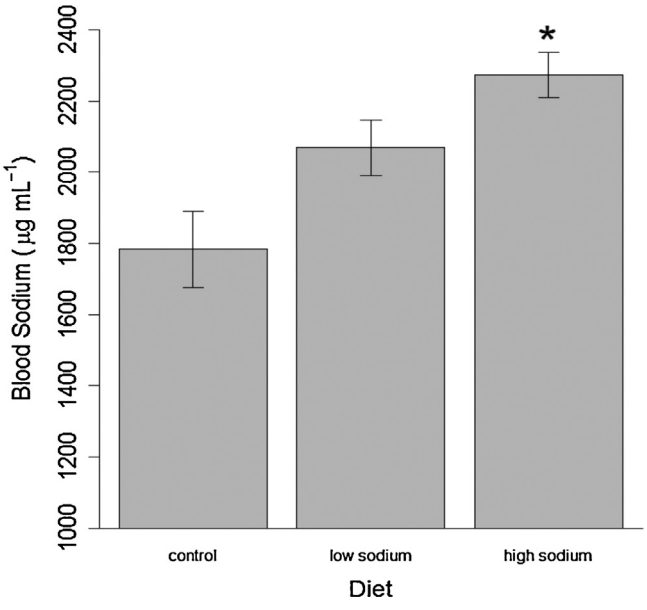

Fig. 3. Mean blood sodium concentrations (+/−SEM) of rainbow trout fed with different diets; normal, low sodium and high sodium. The asterisk denotes a significant difference between the blood sodium concentrations of fish fed with high sodium diet from the blood sodium concentration of fish fed with control food, $p \leq 0.05$.

After 24 h 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 S1). Considering the function of these genes (Table S2), 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 S1). 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 1000 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. 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 h of exposure to copper confirmed the microarray results (Fig. 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 h of exposure to copper. However, when fish were exposed to copper for 3 h, 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 h) did not change. The results of the current study indicate that copper requires more than three 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 min) (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 h) 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 h 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 h 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 (particularly olfactory neurons of fish) (previously discussed) 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. 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).

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. 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. 3).

When the olfactory acuity of the experimental fish was tested, exposure to 10 μg/L of copper for 24 h 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 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 tis-

sues 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 odors.

5. Conclusions

This study demonstrates that 24 h 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2015.01.017.

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