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Contaminant-specific targeting of olfactory sensory neuron classes: Connecting neuron class impairment with behavioural deficits

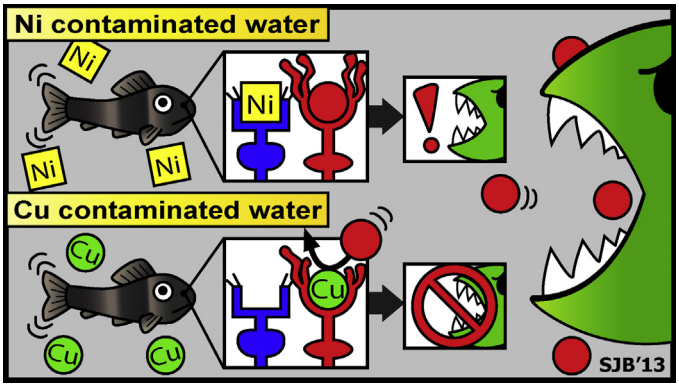
William A. Dew [b], Ali Azizishirazi [a], Greg G. Pyle [a,b,*]

[a] Department of Biology, Lakehead University, Thunder Bay, Ontario P7B 5E1, Canada
[b] Biological Sciences, University of Lethbridge, Lethbridge, Alberta T1K 3M4, Canada

HIGHLIGHTS

- Copper and nickel impair the olfactory system of fathead minnows and yellow perch.
- Copper and nickel have a different effects on ciliated and microvillous OSNs.
- Copper, but not nickel, impairs antipredator response in fathead minnows.
- Response to an antipredator cue in fathead minnows depend on ciliated OSNs.

GRAPHICAL ABSTRACT



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ABSTRACT

The olfactory system of fish comprises several classes of olfactory sensory neurons (OSNs). The odourants L-alanine and taurocholic acid (TCA) specifically activate microvillous or ciliated OSNs, respectively, in fish. We recorded electro-olfactograms (EOG) in fathead minnows (*Pimephales promelas*; a laboratory-reared model species) and wild yellow perch (*Perca flavescens*) whose olfactory chambers were perfused with either L-alanine or TCA to determine if OSN classes were differentially vulnerable to contaminants, in this case copper or nickel. Results were consistent in both species and demonstrated that nickel targeted and impaired microvillous OSN function, while copper targeted and impaired ciliated OSN function. This result suggests that contaminant-specific effects observed in model laboratory species extrapolate to wild fish populations. Moreover, fathead minnows exposed to copper failed to perceive a conspecific alarm cue in a choice maze, whereas those exposed to nickel could respond to the same conspecific cue. These results demonstrate that fathead minnows perceive conspecific, damage-released alarm cue by ciliated, but not microvillous, OSNs. Fish living in copper-contaminated environments may be more vulnerable to predation than those in clean lakes owing to targeted effects on ciliated OSNs.

1. Introduction

The olfactory epithelium of fish comprises a variety of cell types, including olfactory sensory neurons (OSNs) (Zielinski and Hara, 2006). Water bathes the olfactory epithelium and odourants in the water can bind to and activate OSNs, which then transmit

* Corresponding author at: Biological Sciences, University of Lethbridge, 4401 University Drive, Lethbridge, Alberta T1K 3M4, Canada. Tel.: +1 403 332 4048; fax: +1 403 329 2082.
E-mail address: gregory.pyle@uleth.ca (G.G. Pyle).

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information about the external environment to the brain where a behavioural response can be mounted (Hamdani and Døving, 2007). There are three types of OSNs found in the olfactory epithelium of fish; crypt, ciliated, and microvillous (Zielinski and Hara, 2006), which are distinguished by morphological differences and, in some cases, by their response to various odours (Hansen et al., 2003). The response of specific classes of OSNs to different odours can be determined by exploiting a difference in the olfactory signalling pathways in different OSN types, namely that crypt and ciliated OSNs have a cAMP-mediated olfactory signalling pathway while microvillous OSNs have an IP3-based pathway (Hansen et al., 2003; Michel et al., 2003; Rolen et al., 2003). This difference in the olfactory signalling pathways among OSNs was exploited to demonstrate that in the round goby (*Neogobius melanostomus*), taurocholic acid (TCA), a bile salt, induces a response specific to OSNs with a cAMP-mediated pathway (i.e., ciliated and/or crypt OSNs), while L-alanine induces a response specific to OSNs with an IP3-mediated pathway (i.e., microvillous OSNs) (Laframboise and Zielinski, 2011). Bile salts from different fish species do not activate crypt cells, and the specificity of TCA to ciliated cells has been demonstrated via histology (Vielma et al., 2008; Døving et al., 2011; Bazáes and Schmachtenberg, 2012). Work in goldfish has demonstrated that when microvillous cells are intact, fish respond to amino acids but not to TCA (Kolmakov et al., 2009). These results support the conclusion that the olfactory response to L-alanine is mediated by microvillous OSNs, while TCA induces a response specifically in ciliated OSNs. The fact that certain odours induce a response specific to an OSN class means that the status of either ciliated or microvillous OSNs can be measured to determine how each OSN class is affected by contaminants.

Odours can induce a variety of behavioural responses in fish, for example fathead minnows (*Pimephales promelas*) avoid conspecific, damaged-released alarm cue, first described by von Frisch (1938). Chemicals released from fathead minnows whose skin has been ruptured will induce stereotypical antipredator behaviour in other fathead minnows (Smith, 1992; Kats and Dill, 1998). The perception of alarm cues, as well as food, mating, and migratory cues, confer an adaptive benefit to the receiver and is vital to survival. Thus, any impairment of this ability to detect and avoid predators may prevent a prey fish from avoiding predation (Carreau-Green et al., 2008).

A variety of contaminants (e.g., metals and pesticides) are known to impair the olfactory acuity of fish (Scott and Sloman, 2004; Pyle and Mirza, 2007; Tierney et al., 2010). The effect of copper on the olfactory system of fish has been studied at the neurophysiological and, to a lesser extent, the behavioural level. At the neurophysiological level, copper has been shown to reduce olfactory acuity in fathead minnows with exposures ranging from 10 min to 96 h (Green et al., 2010; Dew et al., 2012). Coho salmon (*Oncorhynchus kisutch*) also show decreased olfactory acuity when exposed to low concentrations of copper (Baldwin et al., 2003; Sandahl et al., 2007; McIntyre et al., 2008). The behavioural effects of copper mirror those seen at the neurophysiological level as low concentrations of copper inhibit the antipredator response of coho salmon (Sandahl et al., 2007; McIntyre et al., 2012) and Colorado pikeminnows (*Ptychocheilus lucius*) (Beyers and Farmer, 2001). Copper, therefore, can impair the olfactory system at multiple levels of biological organization. The effect of nickel on olfaction, on the other hand, has been poorly studied even though it is commonly found at elevated concentrations in water bodies near areas receiving anthropogenic input (Pyle and Couture, 2012).

Previously we determined that very low concentrations of copper inhibit olfactory acuity in fathead minnows (Dew et al., 2012). However, it was unclear whether copper had a general effect on all OSN classes, or if each OSN class was differentially affected. Histological work with goldfish demonstrated that when exposed to a high concentration of copper sulphate (16 mg L^{-1}) for a short exposure (10 min), ciliated cells were damaged to a greater extent than microvillous cells (Kolmakov et al., 2009). Corresponding neurophysiological measurements showed that when ciliated cells were damaged and microvillous cells were not, response to two amino acids (L-serine and L-arginine) was intact, and after ciliated cells recovered there was again an intact response to TCA (Kolmakov et al., 2009). It is unknown, however, if OSN-specific effects due to copper exposure would occur at more environmentally-relevant concentrations and exposure durations. To determine if copper specifically affects one or more OSN classes, we first confirmed that L-alanine activates microvillous OSNs and TCA activates ciliated OSNs in fathead minnows by measuring the olfactory response using an electro-olfactogram (EOG), a neurophysiological technique that measures the response of the olfactory epithelium of a fish to an odour. We then exposed fish to increasing concentrations of copper and measured their OSN-specific EOG response to TCA (ciliated OSNs) or L-alanine (microvillous OSNs). Nickel was used as a second contaminant to determine if the effect of copper was a generalized effect of metal exposure, or specific to copper. The effect of copper and nickel on EOG response in wild yellow perch (*Perca flavescens*) was also tested, using native lake water to make up the exposure water. Comparison of laboratory-reared fish with wild fish in their native water demonstrates whether or not any OSN-specific effects of copper and nickel occur in wild fish populations.

The response of fathead minnows to an antipredator cue was measured under copper and nickel exposure for direct comparison to the EOG measurements. By measuring both the effect of copper and nickel on EOG and the behavioural response to an antipredator cue, a direct connection can be made between a behaviour and OSN classes.

2. Materials and methods

2.1. Animals

Adult (1.8–4.1 g) fathead minnows were obtained from the USEPA (Duluth, MN) and housed in static renewal or re-circulatory systems in the Lakehead University Biology Aquatic Facility. Fish were held in dechlorinated Thunder Bay, Ontario municipal water (SI-Table 1) with a 16 h photoperiod. Fish were fed ad libitum once daily with *Artemia* spp., and were allowed to acclimate for a minimum of 2 weeks prior to being used in experiments. Alkalinity was determined as previously described (Pyle et al., 2005). All water samples were collected post-exposure in tubes that were rinsed a minimum of three times with the water to be sampled. For metal analysis, samples were acidified using concentrated trace metals grade nitric acid (Fisher Scientific, Toronto, ON, Canada) and filtered through a 0.45 μm filter. Metal concentrations and dissolved organic carbon were measured by ALS Environmental (Thunder Bay, ON, Canada), a laboratory accredited by the Canadian Association for Laboratory Accreditation (CALA). Metal concentrations were measured using inductively coupled plasma mass spectrometry in accordance to all CALA QA/QC guidelines.

Yellow perch (5.2–8.0 g) were collected from Geneva lake in the Sudbury ON region by angling and acclimated to laboratory conditions for 24 h in Geneva lake water (SI-Table 1). Water collection and analysis was as previously described (Azizishirazi et al., 2013).

2.2. Electro-olfactogram experiments

Electro-olfactogram experiments were performed as previously described (Green et al., 2010). The EOG responses to 10^{-3} M L-alanine (MP Biomedicals, Solon, OH, USA) and 10^{-4} M TCA (Fisher

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Scientific, Toronto, ON, Canada) were measured to determine the response of microvillous and ciliated cells, respectively. Cues were made fresh daily in dechlorinated Thunder Bay, ON municipal water for fathead minnows and in Geneva lake water for yellow perch. Cues were delivered to the olfactory chamber in 2 s pulses at least three times per fish in a randomized order to ensure that olfactory attenuation to any given cue was minimized. After a fish received a cue, delivery of a subsequent cue was delayed by a minimum of 2 min to allow for recovery between cue pulses. The EOG response to a blank (dechlorinated municipal water for fathead minnows or Geneva Lake water for yellow perch) was also measured. The EOG response was determined by measuring the change in amplitude from the baseline to the maximum response to the cue. The EOG response to the respective blank was then subtracted from each EOG response, and the % untreated control response was calculated by dividing each response by the response measured from control animals.

2.3. Transduction pathway determination

The specificity of L-alanine to microvillous OSNs and TCA to ciliated OSNs was confirmed in fathead minnows by exposing the olfactory epithelium to either forskolin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to stimulate the cAMP pathway associated with ciliated cells or U-73122 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to inhibit the IP3 pathway typical of microvillous cells. If there is a reduced olfactory response to a cue during treatment with forskolin, the cue utilizes a cAMP pathway, if there is a reduction in the olfactory response to the cue following a U-73122 treatment, the cue utilizes an IP3-pathway.

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

2.4. Behavioural experiments

Trough mazes measuring 69 cm × 14 cm × 16 cm; $L \times W \times H$ were used to measure the behavioural response of fathead minnows to a chemosensory stimulus (Fig. 1). For each trial 10 L of dechlorinated Thunder Bay municipal water was added per trough. Each trough was divided into three sections; the middle section was the acclimation zone, with two arms extending distally from each side of the acclimation zone. A bottomless, plastic acclimation chamber (19 cm × 13 cm × 11 cm; $L \times W \times H$) was placed into the acclimation zone in each trough, where a subject fathead minnow was placed to acclimate to the maze without having access to the maze arms. At the beginning of each trial 10 mL of the stimulus was slowly injected into to a randomly selected arm (the stimulus arm), and 10 mL of a blank (water matched to the maze) was slowly injected into to the other arm (the blank arm) of each trough. Curtains were placed around the troughs to prevent any motion from the researcher affecting the behaviour of the subject fish. Fish behaviours were recorded by a web camera (RocketFish, Richfield, MN, USA) attached to a MacBook computer from behind the curtain. The acclimation chamber was raised 15 min after the addition of the cues (for acclimation of the fish to the maze conditions and to allow diffusion of the cues throughout the arms) via a string from behind the curtain making sure to not break the surface of the water as dripping water can affect fish behaviour. Fish

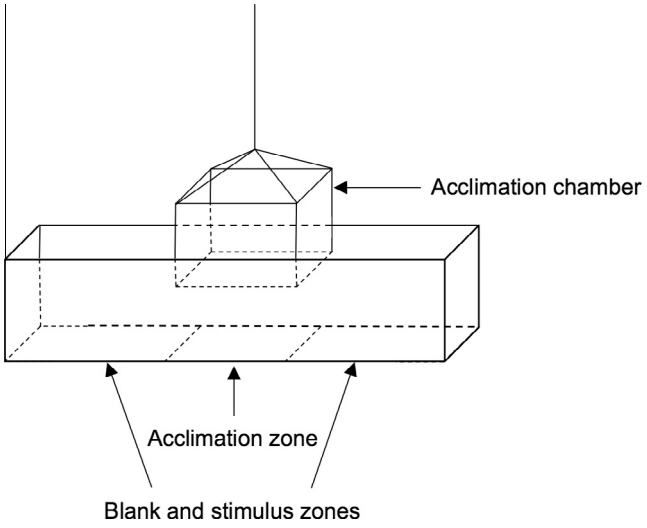

Fig. 1. Behavioural arenas used to measure the response of fathead minnows to conspecific and heterospecific skin extracts.

position in the maze was monitored every 10 s for 8 min. Five mazes, each teasting a single fish, were monitored simultaneously. Time spent in each arm was estimated by establishing the position of the fish at each 10 s monitoring interval and multiplying the frequency of occurrence by 10 s.

The stimulus for behavioural trials was made fresh daily by preparing a skin extract from either a fathead minnow or red swordtail (*Xiphophorus hellerii*). The red swordtail extract was a control for the stimulus, i.e., the smell of damaged fish tissue without the fathead minnow alarm cue. Fish were euthanized using 200 mg L^{-1} MS-222 (Syndel Laboratories Inc., Qualicum Beach, BC, Canada) buffered to pH 7.5. The skin on both sides of the fish was removed and any muscle or other tissue was removed from the skin. The surface area of the skin was measured, placed into a Petri dish containing 1 mL of dechlorinated water, and chopped with dissection scissors for 10 min. The tissue was added to a flask and the Petri dish was repeatedly rinsed with dechlorinated water into the flask to ensure all materials were removed from the Petri dish. Dechlorinated water was added to the flask to bring the final concentration of skin extract to 1 cm^2 per 100 mL. The skin extract in the flask was mixed and allowed to settle for at least 10 min to allow any suspended tissue to sink to the bottom. The top 80% of the volume was used to prevent tissue from being introduced during a trial. Initial behavioural experiments were performed to compare the response of fathead minnows to both the fathead minnow and swordtail skin extracts. If the response of fathead minnows was to the fathead minnow alarm cue, the fathead minnows would be expected to react to the fathead minnow extract, but not the swordtail extract.

2.5. Exposures

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

For EOG experiments, fathead minnows were exposed to one of the following treatments for 48 h: copper (nominal 5, 10, or

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20 μg L^{-1} over background), nickel (nominal 50, 100, or 500 μg L^{-1}), or control water for 48 h. For copper exposures of fathead minnows, there was a background concentration of 6.76 ± 0.89 μg L^{-1} copper in all exposure waters, including the control, which was subtracted from the total copper measured in each sample to give the amount added for each exposure (SI-Table 2). Yellow perch were exposed to nominal concentrations of 20 μg L^{-1} copper, 500 μg L^{-1} nickel, or to a control. Corrected measured values for all exposures were determined as described above, and are found in SI-Table 2.

Initial behavioural experiments were performed comparing the response of fathead minnows to an extract of fathead minnow skin or swordtail skin. In subsequent experiments, fathead minnows were exposed to nominal 5 μg L^{-1} copper, nominal 100 μg L^{-1} nickel, or control water. Corrected measured values were determined as described above and are found in SI-Table 2. All exposures were for 48 h prior to measuring the response of fathead minnows to conspecific alarm cue.

2.6. Statistical analysis

All statistical analyses were performed using R, with the sciplot package for graphs (Morales, 2012; R DevelopmentTeam, 2012). Independent-samples *t*-tests were used to compare corrected EOG responses for each concentration of copper and nickel tested against corresponding controls. A Benjamini–Hochberg *p*-value adjustment was used to compensate for increasing experiment-wise error owing to the multiple *t*-test comparisons. For the yellow perch EOG experiments, an independent-samples *t*-test was used to compare the EOG values under copper and nickel exposures to control values. Any data not conforming to parametric assumptions were transformed using a square-root transformation to recapture parametric assumptions.

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

3. Results

3.1. EOG experiments

Forskolin exposure resulted in a 49% reduction in response to TCA in fathead minnows ($t_3 = -9.150$, $p < 0.01$; Fig. 2A), but not L-alanine ($t_3 = 0.229$, $p = 0.84$; Fig. 2A). In contrast, when U-73122 was added there was a reduced response to L-alanine ($t_3 = -5.101$, $p < 0.05$; Fig. 2B), but not TCA ($t_3 = -2.33$, $p = 0.15$; Fig. 2B).

Fathead minnows exposed to the highest concentration of copper showed an 83% reduction in EOG response to L-alanine relative to the control ($t_{3.83} = 4.19$, $p < 0.05$; Fig. 3A), while exposure to the other two concentrations of copper tested (5 and 10 μg L^{-1}) did not show any statistically-significant differences, despite an apparent reduction in EOG response of approximately 54% when fish were exposed to 10 μg L^{-1}. However, the same 48 h copper exposure resulted in a significant reduction in the fathead minnow's EOG response to TCA at every exposure concentration tested ($t_{3.73} = 2.88$, $p < 0.05$ (5 μg L^{-1}); $t_{5.59} = 7.92$, $p < 0.001$ (10 μg L^{-1}); $t_{6.23} = 7.79$, $p < 0.001$ (20 μg L^{-1}); Fig. 3B). The EOG response to TCA was significantly reduced by 73–91% relative to the appropriate control at every copper concentration tested.

The EOG responses to L-alanine and TCA in fathead minnows exposed to nickel for 48 h (Fig. 3) showed a completely different pattern of effect to those observed after a 48 h copper exposure.

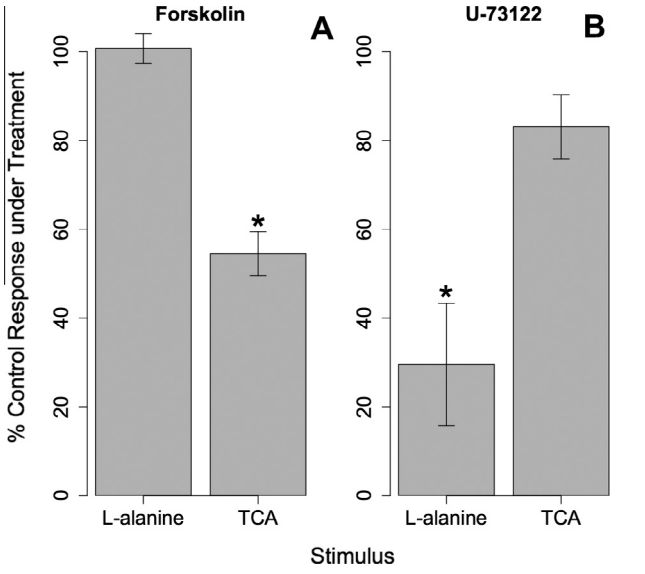

Fig. 2. Percent control response under treatment (mean ± SEM) to L-alanine and TCA under forskolin (A) or U-73122 (B) exposure in fathead minnows ($n = 4$). An asterisk above a bar denotes a significant difference from 100% ($p \leqslant 0.05$).

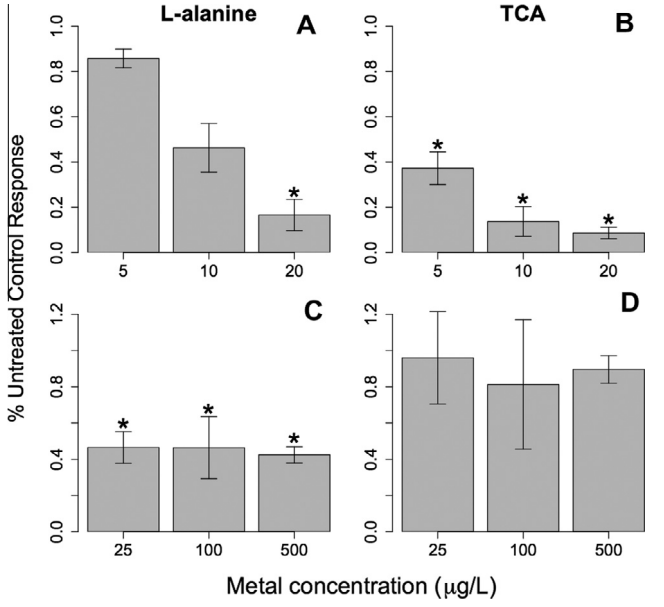

Fig. 3. Corrected EOG response (mean ± SEM) to L-alanine (A) and TCA (B) of fathead minnows treated with copper and corrected EOG response to L-alanine (C) and TCA (D) of fathead minnows treated with nickel. An asterisk above a bar denotes a significant difference from the paired control exposure ($p \leqslant 0.05$), $n = 3$–4 for each bar.

The EOG response to L-alanine was reduced by 53–58% relative to appropriate controls ($t_{3.75} = 3.46$, $p < 0.05$ (25 μg L^{-1}); $t_{5.98} = 2.51$, $p < 0.05$ (100 μg L^{-1}); $t_{3.85} = 3.47$, $p < 0.05$ (500 μg L^{-1}); Fig. 3C). There was no significant reduction in EOG response to TCA at any of the nickel exposure concentrations ($p > 0.05$; Fig. 3D). Representative EOG traces to TCA and L-alanine under nickel and copper exposures are found in Fig. 4.

When wild yellow perch were tested for their EOG responses to L-alanine or TCA after being exposed to copper or nickel, they showed similar patterns to those observed in fathead minnows (Fig. 5). Yellow perch exposed to 20 μg L^{-1} copper showed a 69% reduction in their response to TCA relative to a control

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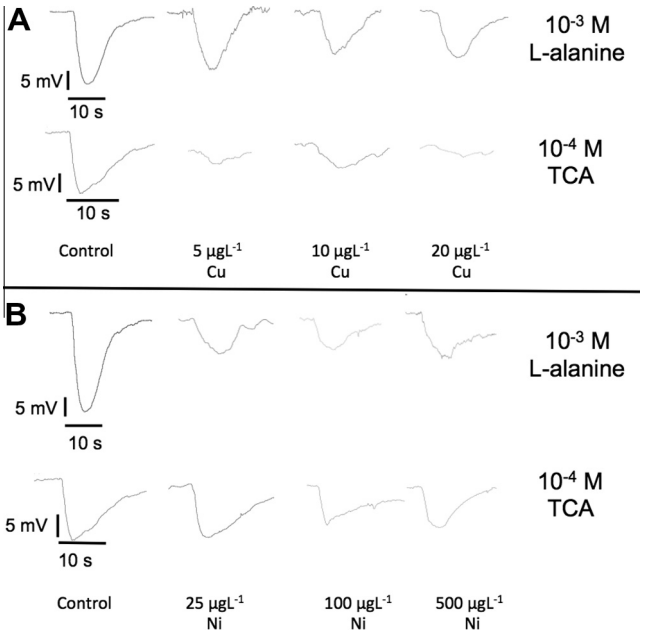

Fig. 4. Representative EOG traces to 10^{-3} M L-alanine and 10^{-4} M TCA for copper (A) and nickel (B) exposures at the concentrations indicated below the traces.

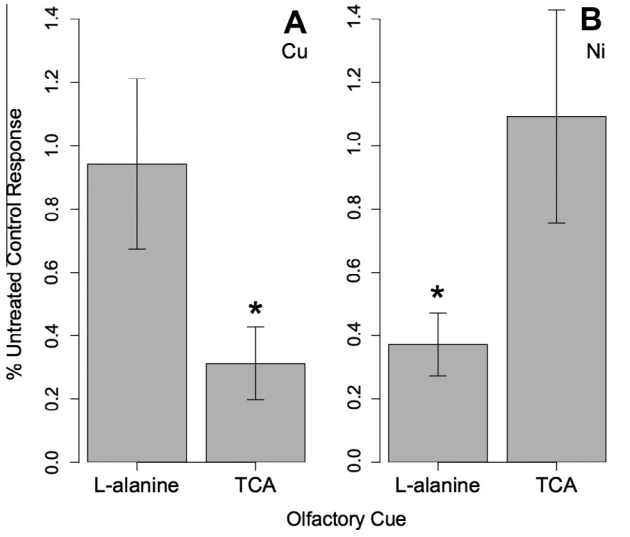

Fig. 5. Corrected EOG response (mean ± SEM) of yellow perch exposed to 20 µg L^{-1} copper (A) or 500 µg L^{-1} nickel (B) to L-alanine or TCA ($n = 4$ for each bar). An asterisk above a bar denotes a significant difference from control exposure ($p \leqslant 0.05$).

($t_{4.73} = 4.15$, $p < 0.05$). However, their response to L-alanine remained intact ($t_{3.74} = 0.20$, $p = 0.85$). On the other hand, yellow perch exposed to 500 µg L^{-1} nickel for 48 h showed an unimpaired response to TCA ($t_{3.71} = -0.26$, $p = 0.85$), whereas their EOG response to L-alanine was impaired by 63% relative to controls ($t_{4.84} = 4.52$, $p < 0.05$).

3.2. Behavioural experiments

When given a choice between an arm containing a skin extract made from fathead minnows or dechlorinated water, fathead minnows avoided the arm with the skin extract, spending 70% less time in arm containing the skin extract relative to the arm containing the blank ($t_8 = 3.42$, $p < 0.02$; Fig. 6). Fathead minnows

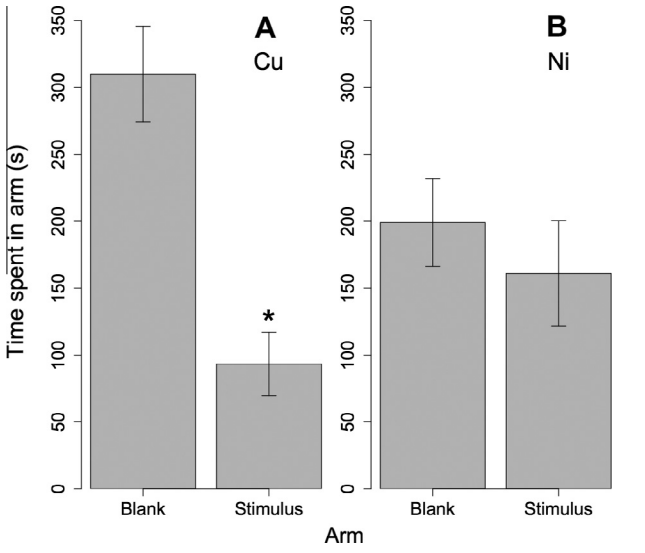

Fig. 6. Time spent by fathead minnows in the blank and stimulus arm (mean ± SEM) when the stimulus was fathead minnow skin extract (A) or swordtail skin extract (B), $n = 9$–10 for each bar. An asterisk above a bar denotes a significant difference between the time spent in the stimulus arm versus the time spent in the blank arm ($p \leqslant 0.05$).

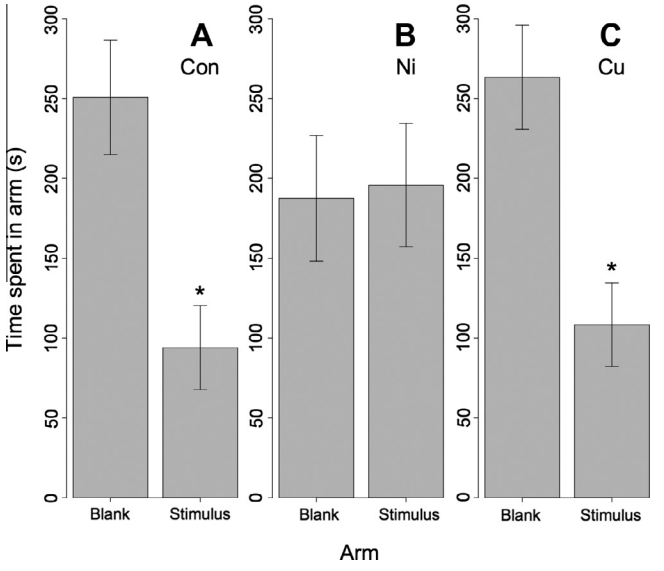

Fig. 7. Time spent by fathead minnows in the blank (dechlorinated water) and stimulus (fathead minnow skin extract) arm (mean ± SEM) under a control (A), 5 µg L^{-1} copper (B) or 100 µg L^{-1} nickel (C) exposure ($n = 12$–15 for each bar). An asterisk above a bar denotes a significant difference between the time spent in the stimulus arm versus the time spent in the blank arm ($p \leqslant 0.05$).

did not distinguish between the blank and stimulus when the stimulus was from a red swordtail control ($t_9 = 0.543$, $p = 0.6$, Fig. 6).

Fathead minnows avoided a fathead minnow skin extract when given a control ($t_{14} = 2.81$, $p < 0.05$: Fig. 7A) or nickel exposure ($t_{11} = 2.71$, $p < 0.05$; Fig. 7C), however, they did not avoid alarm cue when exposed to copper ($t_{11} = -0.12$, $p = 0.92$; Fig. 7B).

4. Discussion

By exploiting the specificity of L-alanine to microvillous OSNs and TCA to ciliated OSNs, we demonstrated that exposure of

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fathead minnows and yellow perch to copper or nickel caused differential impairment of OSN classes. For both species, at all concentrations tested, nickel impaired microvillous and not ciliated OSNs. Copper, on the other hand, had differing effects on fathead minnows depending on the concentration tested. For fathead minnows, at all concentrations of copper tested, ciliated OSNs were impaired, however, only the highest concentration of copper tested impaired microvillous OSNs. The effect of copper on yellow perch mirrored fathead minnows, in that copper impaired ciliated, but not microvillous OSNs. Given that copper impaired both OSN classes in fathead minnows at the highest concentration of copper tested means that at higher concentrations copper is generally toxic to the olfactory system. At lower concentrations, however, copper appears to target ciliated OSNs. Two previous studies with coho salmon demonstrated that the response to TCA (and presumably ciliated OSNs) was impaired by copper at low concentrations (5–10 $\mu g\ L^{-1}$) (Baldwin et al., 2003; Sandahl et al., 2007). The impairment of ciliated OSNs reported in these papers is in line with our study. These reports, however, also note that copper, at most concentrations tested, had a potent effect on the EOG response to L-serine. It is unknown if L-serine specifically activates microvillous OSNs, but if it does then these studies could contradict our study because L-serine may activate both OSN classes in coho salmon (unlike L-alanine in fathead minnows) or because of water chemistry differences. The most likely explanation for the difference, however, is that their exposures were 30–180 min, while ours was 48 h. We recently demonstrated that short, continuous copper exposure resulted in more olfactory impairment in fathead minnows than during long continuous exposures (Dew et al., 2012). It may be that short copper exposures impair both OSN classes, while longer exposures result in significant microvillous OSN recovery. Only one concentration of copper (20 $\mu g\ L^{-1}$) was tested with yellow perch, which resulted in ciliated OSNs being impaired while microvillous OSNs were not. The inter-species difference in sensitivity of microvillous OSNs (perch microvillous OSNs were unaffected at 20 $\mu g\ L^{-1}$ while fathead minnow microvillous OSNs were affected) may be due to inherent differences between the two species, or may be due to differences in water chemistry between laboratory water (fathead minnows) and native lake water (yellow perch). Regardless, the OSN specific effects of nickel and copper seen using laboratory-reared fathead minnows are comparable to the effects of nickel and copper in wild yellow perch using native lake water. The specific activity of copper on ciliated OSNs is also supported by histological work done with goldfish (Kolmakov et al., 2009). The specificity of nickel on microvillous OSNs is novel in that there are no previous studies that investigated the effect of nickel on the olfactory response of fish.

Nickel is taken up into olfactory neurons in fish, however it is unknown into which class of OSNs (Tallkvist et al., 1998). It is possible that nickel entered into microvillous OSNs and inhibited their function. Alternatively, nickel may enter into all OSN types but only impaired the IP3-mediated olfactory signalling pathway found in microvillous OSNs. It is also equally plausible that nickel acts through an entirely different mechanism, suggesting that more work is needed to identify the mode of toxic action. Copper does not enter into OSNs, but instead accumulates in the lamina propria of the olfactory epithelium (Julliard et al., 1995). Therefore, copper appears to exert its effect from the external environment, possibly through interference with cell-surface protein channels or by directly interfering with the electrochemical gradient at the OSN surface. Copper is known to impair gill Na^+/K^+-ATPase, a membrane bound protein that is also involved in olfactory transduction (Laurén and McDonald, 1987; Kern et al., 1991). Copper may impair this pump or another protein at the membrane of ciliated OSNs and exert an inhibitory effect consistent with the inhibitory effects observed in this study. Regardless of the mechanism(s) of action of nickel and copper, they both induce olfactory dysfunction in specific OSN classes.

Fathead minnows avoided a conspecific skin extract, but not a skin extract made from an allopatric heterospecific, indicating that the predator-avoidance response was due to a conspecific alarm cue and not a general odour representing damaged fish skin. When fathead minnows were exposed to copper, this stereotypical avoidance response to a conspecific alarm cue was impaired. However, the response remained intact after fish were exposed to nickel. Copper has been demonstrated to impair the response of at least two other fish species to conspecific antipredator cues, Colorado pikeminnows (Beyers and Farmer, 2001) and coho salmon (Sandahl et al., 2007; McIntyre et al., 2012), at similar concentrations to that used in this study. No work has been done prior to this study investigating the effect of nickel exposure on an alarm cue response, however work done with rainbow trout demonstrated that 50 $\mu g\ L^{-1}$ nickel does not impair agonistic interactions (Sloman et al., 2003). The differential effect of copper and nickel on fright response means that fish in a waterway contaminated with a low concentration of copper may lose their ability to properly evaluate predation risk, while fish in a waterway contaminated with nickel will have this essential ability intact.

Our data allow for a direct connection to be drawn between the neurophysiological and behavioural levels of organization of the olfactory system in fish. Specifically, at the concentrations of copper and nickel tested, copper impairs ciliated OSNs, which are required for detecting conspecific alarm cues, while nickel impairs microvillous OSNs, which are required to detect amino acids but not conspecific alarm cues. It is plausible that other chemosensory-mediated behaviours may also be dependent on a specific OSN class. The connection of the neurophysiological and behavioural levels of organization also allows for predictions to be made of behavioural deficits based on neurophysiological experiments. For example, it is possible that other toxicants target ciliated OSNs, thereby causing an impaired ability in fathead minnows to respond to conspecific alarm cue. Other contaminants and behaviours need to be investigated to fully understand this connection between these two levels of organization and to determine if predictions of behavioural deficits can be made based on neurophysiological experiments.

In summary, this work demonstrates that specific classes of OSNs can be differentially affected by copper and nickel. It also demonstrates that the alarm cue response in fathead minnows first described by von Frisch is dependent on ciliated, but not microvillous OSNs (von Frisch, 1938). Future studies should focus on whether or not other contaminants impair specific OSN classes, which can be related to specific behavioural responses.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2014.02.047.

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