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Differential response of olfactory sensory neuron populations to copper ion exposure in zebrafish

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ABSTRACT

The peripheral olfactory system of fish is in direct contact with the external aqueous environment, so dissolved contaminants can easily impair sensory functions and cause neurobehavioral injuries. The olfactory epithelium of fish is arranged in lamellae forming a rosette in the olfactory cavity and contains three main types of olfactory sensory neurons (OSNs): ciliated (cOSNs) and microvillous olfactory sensory neurons (mOSNs), common to all vertebrates, and a third minor group of olfactory neurons, crypt cells, absent in tetrapods. Since copper is a ubiquitously diffusing olfactory toxicant and a spreading contaminant in urban runoff, we investigated the effect of low copper concentration on the three different OSNs in the olfactory epithelium of zebrafish, a model system widely used in biological research. Image analysis was applied for morphometry and quantification of immunohistochemically detected OSNs. Copper exposure resulted in an evident decrease in olfactory epithelium thickness. Moreover, after exposure, the lamellae of the dorsal and ventral halves of the olfactory rosettes showed a different increase in their sensory areas, suggesting a lateral migration of new cells into non-sensory regions. The results of the present study provide clear evidence of a differential response of the three neural cell populations of zebrafish olfactory mucosa after 96 h of exposure to copper ions at the sublethal concentration of $30 \mu\text{g L}^{-1}$. Densitometric values of cOSNs, immunostained with anti-G $_{\alpha\text{olf}}$, decreased of about 60% compared to the control. When the fish were transferred to water without copper addition and examined after 3, 10 and 30 days, we observed a partial restoration of anti-G $_{\alpha\text{olf}}$ staining intensity to normal condition. The recovery of cOSNs appeared sustained by neuronal proliferation, quantified with anti-PCNA immunostaining, in particular in the early days after exposure. The densitometric analysis applied to mOSNs, immunostained with anti-TRPC2, revealed a statistically significant decrease of about 30% compared to the control. For cOSNs and mOSNs, the decrement in staining intensity may be indicative of cell death, but reduction in antigen expression may not be excluded. In the post-exposure period of 1 month we did not find recovery of mOSNs. We hypothesize that cOSNs are more sensitive than mOSNs to copper treatment, but also more prompted to tissue repair. Anti-TrkA-immunopositive crypt cells appeared not to be affected by copper exposure since statistical analysis excluded any significant difference between the control and treated fish. Comparative studies on OSNs would greatly enhance our understanding of the mechanisms of olfaction.

1. Introduction

Aquatic animals depend on chemical information from the environment in order to carry out vital life processes (Pyle and Mirza, 2007). Olfaction is an essential sense for detection and evaluation of the environment in all vertebrates, including teleosts (Green and

Zielinski, 2013). Other than information on the position and composition of food, this chemical sense permits the recognition of predators and social cues such as pheromones (Wyatt, 2010).

The olfactory system of teleosts can discriminate among an assortment of odorant molecules dissolved in water, including amino acids, bile salts, polyamines, nucleotides, prostaglandins, and sex steroids. Chemical information on the environment is transmitted from the olfactory organ to the brain, influencing alarm response, predator avoidance, food finding, social communication, reproductive activity, and homing migration (Eisthen and Polese, 2007; Hamdani and Døving, 2007; Sorensen and Caprio, 1998).

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Teleost fishes, which are a division of ray-finned fishes, include over 23,000 species (Nelson, 2006). As in the other fishes, the olfactory epithelium in ray-finned fishes is organized in a rosette of lamellae radiating outward from a central raphe. Both the shape and complexity of lamellar organization differ among species (Hansen and Zielinski, 2005; Yamamoto, 1982; Zeiske et al., 1992). Unlike tetrapods, the olfactory system of teleosts lacks a separate vomeronasal organ in addition to a main olfactory system. Teleosts possess a single olfactory epithelium (OE) arranged in lamellae in the olfactory cavities that are positioned dorsally on each side of the head. A great number of olfactory sensory neurons (OSNs) detects odorants and sends odor information into the brain.

The two main types of vertebrate OSNs are ciliated (cOSNs) and microvillous olfactory sensory neurons (mOSNs) (reviewed in Eisthen, 1992) which in teleosts and mammals express olfactory receptors of the OR and TAAR gene families, also named V1R- and V2R-type genes, respectively (Oka et al., 2012). In addition, elasmobranchs, cladistians and teleosts possess one minor group of OSNs, crypt cells (Ferrando et al., 2006, 2007, 2011; Hansen and Finger, 2000), which apparently express only a single olfactory receptor, the V1R-related ORA4 (Oka et al., 2012), believed to be absent in tetrapods (Hansen and Finger, 2000; Laberge and Hara, 2001). Ciliated, microvillous, and crypt-type OSNs are broadly present in ray-finned fishes, but the distribution of each cell type is somewhat variable. By way of the olfactory nerve, axons of ORNs reach the olfactory bulb where they contact dendrites of mitral cells and local interneurons in the glomerular layer, giving rise to complex neuropil structures, glomeruli, which display a stereotyped organization and bilateral symmetry (Baier and Korsching, 1994). In the olfactory bulb, olfactory glomeruli represent the first central nervous station for odor processing, i.e. the first relay station in the olfactory pathway (Gayoso et al., 2012; Hamdani and Døving, 2007).

The discharge of industrial and domestic wastewater is the threat endangering aquatic environments that has the most significant impact on freshwater ecosystems, as well as agricultural production and human health (Katagi, 2010; Klečka et al., 2010; Schwarzenbach et al., 2010). Thus, constant monitoring is essential to ensure prompt intervention whenever hazardous waste discharge events occur. Due to their biodiversity, population structure and health conditions, fish have been judged ideal biomonitoring organisms. Fish have been proposed as organisms for in vivo toxicity assays (Cardwell et al., 1976). Normal embryo conditions, such as egg hatchability and embryo growth and survival, can be also used as monitoring parameters (Lammer et al., 2009a,b). A common approach to assess water quality is also represented by quantifying in fish the activity of enzymatic defenses. Recent works demonstrated that contamination of aquatic environments, i.e. exposure to neurotoxic chemicals such as heavy metals, can impair chemosensory functions, a process recently named 'info-disruption' (Lürling and Scheffer, 2007), and lead to a loss of olfaction in vertebrates (Baldwin et al., 2011; Bondier et al., 2008).

The fish peripheral olfactory system is in direct contact with the external environment. Therefore, it is directly exposed to dissolved contaminants, which can impair peripheral neurological function (Laberge and Hara, 2001; Tierney et al., 2010). In fish, neurobehavioral injuries induced by metals are extremely problematic due to the alteration and possible loss of olfactory-controlled behaviors basic to survival (Baldwin et al., 2003; Sandahl et al., 2007; Scott et al., 2003). Copper is a ubiquitously diffusing olfactory toxicant and a spreading contaminant in urban runoff at low concentrations, varying from 3 to 64 ppb (Soller et al., 2005). Specifically, vehicle emissions, pesticides, and industrial utilizations are major sources of Cu in runoff (Davis et al., 2001; Good, 1993). In particular, copper exerts its neurotoxic effect on fish olfaction (Shaw and Handy, 2011). There is evidence that exposure to sublethal

copper levels results in the loss of chemosensory function, which affects predator-avoidance behavior (McIntyre et al., 2008). The exact mechanisms are not yet completely understood and are still under investigation (Kiaune and Singhasemanon, 2011).

The aim of the present work is to investigate the effect of low concentration of copper ions (Cu^{2+}) on the three different OSNs present in the olfactory epithelium of teleost fish, which are ciliated, microvillous, and crypt neurons. Image analysis quantification of immunohistochemically detected neuronal molecular markers was applied to differentiate neuronal response to Cu^{2+} exposure.

The evaluation of the effect of metals in fish is complicated also by the different toxicity reported in the literature concerning different species and different experimental conditions (Azizishirazi et al., 2013; Song et al., 2015; Tierney et al., 2010; Wang et al., 2013a). The present study was carried out on zebrafish (the freshwater fish *Danio rerio*), which is a model system widely used in embryological, neurological and genetic studies and also possesses numerous advantages in toxicity assessment such as reduced housing costs and small size, which allows quantitative analysis on a large number of slides. (Hill et al., 2005; Miyasaka et al., 2013; Segner, 2009). Furthermore, zebrafish olfactory system, which is histologically comparable to those of other vertebrates, is largely mentioned in the literature (see Tierney et al., 2010), and cells constituting its neuroepithelium are immunologically well characterized (Braubach et al., 2012; Gayoso et al., 2011; Germanà et al., 2007).

2. Materials and methods

2.1. Animals

Forty adults of *Danio rerio* (Hamilton, 1882), 6–8 months old and about 4 cm in total length, were purchased from the Coral Aquarium, Bologna, Italy. They were acclimatized in tanks containing a 1:2 mixture of dechlorinated tap water and distilled water for 30 days, at 25 °C in a 12:12 h light:dark cycle. They were fed twice a day with commercial food distributed by an automatic fish feeder. All procedures conformed to the guidelines of European Communities Council Directive (86/609/CEE), the current Italian legislation regarding the use and care of animals, and the guidelines issued by the US National Institutes of Health. This study was approved by the Scientific Ethics Committee of the University of Bologna (protocol no. 17/79/2014).

2.2. Copper exposure

In the US EPA ECOTOX database (<http://cfpub.epa.gov/ecotox/>), the lethal concentration 50 (LC_{50}) for copper sulfate measured in zebrafish embryos exposed for 48 h was 270 $\mu\text{g L}^{-1}$. In the present study, the concentration of 117 $\mu\text{g L}^{-1}$ of copper sulfate, corresponding to 30 $\mu\text{g L}^{-1}$ of Cu^{2+} , supposed to be sublethal, was employed. Thirty-two fish were moved to 10 L tanks containing 30 $\mu\text{g L}^{-1}$ of Cu^{2+} from copper sulfate (Honeywell, Hanover, D) dissolved in water. A static/renewal system was applied: fish were moved to new tanks containing freshly prepared Cu^{2+} solution every 24 h. Cu^{2+} olfactory toxicity was evaluated after standard 4-day (96 h) exposure, as common use in acute toxicity tests. The water parameters of the acclimatization tank as well as control and treatment tanks were monitored. Representative values are shown in Table 1. The treated animals were divided into four groups, eight specimens each: T0, sacrificed immediately after the 96 h exposure, T3, T10 and T30, sacrificed after 3, 10 and 30 days of recovery, respectively. For recovery, fish were transferred to new 10 L tanks filled with water without addition of copper. Two control fish were sacrificed with every group of treated animals (8 fish in total).

Table 1Water parameters of acclimatization, control, Cu²⁺ exposure, and recovery tanks.

	NO ₃ mg L ⁻¹	NO ₂ mg L ⁻¹	GH dH	KH dH	pH
Acclimatization tanks, new water, before fish insertion	<25	<0.5	14–21°	10	7.4–7.5
Acclimatization tanks, after 24 h fish presence, before water change	<25	<0.5	14–21°	10	7.3–7.4
Control tanks, new water, before fish insertion	<25	<0.5	14–21°	10	7.4–7.5
Control tanks, after 24 h fish presence, before water change	<25	<0.5	14–21°	10	7.3–7.4
Exposure tanks, new water, before fish insertion	<25	<0.5	14–21°	10	7.4–7.5
Exposure tanks, after 24 h fish presence, before water change	<25	<0.5	14–21°	10	7.3–7.4
Recovery tanks, new water, before fish insertion	<25	<0.5	14–21°	10	7.4–7.5
Recovery tanks, after 24 h fish presence, before water change	<25	<0.5	14–21°	10	7.3–7.4

dH, degree of hardness; GH, general hardness; KH, carbonate hardness.

2.3. Tissue

Animals were anesthetized with 0.1% 3-aminobenzoic acid ethyl ester (MS-222, Sigma, St. Louis MO, USA) and killed by decapitation. After removal of the dorsal cranium, the heads were promptly immersed in a modified Bouin's fixative solution, consisting of a saturated aqueous solution of picric acid and formalin (ratio 3:1), for 24 h at room temperature.

After fixation and picric acid removal, the heads were decalcified in 0.25 M EDTA in 0.1 M phosphate buffer, pH 7.4, for 9 days at room temperature, dehydrated and then embedded in Paraplast Plus (Leica Biosystems, Richmond, IL, USA; melting point 55–57 °C). Frontal serial 5 μm-thick sections were collected on silane-coated slides (Sigma). Adjacent slides were used for immunohistochemical detection.

2.4. Immunohistochemistry

Details are reported in Lazzari et al., 2016. Deparaffinized and rehydrated sections were incubated separately overnight with six primary antibodies, in a moist chamber on a floating plate at 4 °C:

a) monoclonal mouse anti-proliferating cell nuclear antigen (anti-PCNA; Clone PC10; P 8825; Sigma; used dilution 1:500) to detect dividing cells (Bettini et al., 2016; Iqbal and Byrd-Jacobs, 2010); b) rabbit polyclonal anti-G_{αolf} (sc-385; Santa Cruz Biotechnology, Santa Cruz, CA, USA; used dilution 1:500), a marker for ciliated olfactory neurons (Braubach et al., 2012; Gayoso et al., 2011); c) rabbit polyclonal anti-calretinin (AB5054; Chemicon International, Temecula, CA, USA; used dilution 1:1,000), detecting a heterogeneous population of ciliated and microvillous olfactory neurons (Bettini et al., 2016); d) mouse monoclonal anti-human neuronal protein HuC/HuD (anti-HuC/D; Clone 16A11; A-21271; Molecular Probes, Eugene, OR, USA; used dilution 1:100), a nuclear marker of immature and mature neurons (Bettini et al., 2016; Iqbal and Byrd-Jacobs, 2010); e) rabbit polyclonal anti-tyrosine protein kinase A (anti-TrkA; sc-118; Santa Cruz Biotechnology; used dilution 1:100), a marker for crypt olfactory cells (Ahuja et al., 2013; Bettini et al., 2016); f) rabbit polyclonal anti-transient receptor potential cation channel, subfamily C, member 2 (anti-TRPC2; LS-C95010; LifeS-pan BioSciences, Seattle, WA, USA; used dilution 1:200), a marker for microvillous olfactory neurons (Bettini et al., 2016). Sections were incubated for 1 h 30 min in the secondary antibody: HRP-conjugated goat anti-mouse IgG (A4416; Sigma; used dilution 1:100) for monoclonal primary antibodies, and HRP-conjugated goat anti-rabbit IgG (PI-1000; Vector Laboratories, Burlingame, CA, USA; used dilution 1:100) for polyclonal primary antibodies. The immunoreaction was visualized using 0.1% 3,3'-diaminobenzidine (DAB; Sigma) as substrate. Nuclear counterstaining with Gill's hematoxylin was carried out to enhance the contrast, with the exception of sections incubated with anti-PCNA antibody. Anti-TRPC2 is specific against zebrafish antigens while all the other antibodies here employed were previously successfully tested by other authors on zebrafish olfactory organ. Sections were then dehydrated in ethanol, cleared in xylene and coverslipped with Per-

mount (Fisher Scientific, Pittsburgh, PA). Negative controls for the specificity of the immunostaining were obtained by omission of the primary antibodies, replaced by 3% normal goat serum. Sections from control fish were used as positive controls.

2.5. Image acquisition, quantification and statistical analysis

Bright-field images of the sections were taken using an Olympus BH-2 microscope (Olympus Italia, Segrate, Italy) equipped with a BEL BlackL 5000 digital camera (BEL Engineering, Monza, Italy). All images used for quantification were taken under the same light conditions. Adobe Photoshop CS3 (Adobe Systems, San Jose, CA, USA) was used to assemble the micrographs. The micrographs were rotated, resized, added with bars, letters and symbols, and adjusted for brightness and contrast, but the content was not altered. Measurements were taken on semi-serial sections using ImageJ 1.50 g software (<https://imagej.nih.gov/ij/>) and restricted to the sensory region of lamellae, easily distinguishable from non-sensory regions by the staining pattern.

In calretinin-immunolabelled sections, epithelial thickness was measured from the basal lamina to the apical surface, excluding cilia and microvilli. For each section, the value of epithelial thickness was calculated as the average of nine measurements, three values from each of three random lamellae. Sensory and non-sensory epithelial areas were calculated in three lamellae per section and averaged. The two regions were easily distinguishable due to both labelling and presence of long cilia on the surface of non-sensory areas.

The densities of TrkA- and PCNA-positive cells were estimated through physical disector method (Bettini et al., 2016). Briefly: serial pairs of sections were collected and only the cells that were observable in the first section of the pair, but not in the second one were counted. Cells were counted in three lamellae per section using the Cell Counter plugin in ImageJ.

In the quantitative analysis of HuC/D, G_{αolf} and TRPC2 immunolabelling, it was very difficult to count positive cells, since G_{αolf} and TRPC2 antigens appear in limited cytoplasmic portions, while HuC/D positive cells were a lot and too close to each other to apply disector method correctly. Therefore, we compared the immunostaining intensity, which was evaluated as an indirect index of the number of immunopositive cells in a specific lamellar region and measured through Optical Density (OD). The OD of three lamellae per section was calculated according to the method of Iqbal and Byrd-Jacobs (2010) and averaged. When the sections were counterstained with hematoxylin, the Color Deconvolution plugin of ImageJ was used for hematoxylin and DAB separation. Average grey values were obtained in the sensory regions. Background values were evaluated from unstained areas of the tissue. Finally, the following formula was applied: OD = log (background grey value/ROI grey value), where ROI is region of interest.

For each antigen, mean values of zebrafish groups were compared using ANOVA and the LSD post hoc test. In the bar graphs, data were reported as mean values ± standard deviations.

3. Results

In zebrafish, the treatment with Cu^{2+} resulted in no micro-anatomical effects: olfactory rosettes preserved their morphology and their lamellae did not show tissue disruption, metaplasia or changes in intraepithelial glands. No animal died during either exposure or recovery.

In the image analysis performed on rosette sections, anti-calretinin antibody, which stains bodies and dendrites of various OSNs, revealed that in T0 and T3 the thickness of the sensory epithelium from the basal lamina to the free surface was decreased, about 7% and 4% compared to the control, respectively (Fig. 1a, b). In T10 and T30, the epithelium thickness was statistically equivalent to the control. In some Cu^{2+} -treated specimens, narrow immunonegative zones, which therefore appeared lacking OSNs, interrupted olfactory epithelial continuity. In anti-calretinin-stained sections, image analysis was also applied to observe the ratio between sensory and nonsensory epithelium areas in the lamellae of olfactory rosette (Fig. 1c, d). In particular, when the ratio was calculated in the lamellae of the dorsal half of the rosette, a significant increase was found in T30 specimens, about 37% compared to the control (Fig. 1c). When the analysis was applied to the lamellae of the ventral half of the rosette, a significant increase of the ratio was already observed in T0, about 84% compared to the control, and the value remained statistically unchanged during recovery (Fig. 1d).

In sections stained with anti-HU antibody, no significant change in total neurons was observed between treated specimens compared to controls (Fig. 2a, b).

The digital image analysis of sections treated with the other antibodies revealed the response of the different neuronal subpopulations of the OE exposed to copper ions.

In the sections treated with anti- $\text{G}_{\alpha\text{olf}}$ antibody, the immunopositivity was mainly located in the dendritic apical knobs and cilia at the free surface of the OE (Fig. 3a). In T0 specimens, the staining density decreases significantly after copper ion exposure, about 59% compared to the control (Fig. 3b). T3-T30

specimens showed significant staining increase compared to T0, but these values remained statistically lower than the control and T3, T10 and T30 were not significantly different between each other.

In the OE, anti-TRPC2 antibody stained dendrite endings and microvilli of mOSNs (Fig. 4a). All treated specimens had densitometric values significantly lower than the control, about 30%, 20%, 10%, and 30% for T0, T3, T10, and T30, respectively (Fig. 4b). Values appeared statistically homogeneous when the comparison was between treated specimens.

The third subpopulation of OSNs consisted of crypt cells. Crypt cells are big neurons, irregularly distributed along the epithelium of the olfactory lamellae. Their number is lower compared to cOSN and mOSN subpopulations. Therefore, in digital image analysis, the choice method was cell counting after immunolabelling with anti-TrKA antibody instead of color densitometry (Fig. 5a). The population of crypt cells showed no statistically significant change in the four stages considered in the post exposure period (Fig. 5b).

In the damaged OE, the recovery of olfactory cell populations is promoted by an increase in proliferative events, just the same occurring also in physiological conditions. Therefore, in the lamellar sensory epithelium, the count of PCNA-immunopositive cells was used to assess mitotic events following copper ion exposure from T0 to T30 (Fig. 6a). Statistical analysis pointed out a significant increase in T0 and T3, about 154 and 167% compared to the control, respectively, whereas T10 and T30 did not statistically differ from the control value (Fig. 6b).

4. Discussion

In the olfactory epithelium of zebrafish, three main populations of OSNs are found by means of immunohistochemical detection of several molecular markers. The results of the present study provide clear evidence of a differential response of the neural cell populations of zebrafish olfactory mucosa to environmental Cu^{2+} present at the sublethal concentration of $30 \mu\text{g L}^{-1}$.

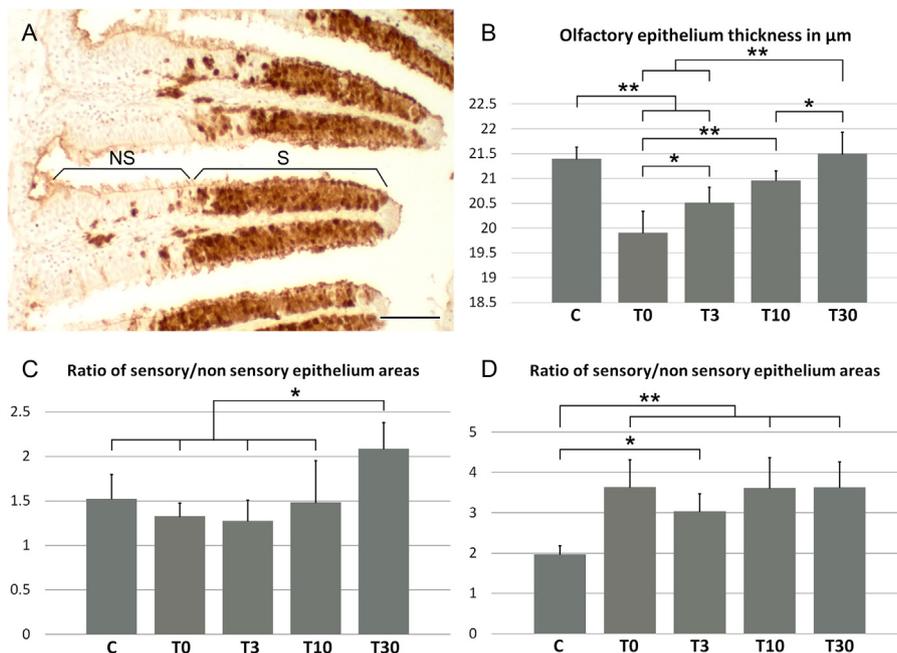


Fig. 1. Morphological analysis of zebrafish olfactory epithelium before and after $30 \mu\text{g L}^{-1} \text{Cu}^{2+}$ exposure; calretinin immunohistochemistry. A) In control fish, the sensory area (S) of the olfactory lamellae shows heavy anti-calretinin immunoreaction; immunostaining is not present in the non-sensory region (NS) of the lamellae. Scale bar: $50 \mu\text{m}$. B) Variation in sensory epithelium thickness during treatments. C) Ratio between non-sensory and sensory areas of the epithelium covering the lamellae in the dorsal half of the olfactory rosette. D) Ratio between non-sensory and sensory areas of the epithelium covering the lamellae placed in the ventral half of the olfactory rosette. C, control; T0, 0 days of recovery; T3, 3 days of recovery; T10, 10 days of recovery; T30, 30 days of recovery. Asterisks indicate significant differences: *, $P < 0.05$; **, $P < 0.01$.

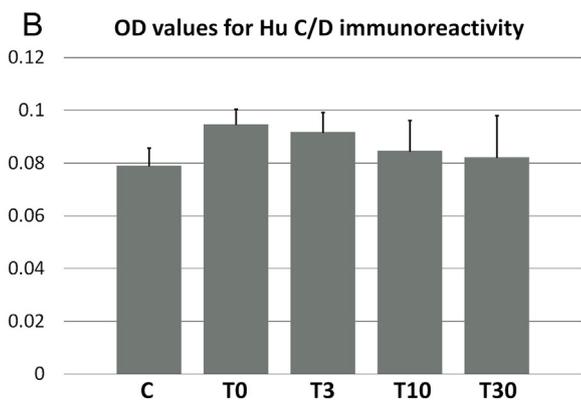
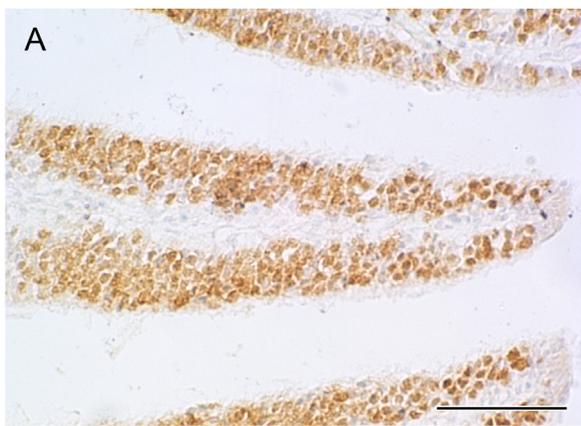


Fig. 2. HuC/D immunohistochemistry in zebrafish olfactory epithelium before and after $30 \mu\text{g L}^{-1} \text{Cu}^{2+}$ exposure. A) Along the sensory regions in the olfactory lamellae of control fish, all the neurons show clear immunopositive cell bodies. Scale bar: $50 \mu\text{m}$. B) Optical density analysis of the olfactory epithelium and comparison between control and treated groups. No significant difference results from the statistical analysis of data. C, control; T0, 0 days of recovery; T3, 3 days of recovery; T10, 10 days of recovery; T30, 30 days of recovery.

In our experiments, cOSNs are affected by Cu^{2+} showing densitometric values of immunostaining decreasing by about 60% compared to the control. The differences in the optical density of the immunostaining could be related to cell death observed in previous works (Kolmacov et al., 2009; Sovová et al., 2014; Wang et al., 2013b). When treated fish are transferred to water without addition of copper, our densitometric measures indicate that a slow recovery takes place and it is already statistically appreciable after 3 days and more evident after 10 days. The recovery rate did not speed up, as T30 is not statistically different from T10. Therefore, we can suppose that the restoration of a normal $G_{\alpha \text{ olf}}$ immunostaining pattern, if possible, would take more than one month.

In goldfish (*Carassius auratus*), sublethal Cu^{2+} exposure ($100 \mu\text{M}$) is reported neither to alter appreciably nor to eliminate mOSNs and their microvilli (Kolmacov et al., 2009). Accordingly, in our treated specimens, these apical processes are still visible and immunostained. However, the densitometric analysis for anti-TRPC2 immunostaining revealed a statistically significant decrease, of about 30%, compared to the control. Also for mOSNs, the differences in the optical density could be indicative of cell death and, therefore, of some differences between the two species. In their review, Tierney et al. (2010) concluded that copper exposure causes morphological changes in the olfactory epithelium of salmonids ranging from slight (ciliary loss) to severe (OSNs death). In zebrafish mOSNs at T0, the densitometric reduction compared to the control is about a half of that measured for cOSNs. Therefore, cOSNs appear more affected than mOSNs, and the difference between

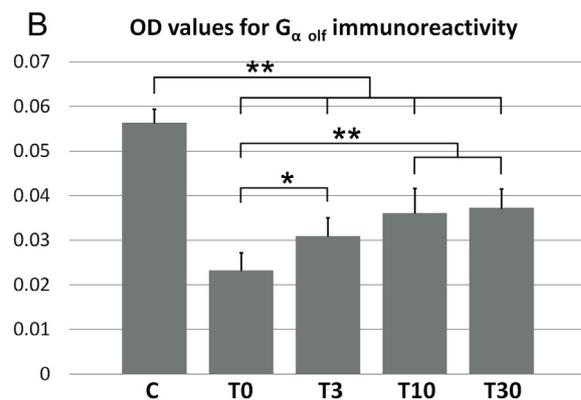
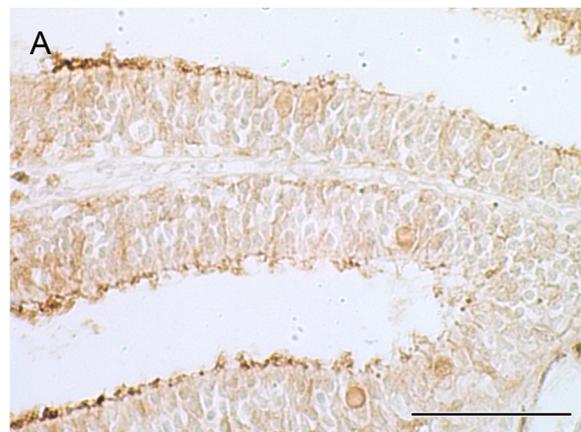


Fig. 3. $G_{\alpha \text{ olf}}$ immunohistochemistry in zebrafish olfactory epithelium before and after $30 \mu\text{g L}^{-1} \text{Cu}^{2+}$ exposure. A) Along the sensory regions in the olfactory lamellae of control fish, the superficial zone of the olfactory epithelium contains the receptor cell knobs which show different staining intensity for $G_{\alpha \text{ olf}}$ immunohistochemistry. Scale bar: $50 \mu\text{m}$. B) Optical density analysis of the olfactory epithelium surface and comparison between control and treated groups. C, control; T0, 0 days of recovery; T3, 3 days of recovery; T10, 10 days of recovery; T30, 30 days of recovery. Asterisks indicate significant differences: *, $P < 0.05$; **, $P < 0.01$.

them turns out to be greater than in goldfish (Kolmacov et al., 2009). The observation that copper affects cOSNs relative to mOSNs has been previously reported by Dew et al. (2014) using a functional approach with electro-olfactograms. In the post-exposure period of 1 month, mOSNs did not show statistically significant increase in immunostaining; consequently, we did not found recovery of mOSNs. Their limited or complete recovery in longer times remains speculative.

In our study, we have an indirect evidence of a decrease in the number of cOSNs and mOSNs since densitometric analysis reveals only a decrease in immunostaining intensity. According to Kolmacov et al. (2009), copper ion exposure could result in alteration or loss of cilia. Therefore, in zebrafish, the decrease in immunostaining signal could be caused by alterations in the morphology of dendrite endings (reduction of cilia and microvilli) and not exclusively by cell death (Jenkins et al., 2009). In accordance with Tilton et al. (2008, 2011) and Wang et al. (2013c), reduction in antigen expression without cell death or morphology alteration could be another explanation.

The third population of OSNs consists of crypt cells (Ferrando et al., 2006; Gayoso et al., 2011, 2012; Hansen and Finger, 2000; Hansen and Zeiske, 1998; Lazzari et al., 2007). Previous immunohistochemical studies on these specific cells were performed using S100 as molecular marker (Braubach et al., 2012; Germanà et al., 2004; Sato et al., 2005). However, anti-S100 antibody was reported to stain not only crypt neurons but also some other cells (Germanà

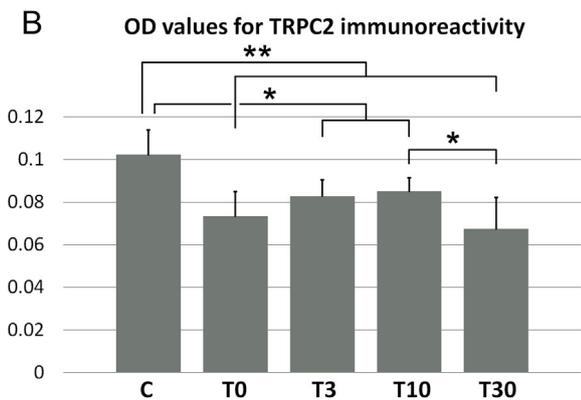
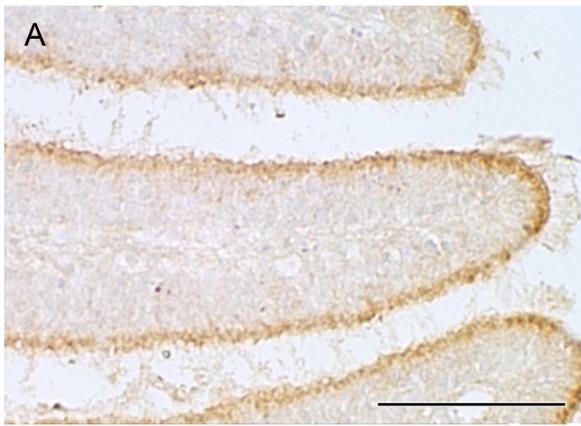


Fig. 4. TRPC2 immunohistochemistry in zebrafish olfactory epithelium before and after $30 \mu\text{g L}^{-1} \text{Cu}^{2+}$ exposure. A) Along the sensory areas in the olfactory lamellae of control fish, the apical region of the olfactory epithelium contains the receptor cell endings which are stained by anti-TRPC2 antibody. Scale bar: $50 \mu\text{m}$. B) Optical density analysis of the olfactory epithelium surface and comparison between control and treated groups. C, control; T0, 0 days of recovery; T3, 3 days of recovery; T10, 10 days of recovery; T30, 30 days of recovery. Asterisks indicate significant differences: *, $P < 0.05$; **, $P < 0.01$.

et al., 2004; Oka et al., 2012; Sato et al., 2005); thus it is considered nonspecific (Ahuja et al., 2013). A strong and specific marker, which could be used to recognize crypt neurons in zebrafish, is anti-TrkA antibody (Ahuja et al., 2013; Bettini et al., 2016), originally used by Catania et al. (2003). Consequently, in the present study, we used anti-TrkA to detect crypt cell population in zebrafish. Although crypt cell counting could suggest an increase in crypt cell number at the end of the exposure, statistical analysis excludes any significant difference in crypt cell number. Therefore, in our experiment, crypt cells appeared not affected by Cu^{2+} exposure at the concentration of $30 \mu\text{g L}^{-1}$. Crypt cells bear both cilia and microvilli, but these apical structures are contained in a deep concavity opening at the apical surface of the cell with a narrow gap and with possible limited access of substances from the mucous layer covering the epithelial surface. In cOSNs and mOSNs, microvilli and cilia, which appear to be the most sensitive structures, are directly exposed to the outer environment. It is possible that this different position protects the sensitive structures of crypt cells from environmental pollutants, such as heavy metals.

In anti-HuC/D immunostaining, statistical analysis of densitometric values showed that there was no significant difference in the number of neurons in the analyzed groups. Although cOSNs and mOSNs showed a clear staining decrease in their specific markers in T0 compared to the control, the statistically unvaried number of total neurons suggests the existence of undifferentiated neurons, i.e. expressing neither $G \alpha_{\text{olf}}$ nor TRPC2.

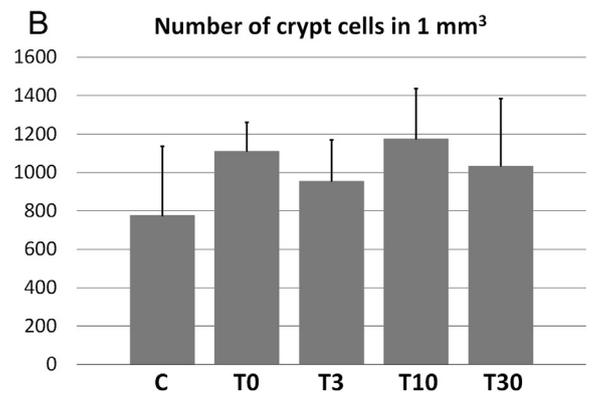
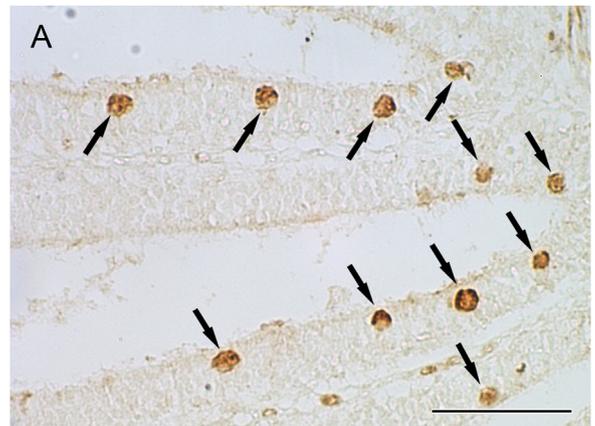


Fig. 5. TrkA immunohistochemistry in zebrafish olfactory epithelium before and after $30 \mu\text{g L}^{-1} \text{Cu}^{2+}$ exposure. A) In control specimen, crypt cells (arrows) are present close to the surface of the olfactory epithelium in the sensory area of the olfactory lamellae. Scale bar: $50 \mu\text{m}$. B) Density of crypt cells and comparison between control and treated groups. No significant differences result from the statistical analysis of data. C, control; T0, 0 days of recovery; T3, 3 days of recovery; T10, 10 days of recovery; T30, 30 days of recovery.

In T0 and T3, anti-PCNA immunostaining increased about 1.5 times compared to the control, whereas in T10 and T30 the immunostaining reverted to values statistically equivalent to the control. These data indicate an increase in the mitotic activity, which coincides with the decrease of $G \alpha_{\text{olf}}$ and TRPC2 immunoreactivity. In the OE, mitotic activity and neuronal differentiation take place physiologically throughout life to substitute OSNs, which come naturally to the end of their life span. In the OE, the mature OSNs downregulate the growth and differentiation of new receptor cells starting from neuronal progenitors (basal cells) in order to keep the size of their populations at a defined level (Wu et al., 2003). During Cu^{2+} exposure, OSNs may decrease their number due to degeneration (decrease in immunostaining) and during recovery, in water without addition of copper, they may gradually increase their number by proliferation and differentiation (increase in immunostaining). The Cu^{2+} exposure has removed the inhibition and consequently activated the response of the germinal layer of the olfactory epithelium, a process already described in mouse (Ducray et al., 2002; Min et al., 2003). In *Poecilia reticulata*, using a monoclonal antibody against GAP-43, a phosphoprotein involved in neuronal differentiation, we demonstrated that, after mitosis, the new differentiating elements migrate from the epithelial basal layer and reach the upper layer in three days. These data from anti-GAP-43 are in agreement with those obtained by 3H-thymidine in mice and anti-BrdU in the lacustrine sockeye salmon (Mackay-Sim and Kittel, 1991; Yanagi et al., 2004).

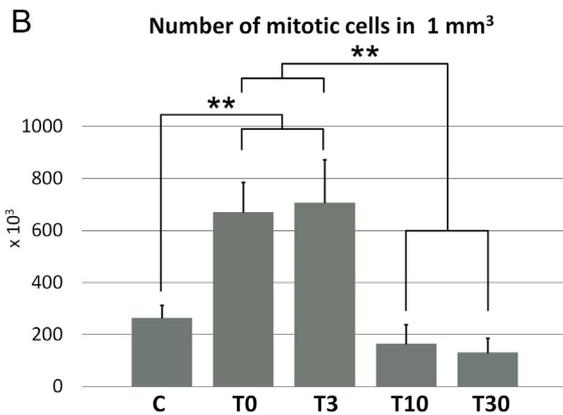
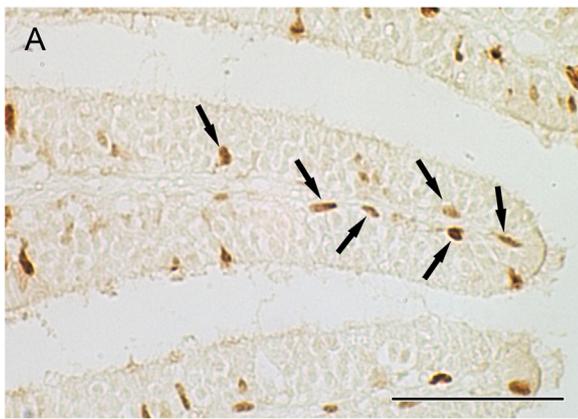


Fig. 6. PCNA immunohistochemistry in zebrafish olfactory epithelium before and after $30 \mu\text{g L}^{-1} \text{Cu}^{2+}$ exposure. A) In control specimen, mitotic cells (arrows) are visible in the sensory area of the epithelium covering olfactory lamellae. Scale bar: 50 μm . B) Density of dividing cells and comparison between control and treated groups. C, control; T0, 0 days of recovery; T3, 3 days of recovery; T10, 10 days of recovery; T30, 30 days of recovery. Asterisks indicate significant differences: **, $P < 0.01$.

In our study, the significantly increased value of anti- $G_{\alpha_{\text{olf}}}$ OD observed in T10 compared to T0 is indicative of the complete differentiation of cOSNs. Ciliated terminal knob differentiation is the final step of maturation of the dendritic branch of new receptor cells. The new olfactory cells require about 10 days to reach their complete maturation (*Tilapia mariae*, Bettini et al., 2006a; *Oncorhynchus mykiss*, Julliard et al., 1996).

The same experimental conditions can induce lesions with different extensions depending on the species (*T. mariae*, Bettini et al., 2006a; *Poecilia reticulata*, Bettini et al., 2006b; *O. mykiss*, Julliard et al., 1996). However, the time of differentiation from basal cells to mature ORNs showed no apparent difference in fish and mammals (Bettini et al., 2006a; Graziadei and Monti Graziadei, 1979; Michel et al., 1999). Moreover, receptor cell differentiation takes the same time in both axotomized and untreated olfactory organs (*Salmo trutta*, Moran et al., 1992; *Oncorhynchus nerka*, Yanagi et al., 2004). The present results for anti- $G_{\alpha_{\text{olf}}}$ and anti-PCNA permit us to hypothesize that a period of about ten days is required for cONS maturation also in zebrafish. Therefore, as previously supposed for *P. reticulata* (Bettini et al., 2006b), the time-course of new receptor cell development does not appear influenced by the type of experimental injury, even when it differently affects neuron populations (Iqbal and Byrd-Jacobs, 2010).

Although our results showing an increase in mitotic activity during recovery should indicate that Cu^{2+} exposure caused reduction in neuronal number, we could not exclude that some affected neurons survived. In this case, the heavy metal simply causes a decrease in

antigen expression, while in the recovery period, in water without addition of copper, neurons gradually increase antigen expression again.

The exposure to $30 \mu\text{g L}^{-1} \text{Cu}^{2+}$ caused no macroscopic effects in zebrafish. Specifically, during exposure and recovery, no animal died, no behavioral alteration was detected, and micro-anatomical and histological analyses revealed that olfactory rosettes and lamellae preserved their morphology. This is in accordance with our previous studies on *Tilapia* (Bettini et al., 2006a), in which $20 \mu\text{g L}^{-1} \text{Cu}^{2+}$ caused no histological alterations of the olfactory mucosa, whereas $40 \mu\text{g L}^{-1} \text{Cu}^{2+}$ were followed by some alterations in the olfactory rosette.

In the present study on zebrafish, calretinin immunostaining showed an evident decrease in olfactory epithelium thickness at T0 stage. This is in accordance with the decrease in $G_{\alpha_{\text{olf}}}$ and TRPC2 immunostaining, which could indicate a certain reduction in the number of cOSNs and mOSNs at T0. The increase in mitotic activity revealed by PCNA immunostaining at T0 and the uniform high amount of total neurons, including still undifferentiated neurons, would be responsible for the gradual restoration of the epithelial thickness to normal values in T10 and T30.

In fish olfactory epithelium, non-neuronal cells are less affected by Cu ions (Julliard et al., 1993, 1996; Moran et al., 1992). In our study, anti-calretinin immunostaining revealed a different behavior of the sensory area between the lamellae of the dorsal half and those of the ventral half of the olfactory rosettes. Whereas in the dorsal lamellae, the sensory area appears increased only in T30 compared to the control, in the ventral lamellae, this increase is already significant in T0 and is maintained up to T30. These results indicate that $30 \mu\text{g L}^{-1} \text{Cu}^{2+}$ treatment causes an increase in the sensory areas of the olfactory lamellae, in accordance with a functional recovery, after continuous Cu^{2+} exposure for up to 96 h, demonstrated by behavioral and electrophysiological assays in *Col-orado pikeminnow* (Beyers and Farmer, 2001) and *fathead minnow* (Dew et al., 2012). However, in zebrafish, the lamellae of the dorsal and ventral halves of the olfactory rosettes show different time of reaction. Calretinin immunostaining indicates a contribution of both cOSNs and mOSNs to the signal, but, considering $G_{\alpha_{\text{olf}}}$ and TRPC2 immunostaining, the delayed increase of sensory areas in dorsal lamellae could be attributed to a delayed increase of the cOSNs with negligible contribution by mOSNs. On the contrary, in the ventral lamellae, the early increase of sensory areas could be ascribed especially to mOSNs. Reduction in epithelium thickness and sensory area increase imply that during epithelium recovery immature neural cells can migrate into adjacent non-sensory regions. This event would especially affect ventral lamellae showing high sensory to non-sensory area ratio.

5. Conclusions

Fish are at risk of exposure to heavy metals introduced in the aquatic environment from various human activities and growing evidence supports that copper, even at low concentration, can cause deleterious effects on olfaction influencing behavior and hence ecology. Different sensitivity to copper exposure not only exists among species as reported in the literature, but also is present among different types of receptor neurons inside the olfactory epithelium as shown by the present study. Immunohistochemical detection of molecular markers revealed a differential response of zebrafish OSNs to $30 \mu\text{g L}^{-1} \text{Cu}^{2+}$ exposure. In particular, cOSNs turned out to be the most sensitive whereas mOSNs had a weaker response. Crypt neurons appeared unaffected.

Alsop and Wood (2011, 2013) reported that the disruption of ion uptake following metal exposure does not appear to be responsible for the acute toxicity, as previously suggested, but rather the toxic-

ity is the result of total ion loss, mostly Na⁺. Moreover, Cu exposure can affect the transcriptions of both subunits of Na/K-ATPase in fish olfactory epithelium (Azizishirazi et al., 2015).

The underlying processes leading to these differences among receptor cells are not fully understood. Different functions were attributed to the three types of OSNs (Ahuja et al., 2013; Bazães and Schmachtenberg, 2012; Biechl et al., 2016; Hamdani and Døving, 2006; Sato and Suzuki, 2001; Vielma et al., 2008). Moreover, many studies have focused on behavioral effects of Cu exposure in fish (Acosta et al., 2016; Poulsen et al., 2014; Scott and Sloman, 2004; Simonato et al., 2016; Sovová et al., 2014; Svecevičius, 2012; Tilton et al., 2011). Comparative studies addressing structure and function of the different OSNs in the different species would greatly enhance our understanding of the mechanisms of olfaction, supplying additional information on neurophysiology, behavior and ecology of fish.

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