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Copper at low levels impairs memory of adult zebrafish (*Danio rerio*) and affects swimming performance of larvae

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ABSTRACT

Metal contamination at low levels is an important issue because it usually produces health and environmental effects, either positive or deleterious. Contamination of surface waters with copper (Cu) is a worldwide event, usually originated by mining, agricultural, industrial, commercial, and residential activities. Water quality criteria for Cu are variable among countries but allowed limits are generally in the μg/L range, which can disrupt several functions in the early life-stages of fish species. Behavioral and biochemical alterations after Cu exposure have also been described at concentrations close to the allowed limits. Aiming to search for the effects of Cu in the range of the allowed limits, larvae and adult zebrafish (*Danio rerio*) were exposed to different concentrations of dissolved Cu (nominally: 0, 5, 9, 20 and 60 μg/L; measured: 0.4, 5.7, 7.2 16.6 and 42.3 μg/L, respectively) for 96 h. Larvae swimming and body length, and adult behavior and biochemical biomarkers (activity of glutathione-related enzymes in gills, muscle, and brain) were assessed after Cu exposure. Several effects were observed in fish exposed to 9 μg/L nominal Cu, including increased larvae swimming distance and velocity, abolishment of adult inhibitory avoidance memory, and decreased glutathione S-transferase (GST) activity in gills of adult fish. At the highest Cu concentration tested (nominally: 60 μg/L), body length of larvae, spatial memory of adults, and gill GST activity were decreased. Social behavior (aggressiveness and conspecific interaction), and glutathione reductase (GR) activity were not affected in adult zebrafish. Exposure to Cu, at concentrations close to the water quality criteria for this metal in fresh water, was able to alter larvae swimming performance and to induce detrimental effects on the behavior of adult zebrafish, thus indicating the need for further studies to reevaluate the currently allowed limits for Cu in fresh water.

1. Introduction

Zebrafish (*Danio rerio*) has been considered a useful model for neurotoxicological tests, adding new tools to classical toxicological studies (Baldissarelli et al., 2012; Linney et al., 2004). Zebrafish is also considered a model for neurological disorders, such as epilepsy (Leclercq et al., 2015), Parkinson's (Fetcho, 2007) and Alzheimer's diseases (Newman et al., 2014). In this regard, during neurodegenerative diseases, copper (Cu) released from nerve cells can reach toxic levels (200–400 μM) (White and Cappai, 2003). Such increase in endogenous Cu levels would affect biochemical as well as behavioral endpoints (Mathie et al., 2006).

Studies focused on fish exposure to Cu are often reported in the literature, particularly those relating to environmental contamination (Brandão et al., 2013; Falfushynska et al., 2011; Hernández and Allende, 2008; Sandahl et al., 2007). However, studies on trace metals using zebrafish as a model generally report biochemical alterations more frequently than behavioral ones (Baldissarelli et al., 2012). Nevertheless, behavioral alterations (Baatrup, 1991; Rehnberg and Schreck, 1986) have been documented for several metals (Baldissarelli et al., 2012; Vieira et al., 2009; Weber and Ghorai, 2013), including Cu (Campbell et al., 2005; Sloman et al., 2003).

Exposure to Cu at low concentrations (μg/L range) can affect neuromodulation in the fish brain. Exposure to a sublethal concentration of Cu (15 μg/L) for 96 h can affect brain extracellular enzymes, such as nucleotidases, decreasing their expressions and activities (Rosemberg et al., 2007). Also, it can inhibit cholinesterase activity (de Lima et al., 2013; Haverroth et al., 2015). Exposure to Cu at 20 μg/L for 96 h inhibited carbonic anhydrase activity and increased the H^+/Na^+ exchange activity in the guppy *Poecilia vivipara* (Zimmer et al., 2012).

Neurochemical effects can be translated into behavioral responses. For instance, Cu (25–50 μg/L) triggers a rapid loss of ciliated mechanoreceptor neurons from neuromasts in the lateral line, presenting the potential for disrupting behaviors in fish that depend on olfaction and

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mechanosensory information (Linbo et al., 2006; Olivari et al., 2008). For example, coho salmon showed an important loss of chemosensory response (McIntyre et al., 2008), which increased predator capture (McIntyre et al., 2012). Low levels of Cu (<10 μg/L), produced a significant inhibition of olfactory activity in fathead minnows, which are below the IC_{50} predicted by the biotic ligand model (BLM) (Dew et al., 2012). Indeed, chemosensory and mechanosensory systems appear to be among the main targets of Cu toxicity, even at low levels (Dew et al., 2012; Linbo et al., 2006; McIntyre et al., 2008, 2012). These examples highlight the marked toxicity of Cu in fish, with potentially deleterious effects at low and environmentally relevant concentrations of this metal.

Like adults, larvae of zebrafish have also been used in toxicological studies. Exposure to Cu (93 μg/L or higher) at 72 h post fertilization (hpf) increased yolk sac area, heart beats, and embryo mortality, as well as decreased hatching and length of zebrafish larvae (Johnson et al., 2007). Median effective concentration for Cu-induced morphological effects was estimated as18 μg/L for zebrafish embryo. These effects were associated with a significant loss of primary and secondary motor neurons (Sonnack et al., 2015). Similarly to the observed in adult zebrafish, the olfactory system (Sandahl et al., 2007) and the lateral line sensory cells of zebrafish larvae were affected by Cu exposure (Hernández et al., 2006; Linbo et al., 2006).

In light of the above, the aim of the present study was to investigate the effects of waterborne Cu exposure in larvae and adult zebrafish. There are reports demonstrating that Cu at low levels (μg/L range) can cause adverse effects in fish (De Boeck et al., 2006; de Lima et al., 2013; Haverroth et al., 2015; Linbo et al., 2006; Moreira-Santos et al., 2008; Sloman et al., 2003). Taken this into account, we investigated if Cu at environmentally relevant concentrations can induce adverse effects on morphological (body length), and behavioral (swimming performance) endpoints in larval zebrafish, as well as behavioral (aggressiveness, social interaction and memory) and biochemical (glutathione reductase, glutathione transferase, and glutathione peroxidase activity) endpoints in adult zebrafish.

2. Materials and methods

2.1. Animals

Adult wild-type zebrafish with Tubingen background of both sexes were obtained from the colony maintained by the Pontifical Catholic University of Rio Grande do Sul (Porto Alegre, RS, southern Brazil). Fish were maintained in automated re-circulating tank system (ZEBTEC, Tecniplast, Buguggiate, Varese, Italy). Fish were maintained under a 14 h light:10 h dark photoperiod at a density of up to five animals per liter of water. Fish were fed three times a day with TetraMin Tropical Flake Fish®, supplemented with brine shrimp larvae.

Zebrafish larvae were obtained by breeding animal from the stock held by the Pontifical Catholic University of Rio Grande do Sul. Just after fertilization, eggs were saved and kept at 26 ± 2 °C and under a 14 h light:10 h dark photoperiod for 72 hpf.

After experiments, adult and larvae of zebrafish were euthanized by hypothermia. Each individual either was used once, for a given behavioral test or for sampling collection for biochemical analyzes. All protocols were in accordance with the local regulation and international guidelines and approved by the institutional Animal Ethics Committee (permits # 12/00,319 and 10/00,196 CEUA PUCRS).

2.2. Water of maintenance and copper exposure

Water used in the experiments was obtained from a reverse osmosis apparatus (18 mohm/cm) and reconstituted with marine salt (Cristalsea™, Marinemix, Baltimore, USA) at 0.4 ppt, resulting in the following composition in mmol/L (Atkinson and Bingman, 1999): Na^+ 5.34, K^+ 0.115, Ca^{2+} 0.115, Mg^{2+} 0.605, Cl^- 6.15 and SO_4^{2-} 0.32. Total organic carbon concentration was 0.33 mg/L. Total alkalinity as CO_3^{2-} corresponded to 0.030 mEq/L. Water pH was 7.4. During fish maintenance, water parameters were monitored daily and kept in the following ranges: pH: 6.5 to 7.5, conductivity: 400 to 600 μS, ammonium concentration: <0.004 ppm, and temperature: 25 to 28 °C.

Copper concentration was measured before and after addition of Cu (nominally: 5, 9, 20, and 60 μg/L) in the water of maintenance. Water samples were collected before and 48 h after introduction of fish in the experimental tanks. Water samples were filtered (0.45 μ-mesh filter) and acidified with HNO_3 (Suprapur, Merck, Darmstadt, Germany) at 1% final concentration. Copper concentration was determined using a high-resolution continuum source atomic absorption spectrometry (HR-CS-AAS; ContrAA 700 Analytik Jena, Germany). Quality assurance controls were performed. A standard copper solution (SpecSol®, QuimLab, Jacareí, SP, Brazil) was used to build a standard curve and check measurement accuracy. Percentage of metal recovery corresponded to 103.5 ± 0.7%.

Adult zebrafish (5 fish/L) were exposed to different Cu concentrations (nominally: 0, 5, 9, 20 and 60 μg/L copper) for 96 h. Copper as $CuCl_2$ was added in the water of maintenance. During copper exposure, fish were not fed with brine shrimp larvae and water was replaced every two days. Fish were kept in a 5-L aquarium (27 cm width × 17 cm height × 12 cm deep) with water of maintenance, which was kept under biological and mechanical filtration, aeration (7.2 mg O_2/L) and temperature control (28 ± 2 °C).

Larvae (72 hpf) were also exposed to different concentrations of dissolved Cu (nominally: 0, 5, 9, 20 and 60 μg/L copper) for 96 h. Copper as $CuCl_2$ was added into the water of maintenance, and gently handled and individually placed in a 12-well plate (n = 10 per treatment). They were maintained under the same conditions described above for adult fish. Control fish were kept under the same conditions used for Cu-exposed fish, but without Cu addition in the water of maintenance.

2.3. Assessment of larvae body length

Body length of larvae was measured by using images obtained from Nikon stereoscopy microscopy using the NIS-Elements D 3.2 software provided by the manufacturer (Nikon Instruments Inc., Japan). Body length was standardized as the distance from the center of an eye to the tail end (Capiotti et al., 2013).

2.4. Locomotor activity of zebrafish larvae

After 96 h of Cu exposure, larvae were placed in fresh media solution and transferred to a 96-well plate. Locomotor activity was recorded during 5 min by a top camera (Quikcam PRO 9000 - Longitec™, California, EUA), and analyzed by ANY-Maze video tracking software (Stoelting Co., Wood Dale, EUA). Total distance (m), average swimming velocity (m/s), and maximal velocity (m/s) were obtained as previously described (Capiotti et al., 2013).

2.5. Spatial memory training-test in adult zebrafish

Spatial memory of adult fish was assessed using the Y maze task. In this context, fish were trained and tested individually in Y-shaped glass aquaria, with three arms (25 cm long × 8 cm wide × 15 cm height): the initial arm, designated the "start arm"; the adjacent arm (always open) was named "other arm", and the new arm (was open only during the test) was designated "novel arm". The external maze walls presented visual clues. During the training session (5 min), fish was individually placed in the start arm with a novel arm closed. After one hour, fish was placed again in the start arm and subjected to an additional test session (5 min). However, in this case, the new arm was opened (Cognato et al., 2012). Training and test sessions were recorded using the Quikcam PRO 9000 camera (Longitec™, California, EUA). The determination of the time spent in each arm was performed using the software

ANY-Maze (Stoelting Co., Wood Dale, IL, USA). The center of the maze (neutral zone) was not computed in the analysis (Dellu et al., 1997).

2.6. Inhibitory avoidance task in adult zebrafish

Analogous to the avoidance test performed with rodents, this test was adapted for testing fish (Blank et al., 2009; McGaugh, 2000). An apparatus measuring 18 cm long × 9 cm wide × 7 cm height, and made of glass was used, as previously reported (Blank et al., 2009). The apparatus was separated by a sliding guillotine-type partition (9 cm height × 7 cm wide) in two equally sized compartments, white and dark. In the training session, adult fish were individually placed on the white side with the partition closed. After 1 min of habituation, the partition was raised 1 cm, allowing fish to cross to the dark side of the tank. Just after fish entered the dark compartment, the slide partition was closed and a pulsed electric shock of 3 ± 0.2 V (intensity measured between electrodes and the center of the dark compartment) was administered for 5 s. Fish were removed from the apparatus and placed in a temporary tank; they were later returned to their housing tank. Twenty-four hours after a training session, the test session was performed, following the same procedure, except that the aversive stimulus (shock) was not applied. The latency to enter the dark compartment was measured as an indicative of memory retention, including the learned association between the aversive stimulus and the dark environment entry.

2.7. Aggressive behavior in adult zebrafish

Aggressive behavior was evaluated in adult fish by the mirror test (Gerlai et al., 2000; Moretz et al., 2006) in a test aquaria (36 long × 25 cm wide and 18 cm height) filled with 6 L of water, where a mirror (45 × 38 cm) was placed at the side of the tank at an angle of 22.5°. After 60 s of fish introduction in the aquaria, aggressive interactions (bites, displays and fast bouts of swimming) were recorded for a period of 5 min. This protocol has been previously validated by actual fish opponents (Ariyomo et al., 2013; Ariyomo and Watt, 2015). Aggressive behavior (boldness and aggressiveness) in zebrafish can be influenced be genetic background (Ariyomo et al., 2013), but aggressiveness was similar between male and female zebrafish (Dahlbom et al., 2011), indicating the appropriateness of using both sexes.

2.8. Social interaction in adult zebrafish

The zebrafish is a social animal. To test social interaction, fish from the same shoal were used in each experiment. Five experimental fish were placed in a small experimental aquarium (30 cm large × 15 cm height × 10 cm W). On one side of the experimental aquarium, an empty tank was placed; and on the opposite side of the aquarium, a "stimulus tank" of identical size containing 15 zebrafish was introduced. After a 30 s of habituation time, the behavior of experimental fish was recorded during 5 min. In order to quantify any inherent preference for the "stimulus" side, the central tank was "virtually" separated into two equal parts. The time that experimental fish spent in the virtual half, adjacent to the conspecific school, was measured (Gerlai, 2003).

2.9. Biochemical analyses

After behavioral tests, gills, muscle and brain tissues were dissected and stored at −80 °C before analyses. Enzyme activities were expressed relative to protein content, as determined by using bovine serum albumin as standard (Bradford, 1976).

For enzyme activity measurements, frozen tissues were homogenized (1:4 weight: volume ratio) in a buffer solution containing 50 mM tris (hydroxymethyl) aminomethane, 0.15 M KCl, and 0.1 mM phenyl-methane-sulfonyl fluoride (pH 7.4). Homogenates were centrifuged at 9000 *g* for 30 min (4 °C) and the supernatant was used as a cytosolic fraction for the enzymatic analyzes.

Enzyme activities were determined spectrophotometrically using a UV/Vis plate reader. GST activity was assayed considering the rate of glutathione conjugation to 1-chloro-2,4-dinitrobenzene at 340 nm and the spontaneous reaction was subtracted from readings (Habig and Jakoby, 1981). GR activity was measured at 340 nm considering the NADPH consumption rate in the presence of oxidized glutathione (Carlberg and Mannervik, 1985). GPx activity was measured at 340 nm through the NADPH consumption rate in the presence of GR, glutathione and cumene hydroperoxide (Wendel, 1981).

2.10. Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). Homogeneity of variance was checked using the Bartlett's test, and outliers were excluded when detected by the Grubb's test. Enzyme activity and social behavior (aggressiveness and conspecific behaviors) were analyzed by one-way analysis of variance (ANOVA) followed by the Dunnett's test, when appropriate. The variance of data regarding the response to novelty, body length and swimming behavior showed significantly different variances. Therefore, non-parametric ANOVA on ranks was used, and differences among groups were detected by the Dunn's *post hoc* test. The inhibitory avoidance task was tested using the unpaired Student's t test (training *vs* test session). In all cases, the level of significance adopted was 95% ($p < 0.05$).

Data on dissolved Cu concentrations were calculated based on values measured before and 48 h after fish introduction in the water of maintenance. Free Cu concentrations were calculated based on the mean values of dissolved Cu concentrations calculated as described above and the water physicochemical parameters observed during the experimental period. Calculations were performed using the Biotic Ligand Model version 2.2.3. (HydroQual, Mahwah, NJ, USA).

3. Results

3.1. Copper concentrations in the experimental media

Measured dissolved Cu concentrations were 0.40 ± 0.09, 5.74 ± 0.51, 7.25 ± 0.08, 16.56 ± 1.18 and 42.30 ± 0.92 μg/L for the nominal concentrations of 0, 5, 9, 20 and 60 μg/L, respectively. They corresponded to 87.2 ± 9.6% (ranging from 114.8 to 70.5%) of the nominal values. In turn, concentrations of free Cu ion calculated using the BLM approach corresponded to 0.002, 0.065, 0.100, 0.727 and 6.548 μg/L, respectively.

3.2. Copper exposure increased swimming velocity and distance and decreased larvae body length

Exposure to copper significantly ($p < 0.01$) reduced (5%) the body length of larvae exposed to the highest Cu concentration (60 μg/L) tested (Fig. 1).

The average distance traveled was increased ($p < 0.01$) from 0.17 ± 0.03 m to 0.38 ± 0.05 m in larvae exposed to 9 μg/L Cu (Fig. 2A), corresponding to a 2.2-fold increase in the swimming distance of Cu-exposed larvae in comparison to those of the control group. Mean swimming velocity was also increased by Cu treatment ($p < 0.01$). Control values of 0.00052 ± 0.00013 m/s increased to 0.0013 ± 0.00019 m/s in larvae exposed to 9 μg/L Cu (Fig. 2B), which corresponded to a 2.5-fold increase with respect to the control group. Maximal velocity (Fig. 2C) was increased by 1.4-fold in larvae exposed to 9 μg/L Cu ($p < 0.01$) and by 1.8-fold in those exposed to 20 μg/L Cu ($p < 0.01$) with respect to the control group. The observed increase in the distance traveled by larvae exposed to 9 μg/L Cu is in agreement with the higher swimming velocity and maximal velocity observed in this group of larvae. At 5 and 60 μg/L Cu, distance traveled, as well as mean and maximal velocity displayed by larvae were not significantly altered by Cu exposure.

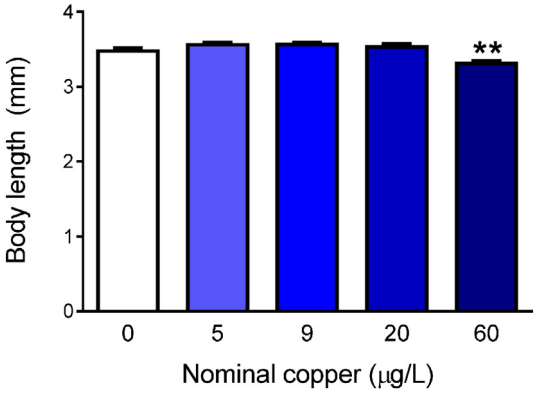

Fig. 1. Body length of zebrafish larvae (72 hpf) exposed to dissolved copper for 96 h. Values are expressed as mean ± S.E.M. (n = 19–21). Significant different mean values with respect to the control group (0) are indicated as ** ($p < 0.01$).

3.3. Behavioral responses of adult zebrafish exposed to copper

Concomitantly to the spatial memory assessment, locomotor activity of adult zebrafish was evaluated considering the distance traveled and the mean velocity. These parameters were not affected by any Cu concentration tested (distance traveled: $p = 0.58$; mean velocity: $p = 0.24$, data not shown), indicating that observed effects in other parameters analyzed are not related to a possible increased locomotor activity. Spatial memory and response to novelty were assessed by Y-maze training-test (Fig. 3). Results showed that exposure to Cu did not alter the time spent in the novel arm, which was homogenous among groups, averaging between 108 ± 21 s and 113 ± 24 s. The time spent by adult fish in the novel arm was significantly increased ($p < 0.05$) with respect to the time spent in the start arm (averaging between 80 ± 13 s and 88 ± 21 s), or in the other arm (averaging between 71 ± 12 s and 95 ± 20 s), except for the group of fish exposed to 60 μg/L Cu. In this case, no significant differences were observed (Fig. 3).

In the aversive memory assessment, fish that retained memory after receiving an electric shock in the training session spent more time in the test session before entering the dark compartment where they previously received the electric shock. Only fish exposed to 9 μg/L Cu failed to acquire memory in the inhibitory avoidance task ($p = 0.21$) since latency did not increase (NS) in the test session (Fig. 4). When comparing the latency time in the test session between fish kept under control conditions and those exposed to 9 μg/L Cu, a significant decrease was also observed. The latency time was reduced from 82.5 ± 21.5 to 25 ± 9.0 s ($p < 0.05$). This response indicates that fish exposed to Cu lost their ability to retain memory. All other fish groups had their latency time increased with respect to those observed in the training session, indicating that their memory retention was preserved.

Aggressive behavior, evaluated by exposing each fish individually to a mirror and recording aggressive display, was not altered by Cu exposure, regardless of the concentration tested (Fig. 5A). In addition, social interaction was not affected by Cu exposure (Fig. 5B).

3.4. Glutathione-related enzymes

The activities of three enzymes related to GSH metabolism (GST, GPx, and GR) were evaluated in gills, muscle, and brain of adult zebrafish. Cu exposure induced changes in GST activity in the gills (Fig. 6B), but not in the brain or the muscle (Fig. 6A and C). GST activity from gills was 67 and 44% lower in fish exposed to 9 and 60 μg/L, respectively. However, no significant changes in GST activity from gills were observed in fish exposed to 5 and 20 μg/L Cu with respect to the control fish (Fig. 6A-C). GPx activity increased 2.1-fold in the muscle of fish exposed to 20 μg/L Cu (Fig. 6D) but showed a lower activity (75%) in the gills of these fish (Fig. 6E). GPx activity remained unaltered in the brain of fish exposed to Cu (Fig. 6F). GR activity was unaltered in muscle, brain, and gills of fish exposed to Cu (5–60 μg/L) for 96 h (Fig. 6G-I).

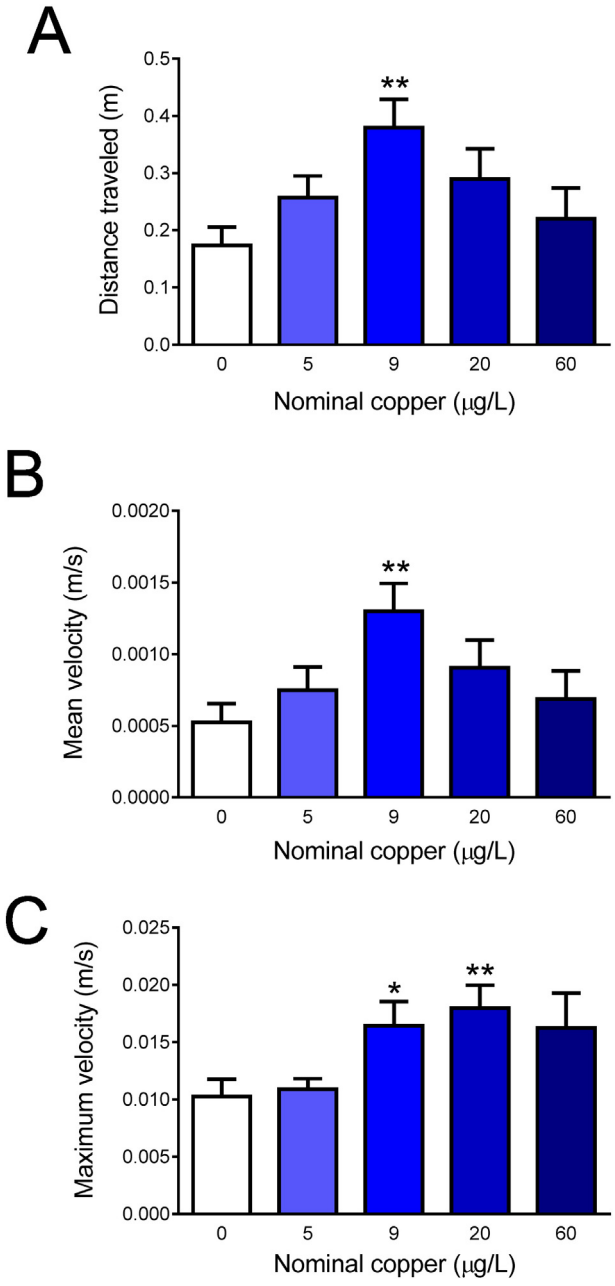

Fig. 2. Swimming performance of zebrafish larvae (72 hpf) exposed to dissolved copper for 96 h. Swimming distance (A), mean velocity (B), and maximum velocity (C) data are expressed as mean ± S.E.M. (n = 16–21). Significant different mean values with respect to the control group (0) are indicated as * ($p < 0.05$) and ** ($p < 0.01$).

4. Discussion

A slightly but significant reduction in body length was observed in zebrafish larvae (96 hpf) exposed to the highest Cu concentration tested (60 μg/L), indicating a detrimental impact of this metal on larvae growth. In a similar study, exposure of *Danio rerio* larvae (72 hpf) to 53 μg/L Cu did not affect embryo mortality, hatching, yolk sac area, heart beats, and body length. However, body length was reduced in larvae exposed to 93 μg/L or higher Cu concentrations (Johnson et al., 2007). Despite the discrepancy in the age of larvae employed by Johnson et al. (1 hpf) and those tested in the present study (96 hpf), body

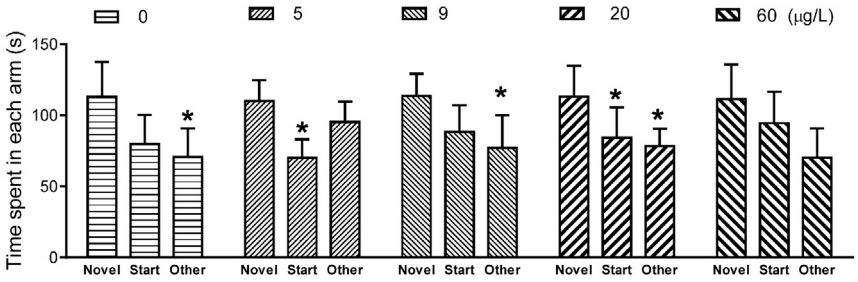

Fig. 3. Spatial memory training-test of adult zebrafish exposed to dissolved copper for 96 h. After being trained, the time spent by fish in each arm of the Y maze was recorded. In the test session, fish are expected to spend more time in the novel or in the other arm of the maze, which was not previously explored (novel), or less explored (other) in the training session. Legends indicate the nominal Cu concentrations. Values are expressed as mean ± S.E.M. (n = 10–12). Significant different mean values with respect to the novel arm are indicated as * ($p < 0.05$).

length was affected at relatively close Cu concentrations in both studies. Therefore, the 5% reduction in body length observed in the present study suggests that 60 μg/L Cu may be close to a threshold limit between toxic and non-toxic levels of Cu for zebrafish larvae.

In addition to the Cu impact on larvae growth, behavioral effects were also observed in both larvae and adult zebrafish tested in the present study. An altered neurological function will generally be behaviorally apparent, and as performed in the present study, it can be analyzed in both larvae and adults of zebrafish (Blank et al., 2009; Irons et al., 2010; Jia et al., 2014). In this context, assessing locomotor activity can indicate compounds that modulate neuron firing rate, thus, presenting potential to affect swimming velocity and distance traveled (Tierney, 2011). In the present study, zebrafish larvae showed an increased mean swimming velocity and distance traveled after being exposed to 9 μg/L Cu for 96 h. In turn, the maximum velocity was affected in larvae exposed to 20 μg/L Cu. Swimming performances of two cyprinids (common and gibel carps) and the rainbow trout were also decreased shortly after (12-24 h) exposure to 30 μg/L (De Boeck et al., 2006). Also, exposure to 105 μg/L Cu induced a reduction in the swimming performance of the rainbow trout (Waser et al., 2009). Increased gill lamellar thickness and ionic imbalance were proposed as a possible mechanism underlying the respiratory distress triggered by Cu exposure, especially in the two cyprinid fish (De Boeck et al., 2007, 2006).

The behavioral alterations observed in the present study after exposure of zebrafish larvae to intermediate concentrations of Cu (9 and 20 μg/L) could be a result from effects of Cu on specific neurochemical circuits such as the cholinergic-mediated signaling, as proposed for

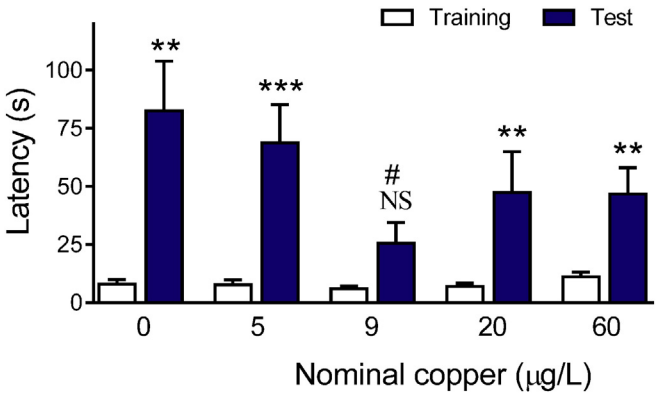

Fig. 4. Inhibitory avoidance task of adult zebrafish exposed to dissolved copper for 96 h. In the training session (Training), fish received an electric shock when entered the dark compartment. The test session (Test) was conducted 24 h later, when fish were resubmitted to the same procedure, except by the absence of shock. The latency to enter the dark compartment was measured. Data are expressed as mean ± S.E.M. (n = 8–11). Significant different mean values with respect to the control group (0) are indicated as NS (non-significant), * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). # indicates significant difference from the Test session of control fish ($p < 0.05$).

adult zebrafish (de Lima et al., 2013; Haverroth et al., 2015). Indeed, Cu effects on the olfactory system have been reported in fish exposed to low concentrations of this metal (<10 μg/L) using a continuous exposure protocol (Dew et al., 2012). Additionally, the number of visible neuromast cells were decreased in *Danio rerio* larvae exposed to 68 μg/L Cu (Johnson et al., 2007). In this case, zebrafish larvae displayed evidence of oxidative stress (Olivari et al., 2008). Interestingly, several oxidative stress parameters were shown to be adequate biomarkers of acute Cu exposure in the euryhaline guppy *Poecilia vivipara* (Machado et al., 2013).

The Cu effects on the sensory system described above were shown to be dependent on the time of exposure to Cu. In fact, neuromast and olfactory cells can be affected within hours of Cu exposure, but show a partial recovery over time (Dew et al., 2012; Linbo et al., 2006). It is worth noting that the swimming performance of the common and gibel carps, as well as the rainbow trout also showed a tendency to recovery over time after had been reduced shortly after (12-24 h) exposure to 30 μg/L Cu (De Boeck et al., 2006). These findings clearly indicate the ability of fish to respond and deal with the acute effects induced by dissolved Cu over time, especially at the range of Cu concentrations tested in the present study. On the mechanism underlying the behavioral responses after Cu exposure, possible effects of this metal on the sensory system of zebrafish larvae seems to be the primary candidates to explain the altered swimming performance observed in the present study. Therefore, future studies focused on the effects of dissolved Cu on the sensory system of zebrafish larvae would be of great interest for a better understanding and interpretation of the behavioral effects of Cu reported in the present study.

Introducing adult fish to new surroundings will induce searching behavior, leading to the formation of cognitive map, which is dependent on neural plasticity. Therefore, the interferences on searching behavior can be used as an index of neurological impairment. Anxiolytic and anxiogenic drugs can interfere with exploratory behavior (Tierney, 2011). In the present study, spatial memory in Cu-exposed adult zebrafish was assessed by using the Y-maze task. Data obtained indicated that exposure to 0, 5, 9 and 20 μg/L Cu did not alter the exploratory behavior of adult zebrafish. Indeed, all fish groups spent more time in the novel arm. Nevertheless, this effect was no longer observed in adult zebrafish exposed to 60 μg/L Cu, given that the time spent in the novel arm of the Y-maze was not significantly different from that observed for the other arms. This result indicates that exposure to a high concentration of dissolved Cu can impair the acquisition and/or consolidation of spatial memory. However, further experiments are necessary to test for reproducibility of these findings and to identify the possible neurochemical targets responsible for the observed effect.

In the present study, two approaches were used to evaluate, social behavior. Firstly, an inclined mirror was used to simulate a conspecific behavior, and to assess aggression. In addition, exposure of a single fish to a group of fish or an empty aquarium was used to assess social interaction. Exposure to dissolved Cu up to 60 μg/L induced no

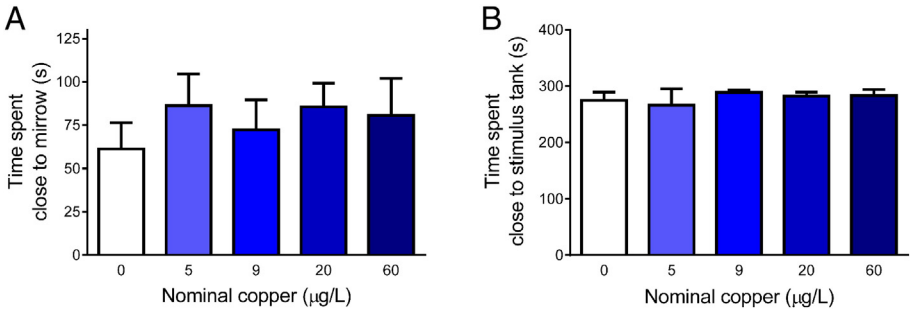

Fig. 5. Aggressive behavior and social interaction of adult zebrafish exposed to dissolved copper for 96 h. Aggressive behavior (n = 12–15) was evaluated by the mirror test, and the number of typical aggressive behaviors recorded. Social interaction (n = 4–5) was evaluated when fish were placed between an empty tank, and a "stimulus tank" containing 15 adult zebrafish. The time spent by fish close to mirrow (A), and time spent close to stimulus tank (B) was recorded. No significant differences were detected between copper treatments and the control group (0).

significant effect on the aggressive behavior or social interaction of adult zebrafish. These findings suggest that exposure to Cu does not disrupt the preference of adult zebrafish to live in groups. Additionally, it is important to stress that the dominant behavior of zebrafish was also shown to be not altered by exposure to 30 μg/L Cu for 48 h (Sloman et al., 2002), thus corroborating with our findings.

The inhibitory avoidance paradigm has been validated for zebrafish and predicts that fish can learn to refrain from swimming from a white to a black compartment to avoid receiving an electric shock (Blank et al., 2009; Moreira-Santos et al., 2008; Ng et al., 2012). The poor performance in this test has recently been associated with decreased levels of genes related to neuroplasticity, the endocrine system, and to stress response (Manuel et al., 2014). Interestingly, exposure of adult zebrafish to 9 μg/L Cu induced a marked loss of memory retention in the inhibitory avoidance task. This effect is clearly detrimental to fish, once it can decrease the animal's fitness to the environment. As observed in the present study, it has been suggested that defensive behaviors are affected by acute exposure of adult zebrafish to Cu. This condition seems to be related to the Cu effects on the locomotor activity and natural tendency to avoid brightly lit environments (Haverroth et al., 2015).

As discussed above for zebrafish larvae, oxidative stress seems to be also involved or associated with the observed effects of acute exposure to Cu on behavioral parameters in adult zebrafish. This seems to be possible at least at Cu concentrations equal or higher than 9 μg/L. This statement is based on the fact that acute (24 h) exposure to 6 μg/L induced no effect on antioxidant enzymes activity (superoxide dismutase and catalase) and thiol levels in zebrafish liver (Haverroth et al., 2015). On the other hand, in the present study GST activity was reduced in gills of adult zebrafish acutely exposed to 9 and 60 μg/L Cu. Additionally, in the present study GPx activity was also reduced in gills of adult zebrafish acutely exposed to 20 μg/L Cu. Furthermore, short-term (48 h) exposure to 15 μg/L Cu also induced increased levels of protein carbonyl in gills and liver, as well as increased expression of the gene encoding for catalase and superoxide dismutase in the liver of zebrafish (Craig et al., 2007). Finally, acute exposure (96 h) of adult zebrafish to an extremely high concentration of Cu (700 μg/L) was shown to increase oxidative stress markers and reduce the activities of antioxidant enzymes, including GPx and GST (Wang et al., 2015). It is also worth noting that these findings are in agreement with previous results

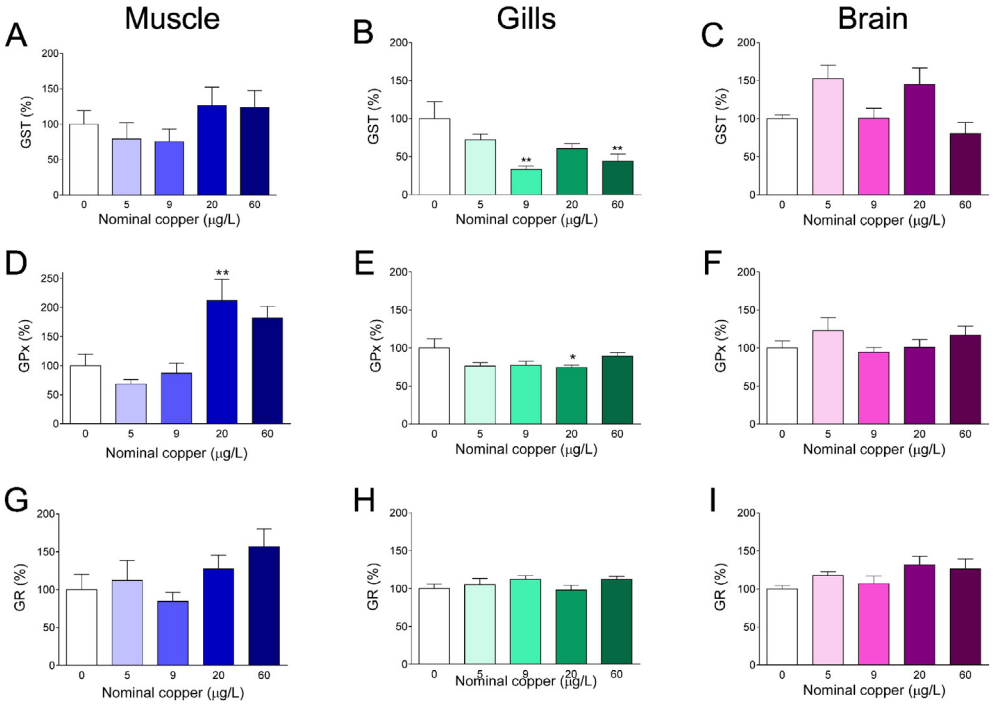

Fig. 6. Activities of glutathione-related enzymes in tissues of adult zebrafish exposed to dissolved copper for 96 h. (A-C) - Glutathione S-transferase (GST), (D-F) glutathione peroxidase (GPx), and (G-I) glutathione reductase (GR) in muscle (A, D, and G), gills (B, E and H) and brain (C, F and I). Values are expressed as percentage of the control group. Data are expressed as mean ± S.E.M (n = 5–6). Significant different mean values with respect to the control group (0) are indicated as * ($p < 0.05$), and ** ($p < 0.01$).

reported for other fish species acutely exposed to similar concentrations of Cu (Machado et al., 2013; Qu et al., 2014; Wang et al., 2014).

As observed in the present study, Cu effects on oxidative parameters are generally dependent on the fish tissue (Feng et al., 2015; Machado et al., 2013; Qu et al., 2014). In the present study, gills showed to be the most affected tissue by Cu exposure. Indeed, activities of enzymes related to glutathione metabolism (GST, GPx, and GR) in the brain of adult zebrafish were not affected by acute exposure to Cu. Additionally, muscle GPx activity was only affected in adult zebrafish acutely exposed to 20 μg/L. These findings are in line with the fact that gills are directly exposed to waterborne Cu. This reinforces the need for further investigation using this tissue to ascertain the mechanisms involved in the effects observed after acute exposure of zebrafish to dissolved Cu, especially in the range of concentrations tested in the present study.

Derivation of adequate water quality criteria for aquatic contaminants, including Cu, is challenging and an ongoing debate worldwide. Studies focused on the identification and characterization of the effects of chemicals on aquatic organisms at low, physiologically and environmentally relevant levels may certainly contribute significantly to a better risk and safety assessment. However, these studies can also generate key information on possible adverse or beneficial effects of aquatic contaminants, which may be directly dependent on the range of concentrations tested (Rietjens and Alink, 2006). In fact, biphasic responses, as observed in the present study for behavioral and biochemical parameters in larvae and adult zebrafish, are often reported after fish exposure to environmentally relevant concentrations of dissolved Cu (Machado et al., 2013).

Regarding biphasic responses, it is important to stress that response of fish to low concentrations of Cu can be largely variable, depending on several factors, including the physicochemical conditions of the water used, as well as the exposure protocol adopted. These conditions make the identification of the exact mechanism involved in a giving effect, as well as the derivation of water quality criteria for aquatic contaminants a real challenging task.

In the present study, the experimental exposure system used was static with complete renew of the experimental media every 48 h. Despite the use of a static exposure system with renewal every 48 h, which would be considered less effective than a continuous exposure protocol, data on measured concentrations of dissolved Cu in the experimental media are in complete agreement with the expected (nominal) concentrations. Indeed, measured dissolved Cu concentrations corresponded to 87.2% of the expected (nominal) concentrations over the exposure period. In addition, concentrations of free Cu ion, the most toxic fraction of dissolved Cu (Paquin et al., 2002), significantly augmented with increasing Cu concentrations. In addition, it is interesting to note that the percentage of free Cu ion corresponded to only 0.4% of the dissolved Cu present in the water of maintenance. However, it increases 2.7- and 3.4-fold at 5 and 9 μg/L Cu, while this increase was of 10.6- and 37.4-fold at 20 and 60 μg/L Cu.

Findings discussed above, together with those of the physicochemical conditions observed during the exposure period, indicate that all responses observed after exposure of larvae and adults of zebrafish to Cu, including the biphasic responses, cannot be ascribed to methodological biases. Indeed, biphasic response is a recognized biological phenomenon that is usually observed in the presence of low levels of aquatic contaminants (Hanekamp and Calabrese, 2006; Calabrese, 2013). However, biphasic responses may not be limited to a simple adaptive response, but accompanied by a transcriptional activation of different pathways that may interact with themselves, increasing the level of complexity in toxicological studies (Steinberg et al., 2008).

On the water quality criteria, regulatory agencies (EPA, 2007, 2012; European Union, 2015) establish a broad range of allowed Cu concentrations in water bodies, ranging from 1 to more than 100 μg/L. The upper limit established for Cu by the Brazilian regulatory agency is 9 μg/L, which is applied to aquatic ecosystems used for aquaculture (CONAMA, 2005). However, dissolved Cu concentrations often exceed the allowed limit. For example, in most rivers of São Paulo State (southeastern Brazil), it was shown to vary from 10 to 20 μg/L Cu (CETESB, 2009). Early life stages of fish are frequently affected by exposure to Cu at concentrations close to 9 μg/L (EPA, 2007). In this context, data from the present study show that environmentally relevant concentrations of Cu (9 and 20 μg/L) affected some biomarkers related to oxidative stress (GST and GPx activities). Furthermore, the current Brazilian water quality criteria for dissolved Cu in fresh water (9 μg/L) cannot prevent against the Cu-induced behavioral alterations in the swimming performance of zebrafish larvae, as well as in the deficits in avoidance memory in adult zebrafish. Furthermore, the dependence of the behavioral response to the concentration of waterborne dissolved Cu resembles a U-shaped curve, indicating that specific or a combination of interactive targets may be affected, clearly showing the need for further mechanistic studies.

Inter-specific interactions such as predation, as well as intraspecific interactions such as schooling and mating behavior, are relevant traits to fish life. Fish survival and reproduction depend on appropriate behavioral performance. Predator avoidance, social and reproductive behaviors are social interactions that are crucial to a successful and adapted life strategy. Exposure to aquatic pollutants, such as Cu, can alter the normal behavioral patterns, thus increasing the chance of a less successful establishment of a fish population. Additionally, it is important to note that this negative biological impact of chemical pollutants usually occurs at levels much lower than those necessary to cause mortality. Indeed, behavioral biomarkers can detect sub-lethal impacts of aquatic pollutants. In the present study, exposure to dissolved Cu at sub-lethal levels was able to disrupt the response to novelty and fear conditioning memory, which are clearly negative outcomes for adult zebrafish. Regarding the larvae response, Cu exposure increased velocity and distance traveled, which are not necessarily negative outcomes; however, they lead to increased energy expenditure, a potential maladaptive response. Therefore, findings from the present study are of highly ecotoxicological relevance, since behavioral disturbances can significantly affect the adaptive performance or even survival of affected fish.

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