The effect of copper on behaviour, memory, and associative learning ability of zebrafish (Danio rerio)

Elsevier Editorial System(tm) for
Ecotoxicology and Environmental Safety
Manuscript Draft

Manuscript Number: EES-19-2640R1

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Article Type: Research paper

Section/Category: Ecotoxicology

Keywords: Novel-tank diving test; T-maze test; Cu accumulation; Behaviour; Habituation; Conditioning.

Corresponding Author: Mr. Ali Pilehvar,

Corresponding Author's Institution:

First Author: Ali Pilehvar

Order of Authors: Ali Pilehvar; Raewyn M Town; Ronny Blust

Abstract: Copper is an essential element in many biological processes, but may exert toxic effects at levels surplus to metabolic requirements. Herein we assess the effect of copper on zebrafish behaviour using two assays, namely the novel tank diving test and a T-maze test with food reward. Novel tank diving tests were conducted on days 0, 4, and 10 of a 10 day Cu exposure (at concentrations of 0.77 µM (25% of the 240 hr LC50) and 1.52 µM (50% of the 240 hr LC50) to assess the alterations of behavioural responses in repeating novel tank diving assays and the effect of Cu on these patterns. Results demonstrate habituation to novelty, which is an indicator of spatial memory. Copper exposure had no effect on the latency of entry into the upper zones of the tank, nor on the total time spent therein, but did cause a greater number of freezing bouts in comparison to the control group. Additionally, Cu exposure had no effect on the habituation responses of zebrafish. Using the T-maze assay, we tested the effect of prior exposure to Cu for 10 days on subsequent behavioural trainings. The T-maze protocol was based on associative learning, where a visual stimulus (colour) was linked with a natural stimulus (food). Results of the control group showed that zebrafish are able to perform associative learning tasks. Moreover, Cu was found to negatively affect the associative learning capabilities. Specifically, while zebrafish in the control group achieved a significant number of correct choices (leading to food reward) throughout the T-maze training, such a trend was not observed for Cu exposed fish. Thus at the exposure concentrations and exposure times considered herein, Cu has no determinative impact on instinctual behavioural responses of zebrafish in repeated novel tank diving assays but does limit the associative learning capabilities.

***Highlights (for review)**

- Zebrafish showed robust habituation responses in novel tank assays.
- Copper exposure did not affect behavioural responses in repeated novel tank assays.
- Zebrafish were able to perform associative learning tasks in the T-maze assay.
- Copper exposure decreased the learning abilities of zebrafish.

The effect of copper on behaviour, memory, and associative learning ability of zebrafish (*Danio rerio*)

Ali Pilehvar[a,*], Raewyn M. Town[a] and Ronny Blust[a]

[a]*Systemic, Physiological and Ecotoxicological Research (SPHERE), Department of Biology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium*

* Corresponding Author

ali.pilehvar@uantwerpen.be
Tel: +3232653779

Abstract

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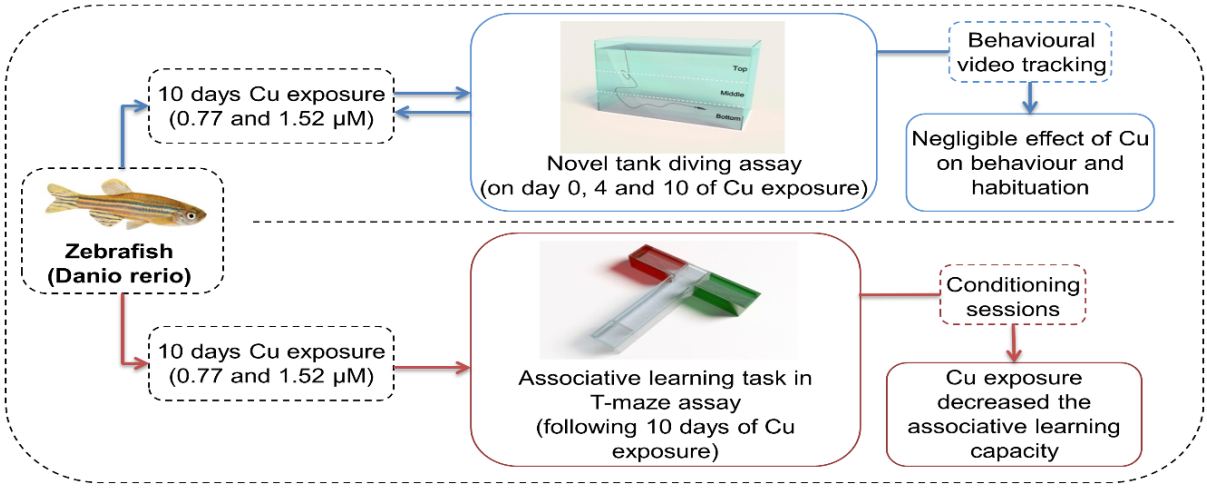
Graphical abstract



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1. Introduction:

Zebrafish has become a valuable vertebrate model organism in a wide range of biological disciplines including neurobehavioural toxicology studies (Kalueff et al., 2016). Multiple behavioural assays have demonstrated the effect of various anxiolytic or anxiogenic compounds, e.g. nicotine (Levin et al., 2007), chlordiazepoxide (Sackerman et al., 2010), and caffeine (Wong et al., 2010) on zebrafish behavioural responses. However, there is a paucity of information on the effect of metals on the behavioural phenotypes of zebrafish. Metals are well known neurotoxicants that can affect animal behaviour (for review see Pyle and Ford, 2017).

The concentration of many metals can be increased in aquatic ecosystems due to anthropogenic activities such as mining, industrial and domestic waste emission, sewage sludge discharge, etc. (Förstner and Wittmann, 2012). Thus, it is crucial to understand the mechanisms of metal toxicity in a wide range of aquatic organisms. Copper (Cu) is one of the essential trace elements contributing as a cofactor in a wide range of biological processes in the body including formation of many enzymes and glycoproteins, cellular respiration, function of nervous system, erythropoiesis and melanin synthesis (Kamunde and Wood, 2004). However, in concentrations above the metabolic requirements it may become toxic for fish in a variety of ways, e.g. by production of reactive oxygen species (ROS) (Bopp et al., 2008) and ionoregulatory disruption, in particular, impairment of branchial sodium (Na) influx through the effect on Na-K-ATPase (Grosell and Wood, 2002). Moreover, it is evident that Cu has detrimental neurological effects: it impairs olfaction via accumulation in the olfactory epithelium and it acts on the molecular signal transduction pathway which inhibits

the signal propagation from the sensory epithelium to the brain (Pyle and Mirza, 2007). Copper also downregulates genes related to calcium channels and ion transport, g-proteins, and olfactory receptors (Tilton et al., 2008). Since the ability to detect the olfactory cues is crucial for processes such as food detection, predator avoidance and mating, Cu induced olfactory impairment may affect many behavioural aspects of fish biology (Grosell, 2011). Indeed Cu has detrimental effects on a wide range of neuro-sensory processes encompassing appetite, vision, olfaction, cognition and etc. (Doria et al., 2018). Aversive memory assessment has shown that Cu contamination at a concentration of9 µg/L(0.14 µM) disrupts the response to novelty and fear conditioning memory in zebrafish (Acosta et al., 2016). Furthermore, a24 h exposure to Cu (0.006 mg/L, 0.09 µM) prior to a behavioural assay (novel tank diving test) was found to significantly decrease the total distance travelled by zebrafish, suggesting Cu induced impairment of locomotor patterns of zebrafish (Haverroth et al., 2015). However, the effect of continuous Cu exposure concomitant with behavioural assays has not yet been characterised.

Behavioural assays have been widely used to evaluate the effect of various pollutants on behavioural paradigms of aquatic organisms (Toft and Guillette, 2005, Cresci et al., 2018). In the present study, we evaluate the effect Cu exposure on behaviour, memory and conditioned learning capacities of zebrafish utilizing two behavioural assays: a novel tank test and a visual discrimination learning test with a T-maze. The novel tank diving test is based on an instinctual tendency of zebrafish to dive to the bottom of a novel environment and remain there until they gradually acclimate to the new environment and feel safe to start exploring and swim in the upper parts of the tank (Levin et al., 2007, Egan et al., 2009). Thus, endpoints such as increased latency to enter the upper zones of the tank, reduced exploration and decreased time spent in the upper zones are considered as indicators of anxiety in fish (Levin et al., 2007). In order to examine the impact of Cu exposure on habituation responses of zebrafish, we have conducted repeated novel tank diving tests during the Cu exposure. Habituation occurs in a wide range of species and neurobehavioral disciplines (Thompson and Spencer, 1966). Moreover, habituation to novelty is often considered as an indicator of spatial memory. Robust habituation responses of zebrafish have already been reported previously within and between the novel tank sessions (Wong et al., 2010).

The effect of Cu on the associative learning and memory abilities of zebrafish was assessed by the T-maze test. The protocol is based on Pavlovian conditioning, a form of learning in which a neutral or arbitrary stimulus (conditioned stimulus; e.g., green colour) becomes associated with a stimulus of some significance to the animal (unconditioned stimulus; e.g., food), so that the conditioned stimulus becomes synonymous with the unconditioned one, thereby evoking the same innate, reflexive behavioural responses, e.g., food seeking (Gould, 2011). Associative learning exploiting food rewards has been studied in detail in zebrafish (e.g. Sison and Gerlai, 2010). To evaluate the effect of Cu exposure on associative learning abilities of zebrafish in present study, zebrafish were exposed to Cu prior to the visual

discriminative learning test conducted based on standard protocol described by Colwill et al. (2005).

Finally, we have measured the whole body metal burden of zebrafish at the end of the experiment to assess the link between the metal accumulation and behavioural phenotypes of zebrafish under metal exposure. By integrating the results of present study we aimed to unveil the effect of Cu exposure (simultaneous and prior) on zebrafish behavioural paradigms.

2. Materials and methods

2.1. Ethical statement

All the experimental protocols of this study were approved by the Ethical Committee for Animal Testing (ECD) of the University of Antwerp and conducted according to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA).

2.2. Animal maintenance

Adult wild type and experimentally naive zebrafish (*Danio rerio*) were obtained from the University of Antwerp zebrafish facility and were given 3 weeks to acclimate to laboratory conditions. They were housed in glass aquariums filled with US-EPA medium hard water ($NaHCO_3$: 96 mg/L; $CaSO_4.2H_2O$: 60 mg/L; $MgSO_4$: 60 mg/L; KCl 4 mg/L; pH: 7.4-7.8; water hardness: 80-100 mg/L $CaCO_3$) at 28 °C with the density of 2 fish/L (Lawrence, 2007). The pre-acclimation as well as experimental procedures were conducted in a temperature controlled chamber (Type WT15'/+5DU-WB, Weiss Technik, Reiskirchen-Lindenstruth, Germany) with a photoperiod of 14h light: 10h dark (lights on at 8:00 am) and the temperature set at 28 °C. The aquarium water was constantly aerated using an air stone, mechanically and biologically filtered, and ammonia, nitrite and nitrate levels were monitored using Tetratest (Tetra®, Melle, Germany) and always kept under the harmful level for zebrafish (Lawrence, 2007). The fish were fed once a day, ad libitum with Sera vipan® (Heinsberg, Germany) flakes and after 15 minutes the remaining food was removed.

2.3. Experimental procedure

2.3.1. Novel tank diving test

2.3.1.1. Chemicals and metal exposure

After the pre-acclimation period, a total number of 30 zebrafish were divided into 3 experimental treatments: (i) Control (no added metals in the exposure water), (ii) 0.77 µM Cu (*ca.* 50 µg Cu/L; as $CuSO_4.5H_2O$, Sigma-Aldrich®, MO, USA) and (iii) 1.52 µM Cu (*ca.* 100 µg Cu/L) for 10 days. Throughout the remainder of the text, we use Cu to refer to dissolved copper. Previous work in our lab has determined the 240 hr LC50 of Cu in the same medium hard water to be 2.9 µM; thus the selected Cu concentrations correspond to 25% and 50%

of the 240 hr LC50 value. The 10 day duration of metal exposure is sufficient to ensure attainment of the incipient lethal level in fish (e.g. Stubblefield et al., 1999). The metal exposures were conducted in polypropylene aquaria concomitant with the behavioural assays. The water was constantly aerated using an air pipe but not filtered and it was 100% renewed every second day with water of the same metal concentration. The zebrafish were fed minimally (≈ 1% of body weight) during the 10 days of metal exposure to avoid increased levels of ammonia and dissolved organic matter. During the 10 days of metal exposure mortalities were recorded every 12 hours and dead fish were removed and kept frozen at -20 °C until the end of the experiment.

2.3.1.2. Apparatus and behavioural testing

The novel tank diving test was conducted according to the previously described standard protocols (Cachat et al., 2010, Levin et al., 2007, Sackerman et al., 2010). During the experimental phase (metal exposure and behavioural assay) zebrafish were housed individually to enable the behaviour of each individual fish to be tracked throughout the experiment and also to reduce the schooling behaviour (Gleason et al., 1977). Thus each individual serves as a replicate, i.e., n = 10 for each treatment. One novel diving tank test was conducted on day 0, day 4 and day 10 of Cu exposure. The procedure involved gently removing fish from the exposure aquarium and introducing them to the rectangular test tank (29×14×19 cm^3; length× width× height), where their behaviour was recorded for 5 minutes by a digital camera (Casio® Exilim EX-F1) placed in front of the observation tank. The test tank was a glass aquarium filled maximally (7 L) with the home tank water (containing Cu for Cu exposed groups) and was virtually divided into 3 equal horizontal zones. On day 0, zebrafish in Cu treatments, introduced to Cu exposed water inside the observation tank for the first time and then transferred to home tank containing Cu for the rest of the experimental phase. The test tank was placed on a stable surface and all the environmental distractions were kept at minimal level. After each session (for each individual fish), the observation tank was rinsed with clean water to remove any chemical cues which may affect the behavioural patterns of the zebrafish and refilled with clean water. On each observation day, the behavioural testing started with zebrafish in the control group, followed by the 0.77 µM Cu exposed fish and then the 1.52 µM Cu group. At the end of the each test day, the observation tank was acid washed to remove any Cu contamination. As previously described by Cachat et al. (2010) and implemented by several others (Egan et al., 2009, Wong et al., 2010) we recorded a combination of behavioural parameters of zebrafish in the novel tank diving test by automated video tracking (Behaviorcloud©, OH, USA) or manual registration including: total distance travelled, average velocity, time spent in the upper 2/3 of the tank, latency of entry into the upper zone of the tank, and the number of freezing bouts. Freezing was defined as a period of immobility for at least 1 s, characterized by total absence of movement, except for the gills and the eyes.

2.3.2. T-maze test

2.3.2.1. Chemicals and metal exposure

The same metal exposure procedure as for the novel tank test was applied (Section 2.3.1.1) with one exception: the zebrafish were exposed to Cu for 10 days prior to the behavioural assays (see section 2.3.2.2), and then were subsequently maintained and tested in clean water. In the T-maze assay, a 10 day exposure to Cu was conducted prior to the behavioural testing in order to evaluate the alterations of the associative learning abilities of zebrafish during a recovery phase of a prior Cu exposure. The selected Cu exposure concentrations resulted in higher levels of Cu in the bodies of exposed fish compared to control fish throughout the duration of the experiment.

2.3.2.2. Apparatus and behavioural testing

Following the metal exposure, all of the fish were transferred to and maintained in a home tank containing clean water throughout the performance of a T-maze visual discrimination learning task. The T-maze assay was conducted according to the protocol defined by Colwill et al. (2005) with some modifications in the choice of the colour pair used for the T-maze sleeves (red and green instead of purple and green or blue and red). The test tank was a custom made T-shaped transparent plexiglass maze equipped with two removable colour sleeves (green and red) to fit around the arms (Fig. 1). The depth of the maze was 10 cm and it was filled to a height of 8 cm with clean water adjusted to the home tank conditions (7.2 L). At the stem of the maze an area measuring 10 cm × 10 cm × 10 cm could be closed off to form a start box. Plexiglass doors were used to isolate the arms of the maze from the stem. Two digital timers were used to time the events and the food rewards (Sera vipan® flakes, Heinsberg, Germany) were delivered using stainless steel tweezers. In the T-maze assay, fish received food only during the daily training sessions and were not fed in the home tank. Additionally, the training sessions were recorded using a digital camera (Casio® Exilim EX-F1) mounted above the T-maze as a means to verify manual observations.

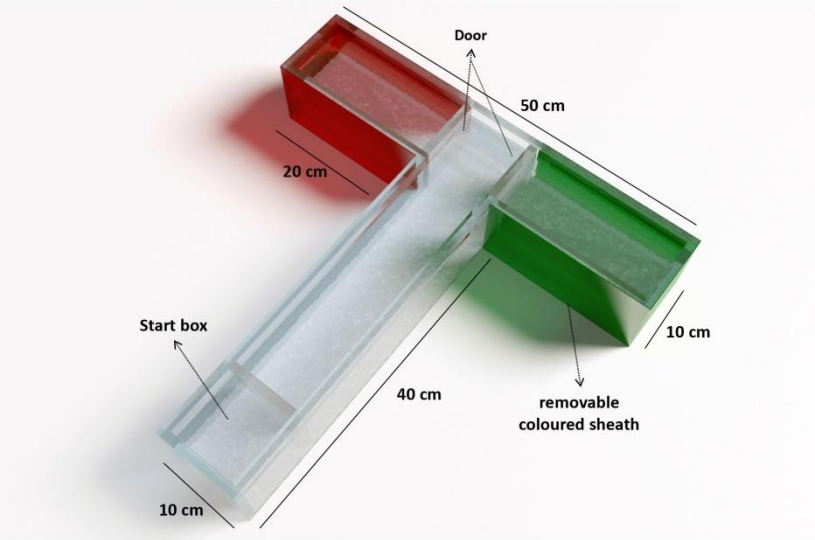

Fig. 1. The schematic view of the configuration and dimensions of the tank used for the T-maze test. The arms of the maze were equipped with removable coloured sheaths (red and green).

The entire behavioural assay was conducted over 25 days and consisted of three phases as follows: (i) Pre-training: a period of two days during which one session per day of conditioning in the T-maze without the colour stimulus was applied for each subject. A session consisted of two subsequent trials with a 30 s interval between each trial. In each of the trials, one of the maze arms was barred while the opposite arm was open. At the start of the trial, 2 minutes were given to the fish to acclimatize to the novel environment in the start box (Colwill et al. 2005). After the acclimation period, the door of the start box was opened and then closed immediately after the fish left the box. As soon as the fish entered the open arm the door was closed and food was rewarded. Whether the fish consumed the food reward or not, latency to leave the start box and the total time taken to complete the trial were recorded for each trial. (ii) Discrimination: a period of 16 days in which the side arms were lined with red and green sleeves and zebrafish were given a choice between the two coloured arms. One discrimination session was conducted on each of the 16 days; each session consisted of 4 trials with a 30 s interval between each trial. For each trial the position of the colour sleeves on the T-maze left and right arms was altered based on the pattern specified in the protocol: RGGRGRRG (Colwill et al. 2005). After the 2 minutes acclimation period in the start box, the door was opened and the fish were allowed to swim into one of the coloured arms. As soon as the fish entered one of the arms the door was closed and a correct colour choice was awarded with food. Incorrect colour choices led to a correction procedure, i.e. the trial was repeated with the wrong colour arm closed, thus the only available option was to swim into the correct coloured arm and receive the food reward. The correction procedure itself did not constitute a trial round. For half of the fish in each treatment (*n = 5*) red was designated as the correct colour while for the other half green was designated as the correct choice leading to food reward. Food consumption, time to leave the start box and enter an arm, as well as the colour of the arm chosen was

recorded in discrimination trials. (iii) Extinction: a period of 7 days in which the same procedure as the discrimination training was performed with two exceptions: no food reward and no correction rounds were given. On each of the 7 days, each subject was tested with one extinction session comprising 4 trials.

In the T-maze assay the fish were also housed individually during the experimental phase (metal exposure and behavioural assay). Thus each zebrafish is a replicate, i.e., n = 10 for each treatment. After each trial the maze tank was rinsed with clean water to remove any remaining food odours and/or chemical cues from the tank, and refilled with clean water.

The T-maze assay was designed to assess the learning abilities of zebrafish in recognition of the colour leading to food reward (discrimination training) and to evaluate the strength of the formed conditioning in the absence of the unconditioned stimulus (extinction training).

2.3.3. Sampling and analytical procedure

During the metal exposure, water samples from the home tank were collected once a day (after feeding and prior to water change on water renewal days) from each tank to determine the dissolved metal concentrations. Water samples were filtered using a 0.2 µm syringe filter (Acrodisc®, Supor Membrane; PALL life sciences), acidified to 2 % H^+ with trace-metal-grade HNO_3 (69 %) and kept at 4 °C until the analysis. The concentrations of Cu and major ions in the exposure media were determined using inductively coupled plasma-mass spectrometry (7700x ICP-MS, Agilent Technologies®) and Inductively coupled plasma optical emission spectrometry (ICP-OES, iCAP6300 Duo, Thermo Scientific®) respectively. At the end of the experiment, zebrafish were euthanized by a lethal dose of tricaine methanesulfonate (MS-222) solution (0.3 g/L) (Matthews and Varga, 2012). The fish samples were then weighed to obtain the fresh weight, oven dried (60 °C, 48 h) and digested by adding 2 ml of nitric acid (HNO_3, 69%) and 0.5 ml of hydrogen peroxide (H_2O_2, 30%) in a hot block (Environmental Express SC154®). Whole body Cu concentration was determined by ICP-MS and calculated on a dry weight basis. Blanks and certificated reference material (SRM-2976, mussel tissue, National Institute of Standards and Technology, Gaithersburg, MD 20899, USA) were included in all series of metal analysis to validate the accuracy of the analytical procedure. Recoveries were within 5% of certified values.

2.4. Statistical analysis

Statistical analyses were conducted using GraphPad Prism 8.0.2 software (GraphPad software, CA, USA). Data were tested for normality of distribution using the D'Agostino-Pearson test. The experiments were conducted in a repeated design: each individual fish was measured 3 times in the novel tank diving test or received several training sessions in the T-maze assay. Accordingly, a two-way repeated measures analysis of variance, RM-ANOVA, with Geisser-Greenhouse correction followed by Tukey's multiple comparisons test was done to analyse the behavioural variables of novel tank test and response time in T-

maze test. To compare the percentage of correct choices between two sessions in T-maze experiment, a two tailed Wilcoxon matched-pairs signed-rank test was used. The whole body Cu burden of the zebrafish in different treatments were compared using two-way ANOVA (with Cu treatment and the behavioural assay as the two factors) followed by Tukey's multiple comparisons test.

3. Results

3.1. Exposure medium composition

The average total dissolved Cu and major ion concentrations measured in the exposure medium during 10 days of metal exposure in the 3 different treatments (for both novel tank and T-maze assays) are presented in Table 1.

Table 1. Mean (±SD) concentrations of dissolved Cu and major ions in the exposure medium during 10 days of exposure (sample size, *n* = 10).

Treatment	Average measured concentration of Cu and major ions (µM±SD)				
	Cu	**Na**	**K**	**Mg**	**Ca**
Control	BQL[(1)]	1025.1± 24.8	65.7± 3.1	520± 10.2	395.5± 17.2
Cu (low)	0.77± 0.07	1028.3± 29.1	64.1± 3.5	518.9± 13.4	396.6± 20.2
Cu (high)	1.52± 0.1	1028.4± 27.3	64.7± 3.7	538.8± 11.7	394.7± 19.6

[(1)] below quantification limit of ICP-MS (1.5×10^{-3} µM).

3.2. Survival

As expected, no mortality was observed in zebrafish exposed to 0.77 µM Cu, while the survival rate decreased to 90% in zebrafish exposed to 1.52 µM Cu in both behavioural assays. Specifically one fish died at 108 hr in the novel tank assay and at 60 hr in the T-maze test. However, no significant difference between mortality curves of the control and exposed fish was detected (Log-rank [Mantel-Cox] test).

3.3. Novel tank diving test

Results of screening behavioural phenotypes of zebrafish in novel tank test are presented in Fig. 2. Neither Cu treatment, nor repetition of the assay affected the total distance travelled by zebrafish (Fig. 2A). Two-way RM-ANOVA showed that repetition of the assay significantly affected the time spent in the upper zone of the tank ($p < 0.0001$), latency to enter the upper zone ($p < 0.0001$), and the number of the freezing bouts ($p < 0.001$). The Cu treatment had a significant impact on freezing bouts only ($p < 0.05$). Moreover, two-way RM-ANOVA revealed a significant interactive effect of Cu treatment and time (repeated tests over subsequent days) on zebrafish average velocity ($p < 0.05$). The 1.52 µM Cu

293 exposed zebrafish had a significantly lower velocity at day 10 in comparison to control fish
294 at the same day (Fig 2B). The time spent in the upper zone significantly increase in all
295 treatments at day 4 and day 10 compared to the same treatment at day 0. No significant
296 effect of Cu exposure was observed at each specific day of the novel tank test between
297 treatments (Fig. 2C). The latency to enter the upper zone decreased constantly over time in
298 all 3 treatments, however, it was only significantly lower at day 10 compared to day 0 (Fig.
299 2D). Finally, the number of the freezing bouts decreased over subsequent days in control
300 fish and it was significantly lower at day 10 compared to day 0, while no significant effect of
301 time was found on freezing bouts in Cu treatments. On day 4, the number of the freezing
302 bouts was significantly higher in the 1.52 µM Cu exposed group compared to control fish at
303 the same day (Fig. 2E).

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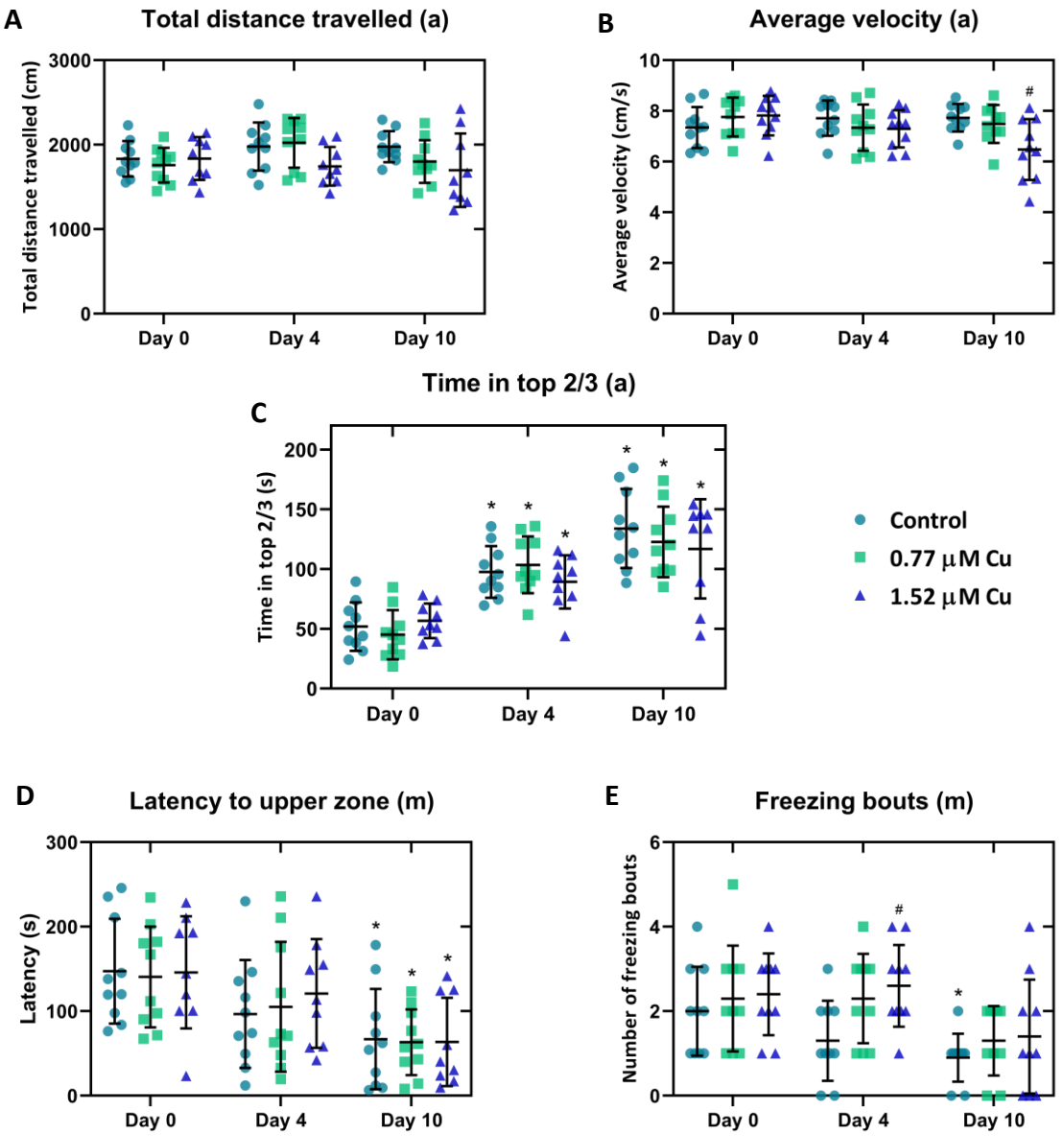

Fig. 2. Behavioural responses of zebrafish in the novel tank test on day 0, day 4 and day 10 of a simultaneous Cu exposure utilizing automated (a) or manual (m) registrations (each data point represents an individual fish and the bars on each group denote the mean± SD for that treatment). Data for surviving fish (replicates, n = 10 for the control and 0.77 µM Cu treatment; n = 9 for the 1.52 µM Cu treatment). * indicates a significant ($p < 0.05$) difference of a treatment compared to same treatment at day (0). # indicates a significant ($p < 0.05$) difference of a treatment compared to control group at the same day (two-way RM-ANOVA).

305

306

307 **3.4. T-maze test**

308 The average time taken for zebrafish to complete the trial for each session is presented in
309 Fig. 3. There was a significant (two-way RM-ANOVA) decrease in the response time of

zebrafish over the 2 pre-training sessions ($p < 0.0001$), in addition Cu treatment also affected this endpoint in this phase significantly ($p < 0.05$). This trend partially occurred in the discrimination sessions as well, where the response time strongly decreased over the first few sessions and then remained approximately constant. Again, both time and treatment significantly affect this endpoint in the discrimination phase ($p < 0.0001$ for both factors). During the discrimination phase in all 3 treatments, the average time taken for zebrafish to complete the trial was significantly lower at the final session 16 compared to session 1 of the same treatment. In the extinction phase, while time was not a determinative factor anymore (i.e. no significant variations over the sessions), Cu treatment still significantly affected the endpoint ($p < 0.0001$). Overall, while all 3 treatments presented a similar trend over the sessions (pre-training, discrimination and extinction), the time taken for the 1.52 µM Cu exposed zebrafish to complete the trial was often higher than that for the 0.77 µM Cu and control groups Fig. 3. While fish in the control and 0.77 µM Cu groups consumed the food rewards in 100% of the trials, the 1.52 µM Cu exposed fish did not eat the food in 8.5% of discrimination trials.

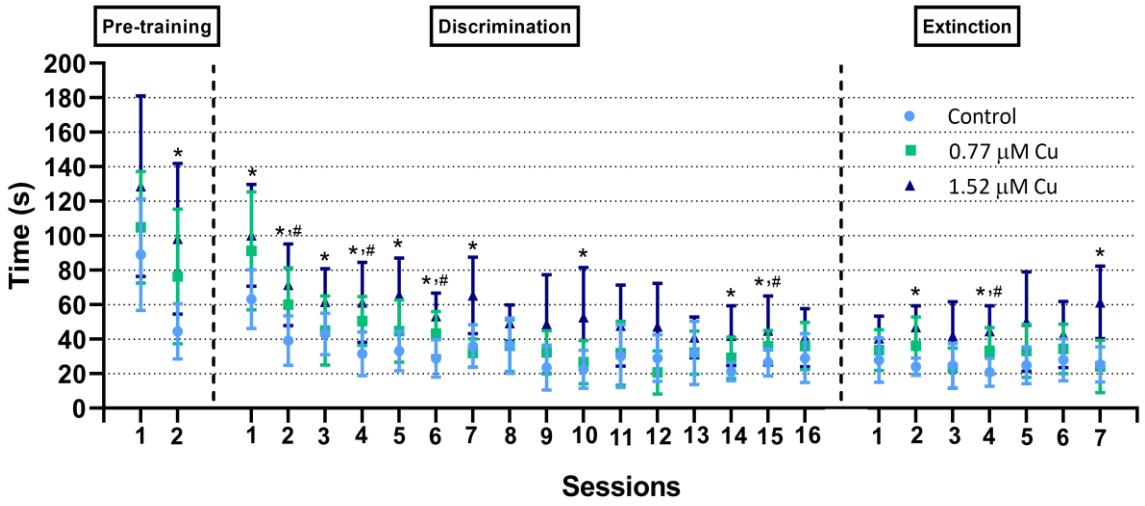

Fig. 3. The average time taken for zebrafish to complete each trial (excl. correction trials) in pre-training, discrimination and extinction sessions (data are presented as means± SD; data for surviving fish; replicates, $n = 10$ for the control and 0.77 µM Cu treatment; $n = 9$ for 1.52 µM Cu treatment). * and # indicate significant ($p < 0.05$) difference of 1.52 µM Cu and 0.77 µM Cu in comparison to the control group at the same session respectively (two-way RM-ANOVA).

An overview of the mean percentage of correct choices among the coloured arms, over the discrimination and extinction sessions is presented in Fig. 4. In each treatment, half of the zebrafish were trained to pick red as the colour for the food reward (Red+) while for the other half green was designated as the correct choice (Green+). In discrimination trainings, in all 3 treatments, zebrafish showed an increased number of correct choices over the subsequent sessions and the percent of mean correct choices were above the chance level

(50%) at the last session. The learning curve had a steeper ascending slope in the control group compared to Cu exposed groups. In all treatments, zebrafish started with a mean correct choice slightly above or below the chance level (depending on the colour trained for) and finished the discrimination trials by reaching up to 80% and 60% mean percent correct choice for the control and Cu exposed groups respectively (Fig. 4A). The statistical comparison (two tailed Wilcoxon matched-pairs signed-rank test) of the mean percentage of correct choices between the first and last discrimination session among each treatment revealed that there is a significant increase in the number of correct choices made by control group ($p < 0.05$) while this was not significant for Cu exposed zebrafish ($p = 0.0938$ and $p = 0.1250$ at Cu concentrations of 0.77 µM and 1.52 µM respectively). Moreover, zebrafish showed a minor instinctual preference towards red colour, however they could overcome this preference over the training sessions and the amount of choices for green or red were comparable at the last discrimination sessions. This preference was not affected by the Cu exposure (Fig. 4). The first extinction session is generally considered as the test session and fish in the control group confirmed that zebrafish can learn a visual discrimination task by performing correct choices up to 80%. For 0.77 µM Cu exposed zebrafish this value decreased in the first session of the extinction phase but was still above the random chance level (50 %). In the 1.52 µM Cu exposed group, the zebrafish which were trained to choose red as the correct choice showed a mean percent correct choice just above the chance level (50%), however, the zebrafish trained to choose green failed the test by obtaining a final mean percent correct choice below the chance level (Fig. 4B). The statistical analyses showed that, the number of correct choices made at the first session of the extinction phase is significantly higher than the first session of the discrimination phase in control group, such an effect was not observed for the Cu treatments. Throughout the extinction sessions and in the absence of food rewards, the mean percent correct choice curves followed a descending slope in all 3 treatments and for both colours (Fig. 4B).

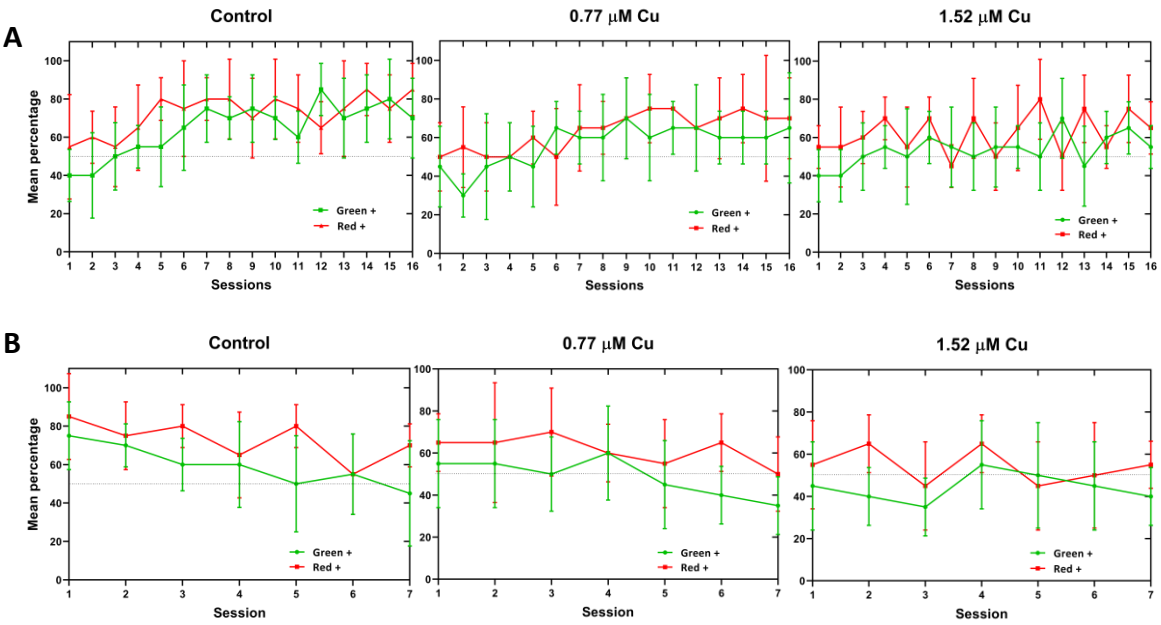

Fig. 4. Mean percentage of correct choice for zebrafish in (A) discrimination and (B) extinction sessions. The dotted line indicates random chance level (50%). Values are means± SD (data for surviving fish; replicates, *n* = 5 for [Green +] and [Red +] groups in the control and 0.77 µM Cu treatment; *n* = 4 for [Green +] and *n* = 5 for [Red +] groups in 1.52 µM Cu treatment).

3.5. Whole body burden of Cu

The whole body burden of Cu in zebrafish trained in the novel tank and T-maze tasks are presented in Fig. 5. The Cu burden was significantly (two-way ANOVA) higher in 0.77 µM and 1.52 µM Cu exposed groups compared to the control group in both assays. Furthermore, the Cu burden in Cu exposed fish in the T-maze test was significantly lower compared to the group exposed to same concentration in the novel tank test (Fig. 5). This effect is mainly linked to the period (25 days) that zebrafish were in clean water (following the 10 day metal exposure) during the behavioural trainings of T-maze assay and subsequent elimination of part of the Cu.

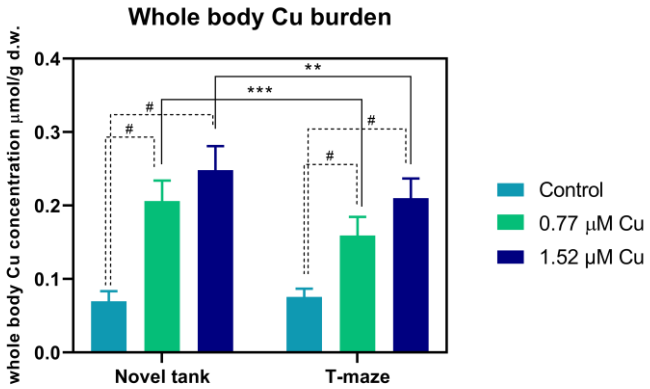

Fig. 5. Whole body Cu burden of zebrafish trained in novel tank and T-maze assays at the end of the experiment. Values are means± SD (data for surviving fish; replicates, n = 10 for the control and 0.77 µM Cu treatment; n = 9 for 1.52 µM Cu treatment). ** p < 0.01, *** p < 0.001 and # p < 0.0001.

4. Discussion

Zebrafish behaviour has been studied extensively in various behavioural assays (e.g. Bault et al., 2015). Moreover, it has been shown that zebrafish have a remarkable capacity to perform learning tasks (Sison and Gerlai, 2010, Arthur and Levin, 2001).

Dissolved Cu in concentrations above the metabolic threshold can affect fish behaviour and learning processes by impairing the neuro-physiological functions of the organism. For example, it has been shown that Cu exposure, at concentrations of 0.22, 0.34, and 0.84 µM for 7 days, decreases serotonin and dopamine levels in the brain of common carp (*Cyprinus carpio*) (De Boeck et al., 1995). These neurotransmitters have wide ranging impacts on fish biology including the locomotion activity and behaviour (Winberg and Nilsson, 1993). Also, the dopaminergic system has been found to modulate different aspects of learning and memory in zebrafish (Naderi et al., 2016). Moreover, exposure to waterborne Cu at concentrations of 0.3 and 1.6 µM for 10 days was reported to cause some histopathological damage to brain tissue of rainbow trout (*Oncorhynchus mykiss*) (Al-Bairuty et al., 2013). The aforementioned cases draw attention to Cu as a neuro-toxicant and its importance in neurobehavioral toxicology. The goal of the present work is to explore whether copper under sub-lethal and close to toxicity threshold scenarios has effects on a number of behavioural traits as manifested in novel tank and T-maze tests.

In the present study, we evaluated (i) the effect of continuous Cu exposure over 10 days on the concomitant behavioural responses of zebrafish to anxiety evoked by novelty via novel tank assays repeated over the exposure period, and (ii) the effect of prior Cu exposure on the learning capacities of zebrafish via T-maze assays.

4.1. Novel tank diving test

We monitored several behavioural traits of zebrafish in the control group (without Cu exposure) upon their introduction to a novel environment. In addition, we evaluated the effect of continuous (10 days) Cu exposure concomitant with repeated novel diving tank assays on behavioural traits of zebrafish. The total distance travelled and the average velocity are typically considered as the indicators of locomotor activity of fish, whereas, the time spent in the upper zones of the tank, latency to enter the upper zones and the number of freezing bouts are linked to the anxiety level and exploratory behaviour (Cachat et al., 2010). In the present study, we have observed no significant effect of Cu treatment or repeated sessions on total distance travelled by fish (Fig. 2A), although, there was a relatively smaller distance covered by the 1.52 μM Cu exposed group at day 4 and 10. On the other hand, whilst the control and 0.77 μM Cu treatments did not show a significant variation in average velocity over the test sessions, exposing zebrafish to 1.52 μM of Cu significantly decreased the average velocity at day 10 (Fig. 2B). In teleosts, decreased swimming performance as a consequence of sub-lethal Cu toxicity has been reported in several studies (e.g. De Boeck et al., 2006). De Boeck et al. (2006) proposed that reduction of swimming performance in carp exposed to Cu could be explained by elevated ammonia accumulation in the plasma and muscle tissue.

Latency to enter the upper zone decreased, and the total time spent therein increased with subsequent sessions (Fig. 2C,D). This could be an indicator of habituation to novelty and decreased anxiety levels in zebrafish as a result of repeated stimulation. Habituation is a central neural process and is termed as “the simplest form of learning” (Rankin et al., 2009). Our results complement the findings of Wong et al. (2010) who examined the intra- (per-minute analysis of zebrafish behaviour during a 6 or 30 minute trial) and inter-session (daily 6 minute trials over 7 days) habituation in zebrafish utilizing the novel tank test. The authors reported significant habituation responses of control groups of zebrafish in both assays. Furthermore, in the same study, the effect of some anxiogenic (caffeine and pentylenetetrazole) and anxiolytic (morphine and ethanol) compounds on the habituation responses of zebrafish was investigated. The anxiogenic compounds supressed the habituation responses while the anxiolytic compounds appeared to have no effect. In the present study, we have observed no significant effect of Cu treatment on these two endpoints (total time spent and latency), nor on the habituation responses of zebrafish. Finally, the number of freezing bouts in the control group decreased over the test sessions and was significantly lower at day 10 which confirms the aforementioned habituation hypothesis. Nevertheless, Cu exposure significantly affected this variable and the number of freezing bouts observed in the 1.52 μM Cu group was significantly higher at day 4 compared to the control group on the same day.

Other studies have shown that a24 h 6 μg Cu/L (0.09 μM) exposure prior to the novel tank test, significantly decreases the distance travelled and maximum speed of the zebrafish

(Haverroth et al., 2015). Additionally, the exposure increased the freezing duration and decreased latency to enter the upper zone and the time spent in the upper zone, although, these alterations were not significant compared to the control group (Haverroth et al., 2015). In another report on the influence of Cu exposure (with concentrations ranging from 5 to 60 μg/L (0.079-0.94 μM) for 96 h) on exploratory behaviour of zebrafish and response to novelty by using a Y-maze test, none of the Cu exposures affected the locomotor activity of adult zebrafish (distance travelled and average velocity). Moreover, only 60 μg/L exposure altered the exploratory behaviour of zebrafish (time spent in the novel arm of the Y-maze), while the other exposures (0, 5, 9 and 20 μg/L) had no significant impact on this behaviour (Acosta et al., 2016).

Comparison of the various literature reports with the results of the present work requires consideration of the different exposure times and Cu concentrations employed. For example, the study of Haverroth et al. (2015) shows that a prior, short term (24 h) exposure to a low concentration of Cu appears to have a more determinative impact on zebrafish behavioural responses in the novel tank diving test. A possible explanation for this effect is the time required for regulatory and detoxification mechanisms to become active. Thus, the minimal effects of a prolonged exposure (4 or 10 days) to high Cu concentrations on instinctual behavioural traits observed in the present work, and by others for 96 hr exposures (Acosta et al., 2016) is ascribed to the efficacy of the activated regulatory and detoxification procedures. The observation of an initial adverse effect at short exposure times, followed by recovery, is a typical response to Cu exposure across many endpoints (e.g. De Boeck et al., 2006).

Overall, in the present experiment, despite the significantly higher Cu accumulation in the body of zebrafish exposed to Cu (0.77 or 1.52 μM) in comparison to the control group (Fig. 5), we have observed no effect of a sub-lethal (0.77 μM) concentration of Cu on the locomotor or behavioural profiles of zebrafish. Although the higher concentration of Cu (1.52 μM) suppressed the locomotor activity (especially velocity) of zebrafish, there was no effect on exploratory variables (time spent in upper zone and latency). The only behavioural variable affected by the 1.52 μM Cu exposure was the number of freezing bouts, which transitionally increased on day 4 and decreased again on day 10 (Fig. 2E). Overall our results indicated robust habituation responses of zebrafish to novelty. Moreover, despite the relatively high Cu concentration applied in this work, no significant influence of Cu exposures was observed on the behavioural traits of zebrafish in repeated novel tank diving assays.

4.2. T-maze test

Discrimination learning capabilities of zebrafish and the effect of Cu exposure were assessed by utilizing a T-maze test. The zebrafish were trained to differentiate the correct colour choice leading to food reward. We used green and red as the pair of colour stimuli. Zebrafish are very visually oriented and their eyes for a large part display the same

morphology and function as other vertebrates including humans (Glass and Dahm, 2004). Zebrafish colour vision is tetrachromatic, that is, their retina possess four types of cone cells sensitive to red, green, blue and ultraviolet (Robinson et al., 1993). Several studies have addressed the inherent colour preference of zebrafish. For example, Jessica et al. (2015) assessed the colour preference in zebrafish using a multiple chamber tank with different environmental colour options, they found that zebrafish preferred blue and green and avoided red and yellow. In another observation, using a two chambered place preference apparatus with colour gravel and T-maze, a strong aversion toward blue colour was observed relative to all other colours (red, yellow and green) when tested in combinations. Moreover, while no biases over the other 3 colour combinations was noted in a place preference assay, red and green were equally preferred and both were preferred over yellow in a T-maze experiment (Avdesh et al., 2012). Spence and Smith (2008) tested foraging biases of zebrafish by raising fish on diets consisting of different coloured food. They reported that zebrafish showed a highly significant innate preference for red, which was modified, but not superseded, by learning. Two explanations were offered for this preference: first, the higher contrast of red colour against background illumination and second, in ecological perspective, the pigmentation of neutral diet of zebrafish which is rich in zooplankton. It has been shown that the predation risk of the zooplankton by zebrafish is proportional to the degree of the red pigmentation in the body of the zooplankton. This could explain the innate bias of zebrafish for the red colour (Spence and Smith 2008). Finally, Bault et al. (2015) evaluated the colour preference of adult zebrafish and the effect of Pb exposure during developmental stages on it by using a three-chambered apparatus and 5 different colour stimuli (orange, yellow, green, blue and purple). Their results showed a general preference for colours of shorter wavelengths, furthermore, they have shown that developmental Pb exposure alters innate colour preference in adult male zebrafish. In the present study, we have used green and red as colour stimulus to elucidate: (1) whether zebrafish have an innate preference for one of the colours, (2) if they can overcome any instinctive tendency by training, and (3) if Cu exposure affects any preference or not.

In the present experiment, the time taken for zebrafish to complete the trials decreased over the pre-training and discrimination sessions until a plateau value was reached (Fig. 3). This finding could be an indicator of habituation and spatial memory in zebrafish. The spatial memory capabilities of zebrafish are well established (Sison and Gerlai, 2010, Arthur and Levin, 2001). Copper treatment significantly affected this endpoint in all 3 training phases and increased the response time. There are two possible explanations for this effect: Cu impairs the spatial memory functions in zebrafish, and/or Cu impairs the locomotor system. The latter effect was observed partially (only average velocity and only at the highest Cu concentration) in the novel tank test, and can make the response time longer.

Throughout the discrimination sessions, the control group confirmed that zebrafish are able to learn to discriminate between the two colour stimuli and to choose the colour leading to food reward (Fig. 4A). The learning curve had an ascending slope in this group and the

average number of correct choices reached up to 80% at the last discrimination sessions. Our findings in this part complement the previous studies on visual discrimination learning capacities of zebrafish (Colwill et al., 2005, Arthur and Levin, 2001). Copper contamination decelerated the learning curve and although the Cu exposed zebrafish (0.77 and 1.52 µM) showed some preference for the correct colour throughout the discrimination sessions (above the chance level, 50%), none of them reached a significant learning level at the end of the discrimination sessions. The first extinction session is considered as the test session (Colwill et al., 2005). In the present study, while the control fish showed a significant acquisition of ability to discriminate colour for food reward, the Cu exposed zebrafish in both groups did not show a significant learning performance in the first extinction session (Fig. 4B). Copper has been suggested to negatively affect the function of the neuro-sensory system of fishes in various aspects. It has been shown that waterborne Cu exposure (at concentrations of 1-50 µM $CuSO_4$) impairs the function of the lateral line system in zebrafish larvae by inducing cellular damage (Hernández et al., 2006). The effects of copper can also be observed as cytopathological changes in the eye cornea of fish, suggesting impairment of the visual system (Baatrup, 1991). Moreover, the Cu induced olfactory impairment is well documented (Grosell, 2011). Finally, it has been suggested that Cu can affect the fish foraging behaviour by causing cessation of feeding or reduced food consumption (Sandheinrich and Atchison, 1990). Any one or combination of these effects may explain the reduced rate of food consumption of 1.52 µM Cu exposed zebrafish in the present study, which in turn can affect the formation of conditioning.

In the present work, the T-maze assay (over 25 days) was conducted in clean water following an initial 10 day exposure to Cu. Thus interpretation of our results is confounded by the potential recovery of the olfactory and other sensory systems during this period. It has been suggested that the Cu induced olfactory impairment occurs rapidly (within minutes) and persists for weeks or longer (Grosell, 2011). Several studies have addressed the recovery time of the olfactory system following Cu exposure. For example, Saucier and Astic (1995) studied the morpho-functional changes in the olfactory system of rainbow trout (*Oncorhynchus mykiss*) during a 40 week exposure to 20 µg Cu/L (0.31 µM) or 40 µg Cu/L (0.63 µM). The time for the olfactory epithelium to recover was 6 weeks for the 20 µg Cu/L exposed group and 14 weeks for the 40 µg Cu/L group. Beyers and Farmer (2001) showed that recovery of olfactory functions is dependent on Cu exposure conditions (time and concentration); based on literature review, they suggested that the regeneration of olfactory cells after Cu exposure occurs within 8 days to 12 weeks. Accordingly, considering the relatively high concentration and the duration of the applied Cu exposures in the present work, the recovery of olfactory functions during the recovery phase (25 days) would be negligible. The number of studies on the recovery of the other neuro-sensory functions following Cu exposure is limited. Hernández et al. (2006) evaluated regeneration of the neuromasts structure in the lateral line of the larval zebrafish during 5 days following 2 h of Cu exposure (1-100 µM $CuSO_4$). The authors reported that, in anterior lateral line (ALL;

551 which covers the head) regeneration occurred in all applied concentrations, while, in
552 posterior lateral line (PLL; which covers the trunk and tail) regeneration did not occur above
553 a threshold concentration (50 µM $CuSO_4$). They also showed that in concentrations below
554 this threshold, the time of recovery was proportional to the applied Cu concentration.
555 Therefore, they concluded that in the PLL regeneration of neuromasts after copper
556 treatment is concentration dependent.

557 The number of correct choices decreased throughout the extinction sessions (Fig. 4B),
558 suggesting that the formed conditioning between the food and colour stimulus was
559 gradually extinguished in all 3 treatment groups in the absence of food reward. In the
560 present study, zebrafish showed a minor innate preference for red colour, however they
561 could overcome this bias throughout the discrimination sessions and the number of choices
562 for both colours was comparable at the end of the discrimination phase (especially for the
563 control group). The Cu exposure did not affect this preference in our study.

564 Overall, we have shown that zebrafish are able to perform associative learning tasks. In
565 addition, we have demonstrated that, prior exposure to both sub-lethal (0.77 µM) and close
566 to toxicity threshold (1.52 µM) concentrations of Cu can significantly limit the associative
567 learning capacities of zebrafish probably by impairing neuro-sensory as well as central
568 nervous system functions. The magnitude of the body burdens generated following
569 exposure to such high Cu concentrations appears to prevent significant recovery of the
570 sensory system within the 25 day test period.

571 ## 5. Conclusion

572 Copper can affect a wide range of biological processes that may impact on behavioural
573 responses. We have assessed the effect of Cu on the behavioural paradigms and learning
574 capabilities of zebrafish by utilizing two well-known behavioural assays: the novel tank
575 diving test and the T-maze test. Our findings in the novel tank test demonstrate the robust
576 habituation responses of zebrafish, i.e. the exploratory activity of the fish increased by
577 repetition of the assay on subsequent days. Nevertheless, despite the concentration
578 dependent Cu accumulation in the whole body of zebrafish, we have observed almost no
579 effect of Cu exposure on behavioural responses of zebrafish observed in repeated novel
580 tank diving assays. On the other hand, zebrafish showed a remarkable associative learning
581 performance in a T-maze assay. Copper exposure at both concentrations (0.77 and 1.52 µM)
582 was found to significantly limit the learning capability of zebrafish. Overall, our data suggest
583 that exposure to Cu at the concentrations and durations used herein, has no determinative
584 impact on behavioural traits of zebrafish in repeated novel diving task assays but does limit
585 the associative learning abilities of zebrafish in T-maze task. The observed outcomes of
586 behavioural assays depend on the interplay between the exposure conditions
587 (concentration of target compound and duration of exposure) and the timescale of biotic
588 handling processes involved in regulation and repair. Such factors also apply to physiological

endpoints. It will thus be of interest to explore the relative sensitivity of behavioural and physiological endpoints for (eco)toxicological risk assessment.

Acknowledgements

This study was funded by the BELSPO Interuniversity Attraction Pole AquaStress (research project P7/31), and was conducted within the framework of the EnviroStress centre of excellence at the University of Antwerp. We thank S. Joosen for technical assistance.

References

ACOSTA, D. D. S., DANIELLE, N. M., ALTENHOFEN, S., LUZARDO, M. D., COSTA, P. G., BIANCHINI, A., BONAN, C. D., DA SILVA, R. S. & DAFRE, A. L. 2016. Copper at low levels impairs memory of adult zebrafish (Danio rerio) and affects swimming performance of larvae. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 185-186**,** 122-130.

AL-BAIRUTY, G. A., SHAW, B. J., HANDY, R. D. & HENRY, T. B. 2013. Histopathological effects of waterborne copper nanoparticles and copper sulphate on the organs of rainbow trout (Oncorhynchus mykiss). *Aquatic Toxicology,* 126**,** 104-115.

ARTHUR, D. & LEVIN, E. D. 2001. Spatial and non-spatial visual discrimination learning in zebrafish (Danio rerio). *Animal Cognition,* 4**,** 125-131.

AVDESH, A., MARTIN-IVERSON, M. T., MONDAL, A., CHEN, M., ASKRABA, S., MORGAN, N., LARDELLI, M., GROTH, D. M., VERDILE, G. & MARTINS, R. N. 2012. Evaluation of color preference in zebrafish for learning and memory. *Journal of Alzheimer's Disease,* 28**,** 459-469.

BAATRUP, E. 1991. Structural and functional effects of heavy metals on the nervous system, including sense organs, of fish. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology,* 100**,** 253-257.

BAULT, Z. A., PETERSON, S. M. & FREEMAN, J. L. 2015. Directional and color preference in adult zebrafish: Implications in behavioral and learning assays in neurotoxicology studies. *Journal of Applied Toxicology,* 35**,** 1502-1510.

BEYERS, D. W. & FARMER, M. S. 2001. Effects of copper on olfaction of colorado pikeminnow. Environmental Toxicology and Chemistry, 20, 907-912.

BOPP, S. K., ABICHT, H. K. & KNAUER, K. 2008. Copper-induced oxidative stress in rainbow trout gill cells. *Aquatic Toxicology,* 86**,** 197-204.

CACHAT, J., STEWART, A., GROSSMAN, L., GAIKWAD, S., KADRI, F., CHUNG, K. M., WU, N., WONG, K., ROY, S. & SUCIU, C. 2010. Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. *Nature protocols,* 5**,** 1786.

COLWILL, R. M., RAYMOND, M. P., FERREIRA, L. & ESCUDERO, H. 2005. Visual discrimination learning in zebrafish (Danio rerio). *Behavioural Processes,* 70**,** 19-31.

CRESCI, A., SAMUELSEN, O. B., DURIF, C. M. F., BJELLAND, R. M., SKIFTESVIK, A. B., BROWMAN, H. I. & AGNALT, A.-L. 2018. Exposure to teflubenzuron negatively impacts exploratory behavior, learning and activity of juvenile European lobster (Homarus gammarus). Ecotoxicology and Environmental Safety, 160, 216-221.

DE BOECK, G., NILSSON, G. E., ELOFSSON, U., VLAEMINCK, A. & BLUST, R. 1995. Brain monoamine levels and energy status in common carp (Cyprinus carpio) after exposure to sublethal levels of copper. *Aquatic Toxicology,* 33**,** 265-277.

633 DE BOECK, G., VAN DER VEN, K., HATTINK, J. & BLUST, R. 2006. Swimming performance and energy
634 metabolism of rainbow trout, common carp and gibel carp respond differently to sublethal
635 copper exposure. *Aquatic Toxicology,* 80**,** 92-100.
636 DORIA, H. B., FERREIRA, M. B., RODRIGUES, S. D., LO, S. M., DOMINGUES, C. E., NAKAO, L. S., DE
637 CAMPOS, S. X., DE OLIVERIA RIBEIRO, C. A., RANDI, M. A. F. 2018. Time does matter! Acute
638 copper exposure abolishes rhythmicity of clock gene in *Danio rerio. Ecotoxicology and*
639 *Environmental Safety,* 155, 26-36.
640 EGAN, R. J., BERGNER, C. L., HART, P. C., CACHAT, J. M., CANAVELLO, P. R., ELEGANTE, M. F.,
641 ELKHAYAT, S. I., BARTELS, B. K., TIEN, A. K. & TIEN, D. H. 2009. Understanding behavioral and
642 physiological phenotypes of stress and anxiety in zebrafish. *Behavioural brain research,* 205**,**
643 38-44.
644 FÖRSTNER, U. & WITTMANN, G. T. 2012. Metal pollution in the aquatic environment, second ed.
645 Springer, Berlin, Heidelberg.
646 GLASS, A. S. & DAHM, R. 2004. The zebrafish as a model organism for eye development. *Ophthalmic*
647 *research,* 36**,** 4-24.
648 GLEASON, P. E., WEBER, P. G. & WEBER, S. P. 1977. Effect of group size on avoidance learning in
649 zebra fish,Brachydanio rerio (Pisces: Cyprinidae). *Animal Learning & Behavior,* 5**,** 213-216.
650 GOULD, G.G., 2011. Modified Associative Learning T-Maze Test for Zebrafish (Danio rerio) and Other
651 Small Teleost Fish. In: Zebrafish Neurobehavioral Protocols. *Neuromethods*. Kalueff A.,
652 Cachat J. (eds), vol 51, Humana Press, New York, pp. 61-74.
653 GROSELL, M. 2011. Copper. In: Homeostasis and Toxicology of Essential Metals. *Fish Physiology*.
654 WOOD, C. M., FARRELL, A. P. & BRAUNER, C. J. (Eds.), vol. 31A, Academic Press, Elsevier,
655 California, pp. 53-133. doi: https://doi.org/10.1016/S1546-5098(11)31002-3
656 GROSELL, M. & WOOD, C. M. 2002. Copper uptake across rainbow trout gills. *Journal of Experimental*
657 *Biology,* 205**,** 1179.
658 HAVERROTH, G. M. B., WELANG, C., MOCELIN, R. N., POSTAY, D., BERTONCELLO, K. T.,
659 FRANSCESCON, F., ROSEMBERG, D. B., DAL MAGRO, J. & DALLA CORTE, C. L. 2015. Copper
660 acutely impairs behavioral function and muscle acetylcholinesterase activity in zebrafish
661 (Danio rerio). *Ecotoxicology and Environmental Safety,* 122**,** 440-447.
662 HERNÁNDEZ, P. P., MORENO, V., OLIVARI, F. A. & ALLENDE, M. L. 2006. Sub-lethal concentrations of
663 waterborne copper are toxic to lateral line neuromasts in zebrafish (Danio rerio). *Hearing*
664 *research,* 213**,** 1-10.
665 JESSICA, O., MAYARA, S., DIANA, C. & ANA, L. 2015. The Zebrafish World of Colors and Shapes:
666 Preference and Discrimination. *Zebrafish,* 12**,** 166-173.
667 KALUEFF, A. V., ECHEVARRIA, D. J., HOMECHAUDHURI, S., STEWART, A. M., COLLIER, A. D.,
668 KALUYEVA, A. A., LI, S., LIU, Y., CHEN, P. & WANG, J. 2016. Zebrafish neurobehavioral
669 phenomics for aquatic neuropharmacology and toxicology research. *Aquatic Toxicology,* 170**,**
670 297-309.
671 KAMUNDE, C. N. & WOOD, C. M. 2004. Environmental chemistry, physiological homeostasis,
672 toxicology, and environmental regulation of copper, an essential element in freshwater fish.
673 *Australasian Journal of Ecotoxicology* 10, 1-20.
674 LAWRENCE, C. 2007. The husbandry of zebrafish (Danio rerio): A review. *Aquaculture,* 269**,** 1-20.
675 LEVIN, E. D., BENCAN, Z. & CERUTTI, D. T. 2007. Anxiolytic effects of nicotine in zebrafish. *Physiology*
676 *& Behavior,* 90**,** 54-58.
677 MATTHEWS, M. & VARGA, Z. M. 2012. Anesthesia and Euthanasia in Zebrafish. *Ilar Journal,* 53**,** 192-
678 204.
679 NADERI, M., JAMWAL, A., CHIVERS, D. P. & NIYOGI, S. 2016. Modulatory effects of dopamine
680 receptors on associative learning performance in zebrafish (Danio rerio). *Behavioural brain*
681 *research,* 303**,** 109-119.
682 PYLE, G. & FORD, A. 2017. Behaviour revised: contaminant effects on aquatic animal behaviour.
683 *Aquatic Toxicology,* 182**,** 226-228.

PYLE, G. G. & MIRZA, R. S. 2007. Copper-Impaired Chemosensory Function and Behavior in Aquatic Animals. *Human and Ecological Risk Assessment: An International Journal,* 13**,** 492-505.

RANKIN, C. H., ABRAMS, T., BARRY, R. J., BHATNAGAR, S., CLAYTON, D. F., COLOMBO, J., COPPOLA, G., GEYER, M. A., GLANZMAN, D. L., MARSLAND, S., MCSWEENEY, F. K., WILSON, D. A., WU, C.-F. & THOMPSON, R. F. 2009. Habituation revisited: An updated and revised description of the behavioral characteristics of habituation. *Neurobiology of Learning and Memory,* 92**,** 135-138.

ROBINSON, J., SCHMITT, E. A., HAROSI, F. I., REECE, R. J. & DOWLING, J. E. 1993. Zebrafish ultraviolet visual pigment: absorption spectrum, sequence, and localization. *Proceedings of the National Academy of Sciences,* 90**,** 6009-6012.

SACKERMAN, J., DONEGAN, J. J., CUNNINGHAM, C. S., NGUYEN, N. N., LAWLESS, K., LONG, A., BENNO, R. H. & GOULD, G. G. 2010. Zebrafish Behavior in Novel Environments: Effects of Acute Exposure to Anxiolytic Compounds and Choice of Danio rerio Line. *International journal of comparative psychology,* 23**,** 43-61.

SANDHEINRICH, M. B. & ATCHISON, G. J. 1990. Sublethal toxicant effects on fish foraging behavior: empirical vs. mechanistic approaches. *Environmental Toxicology and Chemistry,* 9**,** 107-119.

SAUCIER, D. & ASTIC, L. 1995. Morpho-functional alterations in the olfactory system of rainbow trout (Oncorhynchus mykiss) and possible acclimation in response to long-lasting exposure to low copper levels. *Comparative Biochemistry and Physiology Part A: Physiology*, 112, 273-284.

SISON, M. & GERLAI, R. 2010. Associative learning in zebrafish (Danio rerio) in the plus maze. *Behavioural Brain Research,* 207**,** 99-104.

SPENCE, R. & SMITH, C. 2008. Innate and Learned Colour Preference in the Zebrafish, Danio rerio. *Ethology,* 114**,** 582-588.

STUBBLEFIELD, W. A., STEADMAN, B. L., LA POINT, T. W. & BERGMAN, H. L. 1999. Acclimation-induced changes in the toxicity of zinc and cadmium to rainbow trout. Environmental Toxicology and Chemistry, 18, 2875-2881.

THOMPSON, R. F. & SPENCER, W. A. 1966. Habituation: a model phenomenon for the study of neuronal substrates of behavior. *Psychological review,* 73**,** 16.

TILTON, F., TILTON, S. C., BAMMLER, T. K., BEYER, R., FARIN, F., STAPLETON, P. L. & GALLAGHER, E. P. 2008. Transcriptional Biomarkers and Mechanisms of Copper-Induced Olfactory Injury in Zebrafish. *Environmental Science & Technology,* 42**,** 9404-9411.

TOFT, G. & GUILLETTE, L. J. 2005. Decreased sperm count and sexual behavior in mosquitofish exposed to water from a pesticide-contaminated lake. Ecotoxicology and Environmental Safety, 60, 15-20.

WINBERG, S. & NILSSON, G. E. 1993. Roles of brain monoamine neurotransmitters in agonistic behaviour and stress reactions, with particular reference to fish. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology,* 106**,** 597-614.

WONG, K., ELEGANTE, M., BARTELS, B., ELKHAYAT, S., TIEN, D., ROY, S., GOODSPEED, J., SUCIU, C., TAN, J., GRIMES, C., CHUNG, A., ROSENBERG, M., GAIKWAD, S., DENMARK, A., JACKSON, A., KADRI, F., CHUNG, K. M., STEWART, A., GILDER, T., BEESON, E., ZAPOLSKY, I., WU, N., CACHAT, J. & KALUEFF, A. V. 2010. Analyzing habituation responses to novelty in zebrafish (Danio rerio). *Behavioural Brain Research,* 208**,** 450-457.