Sub-lethal effects of waterborne copper in early developmental stages of rainbow trout (Oncorhynchus mykiss)

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1 **SUB-LETHAL EFFECTS OF WATERBORNE COPPER IN EARLY DEVELOPMENTAL**

2 **STAGES OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)**

3

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5 Shannon Weeks Santos[1], Jérôme Cachot[1], Pierre-Yves Gourves[2], Christelle Clérandeau[1],

6 Bénédicte Morin[1], Patrice Gonzalez[2,*]

7

8 [1]UMR CNRS 5805 EPOC, University of Bordeaux, Avenue des Facultés, 33405, Talence

9 Cedex, France

10 [2]UMR CNRS 5805 EPOC, University of Bordeaux, Place du Dr B. Peyneau, 33120

11 Arcachon, France

12

13 ***Corresponding author: patrice.gonzalez@u-bordeaux.fr**

ABSTRACT

The aim of this work was to study the impact of copper during a sub-chronic exposure to environmental concentrations in the early life stages of rainbow trout (*Oncorhynchus mykiss*). Eyed-stage embryos of rainbow trout, at 265°D, were exposed in semi-static conditions to sub-lethal concentrations of $CuSO_4$ up to the larval stage (528 °D) under laboratory-controlled conditions. During 3 weeks, they were exposed to the environmentally-realistic concentration of 2 µg/L Cu and to a 10-fold higher concentration, 20 µg/L Cu. Several biological (survival, hatching success, malformation, growth) and behavioral (swimming activity) and molecular endpoints (genotoxicity and gene transcription) were studied. Exposure to 20 µg/L Cu had an inhibitory effect on hatching and increased half-hatched embryos (25%). At the end of the exposure, no significant differences were observed in growth of the larvae exposed to the highest Cu concentration. However, larvae exposed to 2 µg/L Cu exhibited increased growth in comparison with non-exposed larvae. The percentage of malformed larvae was significantly higher for both copper conditions, with skeletal malformations being the most observed. Expression of several genes was evaluated in whole larvae using quantitative real-time PCR. Genes involved in detoxification (*gst*, *mt1* and *mt2*) and in cell cycle arrest (*p53*) were significantly repressed in both copper conditions when compared to control. In addition, potential genotoxic effects on larvae were investigated by the comet assay on blood cells, but this test did not demonstrate any significant DNA damage on larvae exposed to copper. This study confirms the adverse effects of copper on early life stages of rainbow trout even at the lowest environmentally relevant tested concentration.

Keywords: copper, *Oncorhynchus mykiss*, early life-stage, developmental toxicity, genotoxicity, gene transcription.

41 **1. Introduction**

42 Copper is one of the few metals that occurs naturally in ecosystems, as well as being one of
43 the first used by humans since 8000 B.C. Over 95 % of all copper in circulation today has been
44 extracted since 1900. It is an essential trace element involved in numerous biological
45 processes, such as photosynthesis in plants. It also plays an essential role in certain enzyme
46 functions in animals, including copper-zinc superoxide dismutase and cytochrome c-oxidase
47 (Eisler, 1998). Copper is widely used in industrial, agricultural and urban activities, and
48 consequently is one of the priority substances listed in the Water Framework Directive (WFD).
49 With its significant anti-microbial and anti-fungal properties, copper has been used extensively
50 in vineyards, resulting in high levels of transfer to soils and aquatic ecosystems (Devez et al.,
51 2005; El Azzi et al., 2013; Komárek et al., 2010; Ruyters et al., 2013). Recent reports have
52 shown that Cu-containing fungicides and boat antifouling paints appear to be the main
53 anthropogenic sources of Cu in aquatic ecosystems (Babcsányi et al., 2014; Devier et al.,
54 2005; Petit et al., 2013; Singh & Turner, 2009). Monitoring this element in the coastal marine
55 environment by the Chemical Contamination Observation Network (ROCCH, IFREMER) using
56 the Pacific oyster (*Magallana gigas*) as a sentinel species showed an increase in its
57 concentration at certain points along the French coast, such as Arcachon Bay (Claisse and
58 Alzieu, 1993; Gamain et al., 2016).

59 Pollution of surface water and groundwater is one of the biggest environmental issues. The
60 background copper concentrations in unpolluted water bodies varied between 0.2 to 6 µg/L
61 (Flemming and Trevors, 1989; Willis and Bishop, 2016). Levels of copper can increase from
62 anthropogenic pollution as a result of urban and agricultural runoffs (Davis et al., 2001; Devez
63 et al., 2005). Copper concentration in Beysehir lake (Turkey) was up to 100 µg/L in spring 2004
64 (Tekin-Özan, 2008). The river Lot (southern France), with a large history of metal pollution,
65 concentrations of copper in water column was 17 µg/L in 1987; but, this concentration
66 decreased in 2006 to 3.2 µg/L (Shinn et al, 2009). In several Asturians rivers (Spain), copper

67 concentrations were between 2.5 and 4.9 µg/L (Linde et al., 1998), and in Boulder river
68 (Montana, USA) was ranged from 1.4 to 23 µg/L (Farag et al., 2007).

69 High concentrations of copper in ecosystems may be harmful to aquatic organisms. Indeed,
70 although essential, Cu induces toxic effects during chronic exposure and over-accumulates in
71 living organisms (Marr et al., 1996; Kamunde et al., 2002; Vardy et al., 2013). Since copper
72 can participate in Fenton and Haber-Weiss reactions, it can produce reactive oxygen species
73 (ROS), which react with H_2O_2 and cause cellular damage to lipids, nucleic acids and proteins
74 that can lead to cell death (Manzl et al., 2004; Prousek, 2007; Bopp et al., 2008). Many studies
75 have highlighted the toxic effects of copper on fish and mollusks (Vardy et al., 2013; Gamain
76 et al., 2016; Boukadida et al., 2017). Sensitivity to copper can vary greatly from one fish
77 species to another. For example, Vardy et al. (2013) determined the lethal concentrations
78 (LC_{50}) at 96 h of Cu exposure at different life stages of fish, which for swim-up larvae of white
79 sturgeon and rainbow trout was 10 and 21 µg/L of Cu, respectively. McKim et al. (1978)
80 examined embryo mortality of eight different fish species after Cu exposure and observed
81 significant mortality between 37 and 555 µg/L. Besides, this sensitivity to metals could also
82 differ according to the development stage (McKim et al., 1978; Vardy et al., 2013) and water
83 chemistry parameters like hardness, pH, organic carbon and alkalinity (Brix et al., 2017;
84 Crémazy et al., 2017; Eisler, 1998).

85 Several reports have revealed that early life stages of fish are a great deal more vulnerable to
86 pollutants than juveniles and adults (Eaton et al., 1978; Hutchinson et al., 1998; Van Leeuwen
87 et al., 1985; Vardy et al., 2013). Embryos and larvae organs, such as liver, gills and kidney,
88 are not entirely developed, which can result into a higher sensitivity to toxicants (Mohammed,
89 2013). Additionally, larvae have a permeable skin that could allow the uptake of pollutants
90 (Mohammed, 2013). Because fish embryos and larvae have an important role in the trophic
91 chain and, in the persistence of population as well, the use of these stages are relevant for the
92 assessment of toxicity in the aquatic environment. Several reports have studied copper toxicity

93 in early life stages of fishes (Barjhoux et al., 2012; El-Fiky, 2001; Kazlauskienë, 2002;
94 Stasiūnaitė, 2005; Witeska et al., 2010).

95 Early life stages of rainbow trout (*Oncorhynchus mykiss*) can be easily raised under laboratory
96 conditions, and it is considered, along with other species, as a model animal in ecotoxicology
97 (OECD, 2013). Due to its slow embryo-larval development, toxicity tests allow longer sub-
98 chronic exposures to toxicants. This species has been extensively studied and, because of its
99 low tolerance to poor water quality, is considered as one of the most commonly used sentinel
100 species for aquatic ecotoxicology (De Boeck et al., 2004; Hansen et al., 2002; Kamunde et al.,
101 2002; Olsson et al., 1990).

102 The purpose of the present work was to evaluate and to characterize the sub-lethal effects of
103 environmental concentrations of copper to early developmental stage of rainbow trout. At the
104 end of the 3-week Cu exposure, several end-points like viability, hatching rate, developmental
105 abnormalities, larval swimming behavior, genotoxicity (DNA damage), lipid and protein
106 peroxidation, and gene expression were analyzed.

107

108 ## 2. Materials and methods

109 ### 2.1. Chemicals

110 Copper sulfate ($CuSO_4 \cdot H_2O$, CAS 7758-99-8, 99.99%) and low melting point agarose (LMPA)
111 were purchased from Sigma Aldrich (Lyon, FR). Phosphate-buffered saline (PBS), nitric acid
112 65% and normal melting point agarose (NMPA) were purchased from Fluka Chemie
113 (Buchs, CH).

114

115 ### 2.2. Exposure condition

116 Rainbow trout embryos (*Oncorhynchus mykiss*) at eyed stage were provided by INRA-PEIMA
117 (Sizun, FFR). They were incubated in total darkness under a temperature of 12°C. Embryos

118 were exposed to copper from 265 DD (degrees days) up to the larval stage of 528 DD for a
119 total of 23 days of exposure. Two concentrations of exposure for copper were chosen: 2
120 (environmental concentration) and 20 µg/L. These solutions ~~Copper solutions (2 and 20 µg/L
121 Cu)~~ were prepared using $CuSO_4·5H_2O$ and the required concentrations were calculated
122 according to the quantity of copper ions (Cu^{2+}). Each studied condition, and a control without
123 Cu, involved 3 replicates with 100 embryos in 1 L aquaria. Exposure water was prepared in
124 three 5 L tanks of Saint-Etienne spring water (63 mg/L Ca, 10.2 mg/L Mg, 1.4 mg/L Na, 0.4
125 mg/L K, 173.2 mg/L HCO_3^-, 51.3 mg/L SO_4^{2-}, 2 mg/L NO_3^-, pH 7.6, <1 mg/L Cl^-, <1.5 mg/L
126 DOC). A peristaltic pump (Watson Marlow, USA) was used to maintain a continuous flow rate
127 of water (9 mL/min) into the incubation chambers. To keep the require copper concentration in
128 aquaria, water was renewed every 48 h. At the end of the experiment, larvae were sampled
129 per replicate as follow: 10 larvae to record for biometrics and morphological anomalies, 6
130 larvae for the study of swimming behavior, 6 larvae for blood sampling for genotoxicity test.
131 The remaining larvae were pooled and stored at -80°C for biochemical analysis and gene
132 transcription.

133

134 **2.3. Analysis of copper in water and larvae**

135 Fish and water samples were analyzed to determine Cu concentrations. Fish samples (2 pools
136 of 5 larvae from each replicate) were lyophilized and digested by nitric acid (0.3 mL of HNO_3
137 67 %). Four tubes containing the international certified reference material, Dolt-5 samples
138 (Fish liver), were prepared in order to verify the accuracy of the method. Approximately 80 mg
139 of this material was digested by 3 mL of nitric acid in polypropylene tubes. Then, this mixture
140 was heated by Hot Block for 3 h at 100°C, and after cooling 1.5 mL and 15 mL of ultrapure
141 water (Milli-Q®) was respectively added to sample larvae and certified reference. Finally, 3
142 blank samples were also prepared in the same conditions (3 mL of HNO_3 before heating and
143 15 mL of Milli-Q water added after cooling). 40 mL of water sample of each condition were
144 collected in 3 random days through the experiment, and acidified with 5 % of nitric acid (Nitric

145 acid 65 %, Fluka). Copper concentrations in water of condition “20 µg/L” and fish samples were
146 analyzed by inductively Coupled Plasma Optic Emission Spectrometry (ICP-OES 720, Agilent
147 Technologies), whereas copper concentrations in water of controls and “2 µg/L” conditions
148 were analyzed by atomic absorption spectrophotometer (Varian SpectrAA 240Z, Agilent
149 Technologies, Santa Clara, USA). Detection limits (DL) for ICP-OES was 2.26 µg/L Cu^{2+} and
150 for atomic absorption was 0.5 µg/L Cu^{2+}.

151 **2.4. Phenotypic effects**

152 Dead embryos and larvae were recorded daily and removed immediately to prevent water
153 alterations. Individuals were considered half-hatched when at least a part of the body was
154 inside the chorion. Embryonic and larval mortality refer to the number of dead individual
155 compared to the total number of embryos at the start of the experiment or total number of
156 totally hatched larvae. Half-hatched mortality was only considered when at least a part of the
157 body was inside the chorion. Hatching time refers to the number of degree days (DD: number
158 of days x temperature) from fertilization to hatching. Hatching rate is calculated by dividing the
159 number of half-hatched embryos by the total number of embryos at the beginning of the
160 experiment. The appearance of anomalies was microscopically examined in 10 larvae per
161 replicate randomly chosen. The larvae were placed in a Petri dish with carbonated water and
162 ice to sedate them. Photos were taken for each larva with a stereomicroscope (MZ7.5 Leica)
163 coupled to a camera CCD (DFP420C Leica) and a cold light (Intralux® 4100, Volpi AG,
164 Schlieren, Switzerland). From the photos, we measured total body and head length for each
165 larva using Leica Microsystems software v3.8 (Nanterre, France). Larvae were also observed
166 to locate possible malformations such as edemas, spinal and craniofacial deformities, cardiac
167 anomalies, hemorrhages and yolks sac resorption.

168

169

170

2.5. Genotoxicity test

The comet assay was performed according to the Singh et al. (1988) protocol, adapted by Le Bihanic et al. (2014). Blood sampling were performed in 6 larvae per replicate by decapitation using a heparinized pipette. Samples were stored in a microtube with 200 µL of cryopreservation solution (250 mM sucrose, 40 mM citrate trisodique, 5 % DMSO, pH adjusted to 7.6 with nitric acid 1 M) and immediately frozen in liquid nitrogen until analysis. These storage conditions have already been described by Barillet et al. (2005) and Bony et al. (2008), this protocol has been validated for rainbow trout blood cells in our laboratory. 50 µL of cell suspension was added to 100 µL of LMPA (Low Melting Point Agarose). Two aliquots of 50 µL of the suspension were placed onto slides previously covered with NMPA (Normal Melting Point Agarose, 1 %, w/w) and a coverslip (18x18 mm) was added. The gels were left to harden for 15 min at 4°C and then the coverslips were removed from each slice and a second layer of LMPA was placed on the gels with new coverslips and hardened at 4°C for 15 minutes. The slices were put in a lysis solution (10 mM Tris; 2.5 M NaCl; 100 mM EDTA; 1% Triton x-100; 10% DMSO; pH adjusted to 10 with NaOH) at 4°C for 90 minutes. Slices were placed horizontally on a tray with electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH> 13) for 20 minutes to allow the DNA to unwind. Electrophoresis was performed with a voltage of 25 V and 290 mA for 20 minutes. After electrophoresis, slices were rinsed 3 times with a neutralizing solution (0.4 M Tris, pH 7.5) for 5 minutes at 4°C. Then, slices were soaked with absolute ethanol for 20 minutes and dried at a room temperature for at least 24 hours. Slices were stained with 20 µg/mL of ethidium bromide solution. Comet analysis was performed using an epifluorescence microscope, and DNA damage was measured with Comet Assay IV software on 100 cells randomly selected. Results are expressed as percentage of degradation of DNA tail. Heavily degraded cells, or inexistent head with diffuse tail, were not counted in the tail DNA measurements as proposed by Kumaravel et al. (2009). However, they were counted as hedgehogs on a total of 100 cells per gel.

198 **2.6. Behavioral analysis: swimming test**

199 The swimming behavior of 528 DD larvae was studied using the Daniovision Image analysis
200 system version 10.0 Noldus. Six larvae per replicate were placed in 6-well microplates
201 containing 8 mL of exposure water. The microplates were placed in the recording chamber in
202 the dark at 12 °C for about 30 min to acclimate the larvae. Then the analysis was started. An
203 infrared camera in the chamber recorded the movements of each larvae focusing on their
204 center of gravity. The analysis takes 30 min with dark/light/dark cycle of 10 min each. This
205 cycle allows to analyze the behavior of larvae in response to light stimulation. Ethovision
206 software allows to record the average speed of each larvae every 30 seconds. For each larva,
207 the swimming performance was assessed by determining the total distance moved (cm) in 10-
208 minute period, and time of mobility accumulated (s) on larvae. Mobility is defined as the period
209 of time where the area of total larva is modified albeit central point of larvae had not changed.
210 If more than 60 % of the area was changed, it was considered as highly mobile. Otherwise,
211 less than 60 %, it was considered only as mobile.

212

213 **2.7. Biochemical analysis**

214 *Preparation of supernatant*

215 Pools of 5 larvae were frozen in liquid nitrogen and then stored at -80°C until analysis. At the
216 moment of analysis, yolk sac from larvae were removed and then larvae (approximately 250
217 mg) were homogenized in a chilled phosphate buffer (0.1 M; pH 7.5) using an UltraTurrax®
218 tissue homogenizer fitted with a potter at 3,000 rpm (4°C). The supernatant was centrifuged at
219 9,000 g for 25 min at 4°C to obtain the supernatant S9 fraction. This fraction contains soluble
220 proteins from cytosol and microsomes (Parmentier et al., 2007). Total protein, TBARS levels
221 and protein carbonyls content were analyzed using the S9 fraction.

222

223 *Total protein*

224 The total protein concentration was measured on S9 fraction by the method of Lowry et al.,
225 (1951) using Bovine Serum Albumin (BSA) as a standard and Folin's reagent. Measurements
226 were done using a spectrophotometer microplate reader (Synergy HT, BioTek).

227 *Lipid peroxidation*

228 Lipid peroxidation was determined using the adapted technique of Buege and Aust (1978)
229 adapted to a microplate reader. 500 µL of a solution containing 20 % of trichloroacetic acid
230 (TCA) and 20 % butylated hydroxytoluene (BHT) was added to 500 µL of S9 fraction and then
231 mixture was centrifuged for 10 min at 9,000 g. Afterwards, 600 µL of supernatant with 480 µL
232 of TRISbase (25 mM)-TBA (thiobarbituric acid-100 mM) and 120 µL of 0.6 N HCl heated at
233 80°C for 15 min. The tubes were then cooled and mixed. TBARS levels were read using a UV-
234 spectrophotometer (Biotek Synergy HT) in a microplate at 530 nm. Results were expressed as
235 nmoles of thiobarbituric acid reactive substance (TBARS) equivalents per milligram of protein.

236 *Protein Carbonyl analysis*

237 Protein carbonyl content was assessed using the spectrophotometry method described in
238 Augustyniak et al., (2015). 50 µL of a solution of 11 % streptomycin sulfate – phosphate buffer
239 (100 mM pH 7.4) were added to 500 µL of S9 fraction, then it was mixed and incubated for 15
240 min at room temperature. Afterwards, mixtures were centrifuged for 10 min at 6,000 g. Then
241 200 µL supernatant was added to 800 µL of DNPH (2.4-dinitrophenylhydrazine 10 mM) used
242 as a sample tube, and to 800 µL of HCl 2.5 M used as a control tube; then, incubated for 1 h
243 at room temperature with vortexing every 15 min. Proteins were precipitated with 1 mL of 20 %
244 TCA (trichloroacetic acid), vortexed and centrifuged for 10 min at 10,000 g. The pellets were
245 rinsed with 1 mL of ethanol-ethyl acetate (v:v), vortexed and centrifuged three times. Then
246 pellets were solubilized with 500 µL of 6 M guanidine HCl and centrifuged at 10,000 g for 10
247 min. The carbonyl content was measured using a UV-spectrophotometer (Biotek Synergy HT)
248 at 370 nm. Results were expressed as nanomoles of DNPH incorporated/mg protein.

249 **2.8. Gene expression**

250 6 larvae per replicate were collected in a storage buffer (RNA later, Qiagen). Samples were
251 frozen in liquid nitrogen and stored at -80°C until analysis.

252 *RNA extraction*

253 Total RNA extraction from whole larvae was performed with the kit “SV Total RNA Isolation
254 system” (Promega) following the supplier’s recommendations. Larvae were homogenized
255 using the MP fastprep®-24 (Biorad, 6 m/s, 40s) with 14.4 mm diameter ceramics beads (MP
256 Biomedicals, Lyssing Matrix D Bulk).

257 *Retro-transcription of total RNA into cDNA*

258 The retro-transcription of total purified RNA was carried out with the kit “GoScript Reverse
259 Transcription System” (Promega). To execute the Reverse transcription, 1 µL of oligodT (1
260 µM) and 1 µL of hexanucleotides (1 µM) have been added to 10 µL of total purified RNA (1
261 µg). Then, the mix was heated at 70°C for 5 minutes with a thermocycler (Eppendorf
262 Mastercycler) followed by 5 minutes at 4°C to allow primer annealing. Subsequently, 1 µL of
263 dNTP solution (10 mM), 4 µL of activity buffer, 1.5 µL of $MgCl_2$ (25 mM), 1 µL or reverse
264 transcriptase (1 U/µL) and 0.5 µL or RNAsine were added. The reverse transcription reaction
265 was performed for 1 h at 42°C in a thermocycler. The cDNA thus obtained were stored at -
266 20°C pending analysis by quantitative real-time PCR reaction.

267 *Quantitative real-time PCR*

268 Thirteen genes were investigated and specific primer-pairs designed with primer3plus software
269 (Table 1). All the primer-pairs used in this study have an efficiency upper than 95%. Real-time
270 qPCR was carried out using GoTaq® qPCR Master Mix kit (Promega). PCR reactions
271 contained 17 µL of a mixture of Nuclease-Free Water and GoTaq® qPCR Master Mix
272 containing the SyberGreen fluorescent dye, 2 µL of specific primer pairs mix (200 µM each)
273 and 1 µL of cDNA. Real-time quantitative PCR reactions were performed in a Mx3000P®
274 qPCR System (Stratagene). The amplification program consisted in one cycle at 95°C for 10

275 min, then 45 amplification cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. Specificity
276 was determined for each reaction from the dissociation curve of the PCR products. This
277 dissociation curve was obtained by following the SYBR Green fluorescence level during a
278 gradual heating of the PCR products from 60 to 95°C.

279 Cycle thresholds (Ct) were obtained from MxPro™ qPCR software for each gene. Two different
280 reference genes were investigated (Rpl7 and EF1α) and were found to be stable in our
281 conditions. Consequently, relative quantification of each gene expression level was normalized
282 according to the mean Ct value of these two reference genes according to the 2∆Ct methods
283 described by Livak and Schmittgen (2001). The ratio of transcription level to exposed
284 individuals with the control ones gives the induction factor (>2) or repression factor (<0.5) of
285 studied genes.

286

287 **2.9. Statistics**

288 Each studied condition was triplicated identically and each replicate was considered as an
289 independent sample. All data are represented by the mean ± SE (Standard Error). Statistical
290 analyzes were carried out using R (http://cran.r-projet.org/). The Normality of data distribution
291 was verified on the residues by the Shapiro-Wilk test ($p < 0.01$) and the homogeneity of
292 variances was evaluated by the Levene test ($p < 0.05$). In the case of normal distribution, a
293 one-way ANOVA analysis was used ($p < 0.05$) followed by a Tukey post-hoc test. In the case
294 that data was not normal, data was evaluated by non-parametric tests of Kruskal-Wallis
295 ($p < 0.05$).

296

297 **3. Results**

298 **3.1. Test condition**

299 Dissolved oxygen in water varied between 81.4 and 95.7 % throughout embryonic and larval
300 exposure. Table 2 shows Cu concentrations determined in the exposure water. Water samples
301 were collected in 3 random days. In the control group, Cu concentrations were below the
302 detection limit. Cu concentrations of the treatment at 20 µg/L were close to the nominal
303 concentration. However, results from 2 samples of the treatment at 2 µg/L were below the
304 detection limit (0.5 µg/L Cu).

305

306 **3.2. Embryonic and larval survival**

307 No significant differences in embryonic and larval survival were observed between the control
308 group and the condition at 2 µg Cu /L (Table 3). Regarding the condition of 20 µg/L Cu, a
309 significant decrease of embryonic survival was observed compared to control. Mortality of half-
310 hatched embryos was significantly higher for those exposed to 20 µg/L Cu compared to other
311 conditions. In addition, Figure 1 and Table 3 show a significant lower hatchability for this
312 condition in comparison to control and 2 µg/L Cu. On the other hand, the hatching time (DD)
313 did not show any significant differences between the control and the two Cu exposure
314 conditions (Table 3).

315

316 **3.3. Larvae copper accumulation**

317 After 3 weeks of exposure, larvae exposed to 20 µg/L Cu showed a significantly higher
318 accumulation of Cu ($p < 0.01$) compared to control and larvae exposed to 2 µg/L Cu (Table 3).
319 No significant difference in Cu accumulation was observed in larvae exposed to 2 µg/L Cu
320 comparatively to control.

321

322 ### 3.4. Biometrics

323 Exposure to the higher concentration of Cu (20 µg/L) did not promote significant changes on
324 larvae biometrics compared to control (Table 3). However, larvae exposed to 2 µg/L Cu were
325 significantly longer compared to control larvae. However, this difference is small representing
326 only 4.14 % of increase between the two conditions. No significant differences were observed
327 between the length of head and the ratio between the size of head and the total length for the
328 two studied Cu conditions and the control (Table 3).

329

330 ### 3.5. Malformations

331 Both copper conditions impaired normal development of larvae, where total malformations
332 were significantly higher when compared to control condition (Table 3). Spinal anomalies
333 (kyphosis, lordosis and c-shaped curvature) were the most frequently observed malformation,
334 but its incidence was much higher in the Cu exposed groups than in control. The incidence of
335 all the other recorded abnormalities (edemas, yolk sac malabsorption and craniofacial
336 malformation) tended to increase with increasing Cu concentration in the exposure water,
337 although no significant differences were observed among groups (Table 3).

338

339 ### 3.6. Behavior

340 Responses lo light stimulation of larvae are presented in Figure 2. The results represent the
341 average distance traveled by larvae (Figure 2-A) and the cumulative time of high mobility
342 (Figure 2-B) and mobility (Figure 2-C) of larvae. Over the light period, larvae from all conditions
343 showed similar responses to light stimulation and all larvae increased their distance traveled
344 and their mobility compared to the first dark period. However, no significant differences were
345 observed between conditions on all studied parameters.

346

347 **3.7. Genotoxicity**

348 Results from the comet assay did not reveal any significant DNA damage in both Cu studied
349 conditions when compared to control larvae (Figure 3-A). Nevertheless, the percentage of
350 severely damage cells or ghost cells (“hedgehog”) was significantly increased in larvae
351 exposed to 20 µg/L Cu in comparison to control (Figure 3-B).

352

353 **3.8. Lipid and protein oxidation**

354 Larvae exposed to 2 and 20 µg/L of Cu exhibited no significant changes on the content of
355 thiobarbituric acid (Figure 3-D) and protein carbonylation (Figure 3-C) comparatively to non-
356 exposed larvae.

357

358 **3.9. Gene expression**

359 Cu effects on gene transcription were pronounced on both larvae exposed to 2 and 20 µg/L Cu
360 (Table 4). Indeed, genes involved in detoxification (*gst*, *mt1* and *mt2*) and in cell cycle arrest
361 and apoptose (*p53*) were significantly repressed compared to control larvae. In contrast, gene
362 transcription related to DNA repair system (*ogg1* and *rad51*) was not significantly altered at the
363 end of 3-weeks Cu exposure. Also, no effects were observed on *cat* and *12s* genes for both
364 studied conditions. In the case of larvae exposed to 2 µg/L Cu, the transcription of *sod* was
365 significantly decreased when compared to control larvae.

366

367 **4. Discussion**

368 Copper is a ubiquitous metal that is naturally present in the environment. Although copper is
369 an important trace element for the proper functioning of the organism, strong concentrations
370 may be harmful to aquatic organisms (Flemming and Trevors, 1989; McKim et al., 1978). In
371 the present study, exposure to 2 µg/L Cu did not increase embryonic and larval survival and

372 hatching rate of rainbow trout when compared to control. Regarding the exposure to 20 µg/L
373 Cu, a significant decreased of embryonic viability was observed. However, the embryonic
374 viability was superior to the survival criteria given by OECD (2013) at 75 % for control condition
375 of rainbow trout. On the other hand, our results from this condition show a strong mortality
376 during hatching of rainbow trout. Regarding the literature, our results are comparable to those
377 found by other authors. McKim et al., (1978) demonstrated an almost complete embryo
378 mortality on rainbow trout when exposed to 37 µg/L Cu. Compared to other species, rainbow
379 trout showed greater sensitivity to Cu. For example, for a complete embryo mortality of brook
380 trout (*Salvelinus fontinalis*), lake trout (*Salvelinus namaycush*) and herring (*Coregonus artedi*)
381 about 555 µg/L of Cu were needed (McKim et al., 1978). Vardy et al. (2013) determined that
382 the LC_{50} at 96 h for yolk-sac stages of white sturgeon (*Acipenser transmontanus*), rainbow
383 trout and fathead minnows (*Pimephales promelas*) was 22, 40 and 102 µg/L of Cu respectively.
384 It was also observed that later larval stages of white sturgeon and rainbow trout were even
385 more sensitive to the effects of Cu than yolk sac stages (9 and 22 µg/L of Cu respectively).
386 Another study was performed on two different stages of rainbow trout, embryo and swim-up
387 stages, where lethal responses to Cu were observed from 22 µg/L for both stages after a sub-
388 chronic exposure (Besser et al., 2005). Cu lethality not only depends on the sensitivity of the
389 fish species and stage, but also on the water chemistry parameters such as pH, concentration
390 of DOM (dissolved organic manner), and hardness (Brix et al., 2017; Crémazy et al., 2017).
391 Crémazy et al. (2017) observed that tolerance of juvenile rainbow trout to Cu was augmented
392 when pH, hardness and concentration of DOM were increased.

393 Mortality of half-hatched embryos exposed to 20 µg/L of Cu was significant during hatching
394 time; hence, the hatchability was significantly reduced compared to the control group. Hatching
395 can be a very critical step for fishes. Throughout the embryonic stage, the chorion offers
396 protection to embryos in a contaminated environment. However, during hatching this protection
397 is no longer present and the larvae are in direct contact with the contaminated medium (Arukwe
398 et al., 1997). One hypothesis is that copper may have an inhibitory effect on the activity of the

399 chorionase enzyme, which degrades the inner area of the chorion to facilitate the hatching by
400 mechanical and osmotic process (Johnson et al., 2007; Jezierska et al., 2009; Barjhoux *et al.*,
401 2012).

402 At the end of exposure, control larvae had accumulated 5.66 ± 0.13 µg/g Cu (d.w.). According
403 to Shearer (1984), Cu accumulation in unexposed larvae of rainbow trout can vary between 2
404 and 7 µg/g Cu d.w. Results of Cu accumulation on unexposed larvae from Shearer (1984) and
405 Hansen et al. (2002) are comparable to our results. Marr et al. (1996) revealed that swim-up
406 fry rainbow trout exposed to 9 µg/L Cu accumulated 9.89 µg/g Cu after 20 days exposure and
407 continue to accumulate up to 17.91 µg/g after 60 days exposure. This accumulation is higher
408 than that obtained in our study after 3-weeks of exposure at 20 µg/L Cu. Several authors have
409 suggested that copper accumulation is regulated by the depuration and the slow exchange
410 capacity of metals with the surrounding medium (Carbonell and Tarazona, 1994; Marr et al.,
411 1996). Despite the lethal effects observed, no significant changes on larvae biometry was
412 reported at the end of exposure to 20 µg/L Cu. A decrease on total size of larvae was expected,
413 since the process of metabolism and homeostasis consume energy and the growing rate of
414 fish may be affected as explained by Marr et al. (1996). Several studies have shown a
415 relationship between the metal accumulation and the total size of larvae when they are
416 exposed to metals (Guo et al., 2016; Marr et al., 1996). However, our results seem to be
417 consistent with the low Cu accumulation in larvae exposed to this condition. Regarding Vardy
418 et al (2013) results about the differences in tolerance according to the larval stage, we might
419 presume that the lack of Cu accumulation in our larvae is probably link to the fact that, at the
420 end of the 3-weeks Cu exposure, our larvae were still absorbing their yolk-sac and probably
421 just starting to change to exogenous feeding. Swim-up rainbow trout larvae used in Marr et al.
422 (1996) were fed and were more active leading to a larger exposure to Cu since more water is
423 pumped through the gills. Since our larvae were not fed, there was no contamination or Cu
424 accumulation by trophic way.

425 For larvae in control conditions, the most commonly observed malformations were edema,
426 vertebral abnormalities and craniofacial anomalies. These kinds of malformations in control
427 condition have already been noticed by several authors (Barjhoux et al., 2012; Jezierska et al.,
428 2009; Witeska et al., 2010), and can be attributed to parental factors or laboratory culture
429 conditions. In this study, exposure to 2 and 20 µg/L Cu showed a significant increase on
430 malformed larvae compared to control larvae. Vertebral abnormalities (kyphosis, lordosis,
431 scoliosis and C-shape form) were the most recorded among all observed deformities. In the
432 literature, vertebral deformations are also the most observed malformations (Barjhoux et al.,
433 2012; Jezierska et al., 2009; Witeska et al., 2010). For instance, 80% of malformations
434 recorded on barbel larvae (*Barbus barbus*), after exposure to different concentrations of copper
435 and lead were vertebral abnormalities (Ługowska and Kubik, 2011). Japanese medaka larvae
436 (*Oryzias latipes*) showed 35 and 59 % of spinal deformities following an embryonic exposure
437 to copper and cadmium spiked-sediment (Barjhoux et al., 2012). These defects can be caused
438 by disturbances induced by metal exposure that may occur at early stages of development or
439 difficulties encountered during a laborious hatching (Jezierska et al., 2009).

440 One aim of this work was to study the impact of copper on swimming behavior of larvae.
441 Exposed larvae covered similar distances as control larvae and no significant difference was
442 observed on travelled distance. The study of Haverroth et al. (2015) showed significant
443 reduction on total distance traveled and restricted mobility on zebrafish (*Danio rerio*) exposed
444 to 6 µg/L Cu after 24 hours. This can suggest that locomotor patterns were weakened
445 (Haverroth et al., 2015). Atchinson et al. (1980) described the different ways by which metals
446 can alter the locomotion on fish, either by the influence on the attraction or the evasion to a
447 contaminated area, the impaired sensory perception on hyper or hypoactivity, or reducing the
448 swimming performance.

449 The comet assay did not reveal any significant induction of DNA damage promoted by Cu
450 exposure. However, several studies have reported DNA damage upon exposure to Cu or other
451 metals (Barjhoux et al., 2012; Bopp et al., 2008; Canalejo et al., 2016; Oss et al., 2013; Santos

452 et al., 2010). Barjhoux et al. (2012) demonstrated the genotoxic potential of Cu and Cd on
453 *Japanese medaka* larvae after embryonic exposure to environmental sediments from
454 contaminated sites of the Lot-Garonne-Gironde continuum. ~~Oss et al. (2013) observed an~~
455 ~~increase of micronuclei levels after 30-days exposure to environmental concentrations of Cu~~
456 ~~(0, 25, 50 µg/L) on juvenile fat snook (*Centropomus parallelus*), and these differences did not~~
457 ~~change after 30-days of recovery.~~ In one study, a significant increase of micronuclei levels was
458 also observed on European seabass (*Dicentrarchus labrax*) exposed to Cu (0.01-1 mg/L) for
459 96 h (Canalejo et al., 2016). However, at 24 h exposure, differences were only observed on
460 fish exposed to the strongest Cu concentration (1 mg/L). Canalejo et al. (2016) also observed
461 that micronuclei frequency was even more important for fish exposed to Cu than those exposed
462 to As or Pb at the same concentrations. Environmental concentrations of Cu (3.2 to 128 µg/L)
463 induced DNA strand breaks in a concentration-dependent manner in blood cells of mature
464 male stickleback after exposure for 4-days (Santos et al., 2010). Oss et al. (2013) observed
465 an increase of micronuclei levels after 30-days exposure to environmental concentrations of
466 Cu (0, 25, 50 µg/L) on juvenile fat snook (*Centropomus parallelus*), and these differences did
467 not change after 30-days of recovery. In our study comet assays were performed after 3-weeks
468 exposure which could allow DNA repair system activation and efficient DNA damage repair.
469 However, this is not in accordance to Oss et al. (2013) which did not observed recovery of
470 micronuclei. On the other hand, we found an increase of very damaged cells counted as
471 "hedgehog" in the group of 20 µg/L Cu. An increase of these cells may be explained by the
472 generation of DNA strand breaks through the production of reactive oxygen species by Cu.
473 These "hedgehog cells" were currently considered as apoptotic or necrotic cells (Barjhoux et
474 al., 2012). However, some studies have evidenced that these hedgehog cells could be repaired
475 and should not be considered as apoptotic cells (Lorenzo et al., 2013; Meintières et al., 2003).
476 To determine the real nature of these cells, other analytical techniques would be required such
477 as flow cytometry. ~~In our study case, an increase of these cells may be explained by the~~
478 ~~generation of DNA strand breaks through the production of reactive oxygen species by Cu. To~~

479 ~~confirm this hypothesis, it would be necessary to evaluate ROS production in cells and/or to~~

480 ~~determine the real nature of these cells by flow cytometry.~~

481 One way Cu exerts its toxicity is by the formation of reactive oxygen species (ROS), which at
482 important levels can damage the cell through lipid peroxidation, protein denaturation and DNA
483 damage (Romeó et al., 2000; Sevcikova et al., 2011; Vutukuru et al., 2006). Catalase (cat)
484 and superoxide dismutase (sod) are two of the enzymes that protect cells from the oxidative
485 damage caused by ROS. Sod catalyzes the dismutation of the superoxide anion radical into
486 oxygen and hydrogen peroxide, which could be further decomposed by the *cat* into water and
487 oxygen. In our study, after a sub-chronic exposure to Cu, RT-PCR results for *cat* expression
488 did not reveal any significant modulation on rainbow trout larvae. Woo et al. (2009) observed
489 an important induction of *cat* gene in liver when Javanese medaka (*Oryzias javanicus*) was
490 exposed to different concentrations of Cu, Cd, and Zn for 48 h. In the study of Casanova et al.
491 (2013) *cat* expression on tambaqui fish liver increased after 1h of exposure to copper and
492 decreased to basal levels at 3 h of exposure. The expression level of cytoplasmic *sod* was
493 decreased on larvae exposed to 2 µg/L of Cu when it was compared to control group. No
494 significant induction of *sod* expression was observed on liver of Javanese medaka (Woo et al.,
495 2009) and zebrafish (Craig et al., 2007) after exposure to Cu. However, Craig et al. (2007)
496 stated an important induction on *sod* and *cat* genes expression on liver when zebrafish was
497 under protective treatment of 10 mM Na^+ plus Cu. Activation and deactivation of the antioxidant
498 defense system are determined by the duration and the intensity of the stress applied to the
499 organism (Casanova et al., 2013).

500 Larvae exposed to copper conditions exhibited a significant repression of genes involved in
501 detoxification (*gst*, *mt1* and *mt2*) and in cell cycle arrest and apoptosis (*p53* and *bax*).
502 Regarding detoxification genes, it was surprising since, after 21 days of Cu exposure, it was
503 expected an induction, in particular of those genes encoding the metallothioneins (*mt1* and
504 *mt2*). Metallothionein (MTs) proteins have the capacity to bind xenobiotic heavy metals through
505 their thiol groups providing protection against metal toxicity and oxidative stress. Indeed,

506 previous studies have demonstrated an induction of *mt1* and *mt2* genes when exposing fish to
507 metals including Cu (Gonzalez et al., 2006; Olsson et al., 1989; Sheader et al., 2006; Woo et
508 al., 2006). The study of Doering et al., (2015) also observed a down-regulation response of *mt*
509 on liver explants from white sturgeon exposed for 24 h to Cu. Glutathione-s-transferase (gst)
510 has an important role in detoxification of xenobiotic compounds, catalyzing the conjugation of
511 reduced glutathione (GSH) to a variety of endogenous and exogenous electrophilic functional
512 groups. Induction of *gst* gene expression has been reported in Javanese medaka liver after
513 being exposed to different heavy metals (Cu, Ag, Cd, Ni, and Cr) and in European flounder
514 (*Platichthys flesus*) liver exposed to Cd after 1 and 3 days of exposure (Sheader et al., 2006;
515 Woo et al., 2009). However, we studied gene expression in whole trout larvae and not in a
516 specific fish organ like mostly previous studies refer. Gene repressions of *mt1*, *mt2* and *gst*
517 could be explained by different hypotheses. The first one, it is the high sensitivity that rainbow
518 trout larvae have to Cu could lead to a significant repression of these genes after 3-weeks
519 exposure at these concentrations of Cu. Certain studies had already observed the differential
520 regulation of *mt* gene expression in fish exposed to metals like Cu and Cd (Minghetti et al.,
521 2008; Tiwari et al., 2010). Differential expression of *mt* gene was observed in intestine, gill,
522 kidney and liver of sea bream (*Sparus aurata*) after exposure to dietary (0.3-130 mg/kg) or
523 waterborne (300 µg/L) copper for 30 days (Minghetti et al., 2008). Tiwari et al. (2010) studied
524 the kinetics of *mt* expression on freshwater murrel (*Channa punctata*) after exposure to
525 3.74 mg/L of Cd indicating that the expression varied according to the time of exposure. At this
526 study, Tiwari et al. (2010) observed induction of *mt* expression on liver and repressions on
527 kidney and gill within the first 8 h of exposure, and induction on kidney and repressions of *mt*
528 expression on liver and gill after 14 days of exposure. Both studies revealed that *mt* expression
529 was tissue specific. Besides, rainbow trout larvae could also be more sensitive during the
530 transition from endogenous yolk-sac reserve to the exogenous feeding as hypothesized by
531 Vardy et al. (2013). Indeed, Vardy et al. (2013) found that later larval life stages of white
532 sturgeon and rainbow trout were more sensitive to Cu than at yolk-sac stage. In our study, at
533 the end of these 3-weeks of Cu exposure, rainbow trout larvae were probably starting to turn

534 to exogenous feeding, and since their biological activity was increased more water passed
535 through the gills increasing their exposure to Cu (Vardy et al., 2013). Another hypothesis could
536 be that larvae may have implemented other mechanisms of detoxification. Indeed, other
537 proteins like ABC transporters have been previously described to be efficient for trace metal
538 detoxification (Gonzalez et al, 2006).

539 Cell cycle arrest is one of the principal functions of p53 when it recognizes DNA damage
540 (Verhaegh et al., 1997). Having a defective *p53* gene could lead to tumorigenesis and cancer.
541 In our study, transcription of *p53* was repressed in both Cu conditions. According to several
542 studies (Verhaegh et al., 1997; Sandrini et al., 2009) *p53* gene expression could be regulated
543 by the activation of p53 protein. Therefore, a possible inactivation of p53 protein, after Cu
544 exposure, could lead to the inactivation of *p53* gene. Additionally, the study of Verhaegh et al.
545 (1997) reported the inactivation of p53 protein after direct interaction with copper on human
546 cell line. Regarding the expression of *bax* gene, only a slight non-significant repression (p=
547 0.0656) was observed in larvae exposed to 20 µg/L of Cu. Interestingly, Sandrini et al. (2009)
548 observed that *bax* gene was induced and *p53* gene was repressed on zebrafish liver cell line
549 after 24 h of exposure to Cu. Concerning the DNA repair-related genes *ogg1* and *rad51*, we
550 did not see any significant modulation. But since we did not observe genotoxicity on blood cells
551 with the comet assay, a possible explanation is that DNA strand breaks may have been
552 repaired prior the comet analysis at 21 days of Cu exposure.

553 Malondialdehyde (MDA), measured as TBARS (thiobarbituic acid reactive substances), is one
554 of the end-products of lipid peroxidation and it is a biomarker of oxidative stress. ROS degrade
555 polyunsaturated lipids forming MDA, which appears to be potentially mutagenic since it reacts
556 with deoxyadenosine and deoxyguanosine, arising DNA adducts (Janero, 1990). In our study,
557 both Cu concentrations did not increase MDA levels in larvae. However, several authors have
558 reported the effects of high concentrations of metals and other xenobiotics on lipid peroxidation
559 in aquatic organisms (Baker et al., 1998; Doyotte et al., 1997; Radi and Matkovics, 1988;
560 Romeó et al., 2000; Thomas and Wofford, 1993; Vutukuru et al., 2006). For example, Vutukuru

561 et al. (2006) showed an increase of lipid peroxidation in the viscera of *Esomus danricus*
562 exposed to 5.5 mg/L of Cu. Baker et al. (1998) fed juvenile grey mullet (*Chelon labrosus*) with
563 a high-Cu diet for 4 weeks and found a rise of 3-fold in hepatic MDA. Pandey et al., (2001)
564 observed that *Channa punctatus* exposed to 10 mg/L of Cu for 4 weeks did not induced lipid
565 peroxidation in liver, kidney and gill. Interestingly, Cu showed to have a protective role on lipid
566 peroxidation in liver when fish were exposed to endosulfan for 24 h (Pandey et al., 2001).

567 Regarding the carbonyl protein content, non-significant increase was observed on larvae after
568 3 weeks of copper exposure. Protein carbonyl content is one of the most used biomarker of
569 protein oxidation (Stadtman and Berlett, 1998) because of its irreversible alteration. Craig et
570 al. (2007) observed an induction of protein carbonyl levels in liver and gills after 48 h of
571 exposure to 15 µg/L of Cu on zebrafish. Besides, Craig et al. (2007) also remarked the
572 protective actions of Ca^{2+} and Na^{2+} ions in generation of protein carbonyl levels in gills and
573 liver when they were added during Cu-treatment. Ransberry et al., (2015) alluded tissue-
574 specific responses, since exposure of 50 and 200 µg/L of Cu on zebrafish induced protein
575 carbonyls in intestines, but not in the gills and livers.

576 Cu is known to cause oxidative damage in lipids and proteins, with a potential to induce
577 genotoxic effects. However, in our study no significant alterations were observed on lipid
578 peroxidation, protein contents and DNA damage. We could hypothesize that the
579 concentrations of Cu used in this study were not enough to cause oxidative alteration in lipid,
580 protein and DNA of rainbow trout.

581

582 ## 5. Conclusion

583 Copper is an essential trace element important for the metabolism of the body. However, at
584 high concentrations it can interfere with certain processes such as protein synthesis and
585 cellular damage. This study provides an extensive evaluation of Cu impact or early
586 developmental stages of rainbow trout that includes phenotypic, behavioral, genetics and

biochemical endpoints. Results revealed sub-lethal effects of copper on development mainly on embryos exposed to the higher copper concentration. Defects included increase of half-hatched embryos, spinal deformities, genotoxicity and deregulation of genes involved in detoxification and cell cycle arrest. Mortality was higher at hatching but larvae appeared to adapt to the contaminated condition. However, alteration on gene expression, primarily those involved in detoxification (gst, mt1 and mt2) could compromise the survival of this species under natural conditions, since environmentally realistic concentration of copper were used in this study.

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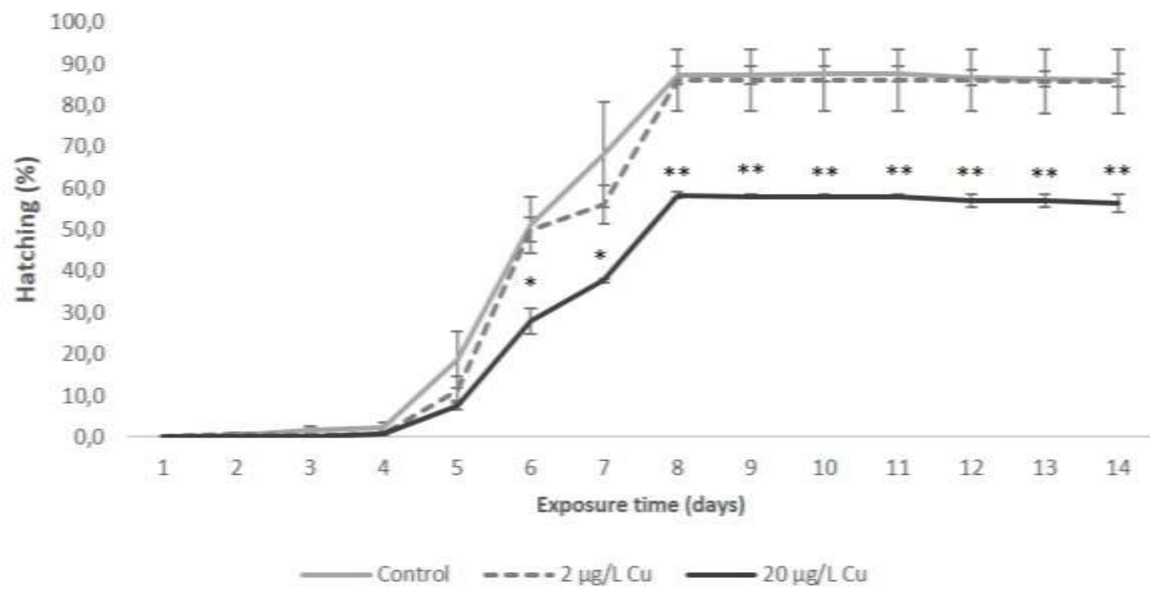
FIGURE CAPTIONS

Figure 1. Hatching rate for each studied condition. Asterisks indicate significant differences compared to control condition. Values are Mean ± SE. N=3, ANOVA, *p<0.05, **p<0.01

Figure 2. Total distance travelled by rainbow trout larvae (A), and cumulative time of high mobility (B) and mobility (C) of larvae. Locomotion was recorded in 30 minutes video analysis. Values represent average over 10 minutes interval. Values are Mean ± SD, N=3, ANOVA.

Figure 3. Percentage of DNA tail intensity (A) and hedgehog (B) from blood cells of rainbow trout larvae assessed with the comet assay. Protein carbonyl content (C) and lipid peroxidation expressed as TBARS (D) of whole-body rainbow trout larvae. Protein carbonyl is expressed as nanomoles of carbonyl/mg of protein, and lipid peroxidation is expressed as nanomoles of TBARS/mg of protein. All values are expressed as Mean ± SE, N=3, ANOVA.

926 Figure 1.



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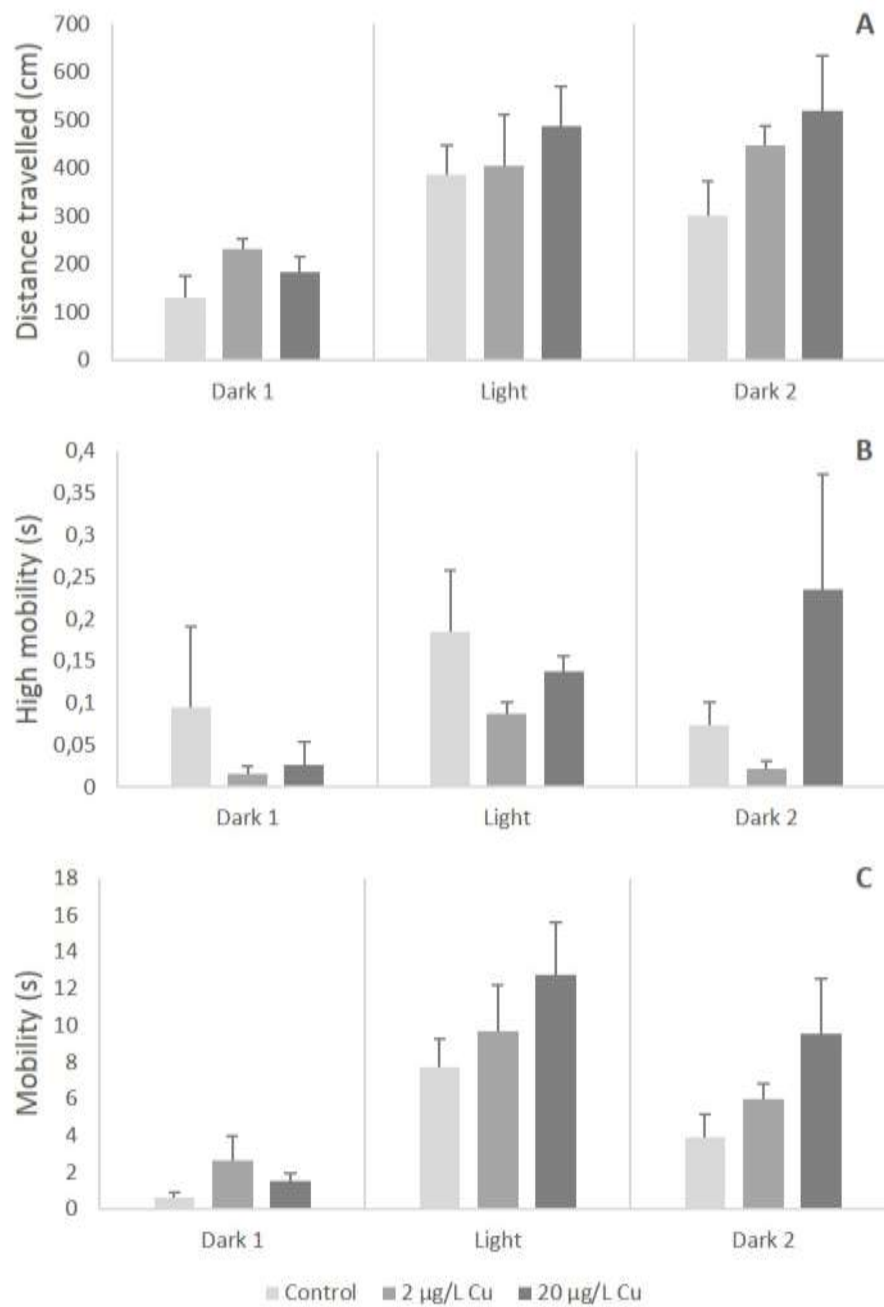
929 Figure 2.



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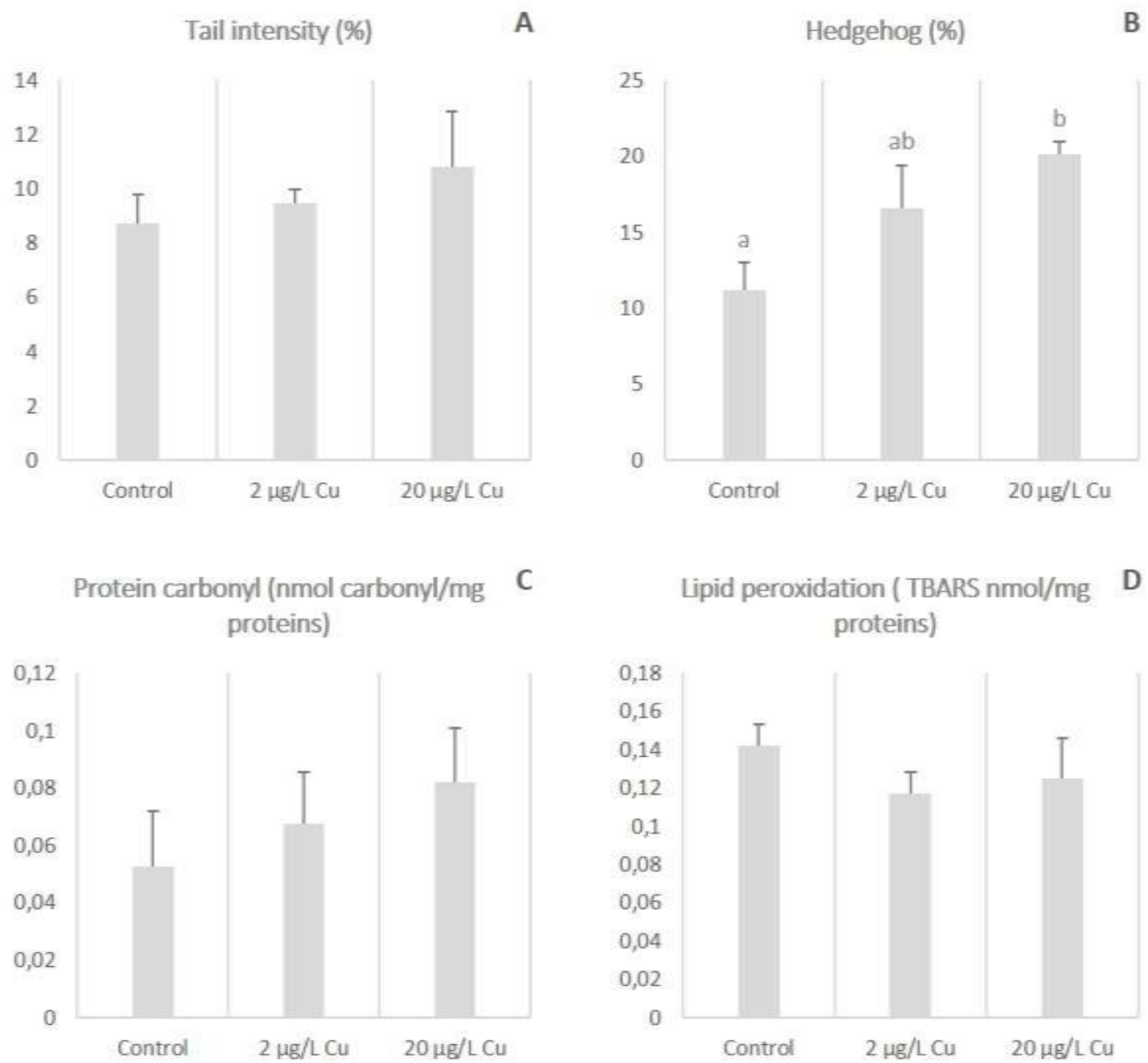
932 Figure 3.



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934

935 **Table 1:** Accession number and specific primer pairs for *Oncorhynchus mykiss* used in our study.

Gene	Accession number	Primer (5' – 3')
Rpl7	NM_001160672.2	GGTCGCTCTCACAGACAACA[a] TTATGTCCGTCCCTCTGGGT[b]
EF1α	NM_001124339.1	ATGGGCTGGTTCAAGGGATG[a] GATCATACCGGCCTTCAGGG[b]
Cat	FJ226382.1	CAGGTGTCTTTCTTGTTCAG GTCCAGGATGGGAAGTTGC
Sod	NM_001124329.1	TGATTGGGGAGATCTCGGGT[a] CGGGTCCAGTGAGAGTCAAC[b]
Gst	BT073173.1	ATTTTGGGACGGGCTGACA CCTGGTGCTCTGCTCCAGT
Mt1	M18104.1	GTGGATCCTGCAAGTGCTCA[a] GTAATGCACCAGGCCTCACT[b]
Mt2	XM_021597409.1	ATCTTGCAACTGCGGTGGAT[a] CCTCACTGACAACAGCTGGT[b]
Cox	KP013084.1	TCGTTTGAGCCGTGCTAGTT[a] CTTCTGGGTGGCCGAAGAAT[b]
12S	KY798500.1	GCGCCAGCTTAAAACCCAAA[a] GCCCATTTCTTCCCACCTCA[b]
Ogg1	XR_002474791.1	CTGATGGACAAGGCCAGTGT[a] GTAAGGACCCCATGGCTGTC[b]
Rad51	XM_021612309.1	AGGCTGGAGGAGGACATCAT[a] GTATTTGAGGGTGGCAGCCT[b]
Bax	BT074328.1	CAGAAAACCCAGGGAGGCAT[a] AGAACACATCCTGGGCACAG[b]
p53	NM_001124692.1	CCTACCAATGAGTAAGACAT[a] CCCGAAACATCCCGCT[b]

[a]Forward primer

[b]Reverse primer

936

937 **Table 2:** Mean copper concentrations measured in exposure chambers (μg/L).

	Nominal Cu concentration (μg/L)		
	0.0 (Control)	*2*	*20*
T1	<0.5	<0.5	20.34
T2	<0.5	<0.5	18.73
T3	<0.5	1.45	18.71

938

939

940 **Table 3:** Developmental endpoints in embryos and larvae of rainbow trout following exposure to waterborne Cu.
941 Values are Means ± SE. Different letters in the same row indicate significant differences between conditions. N=3,
942 ANOVA/Kruskall-Wallis, $p<0.05$.

	Control	2 µg/L Cu	20 µg/L
Acute toxicity			
Embryo survival (%)	**97.3 ± 1.7**[a]	**97.3 ± 1.7**[a]	**88.3 ± 1.8**[b]
Larval survival (%)	92.9 ± 0.6	94.7 ± 1.1	90.3 ± 3.8
Half-hatched mortality (%)	**7.0 ± 1.5**[a]	**9.7 ± 5.7** [a, b]	**25.0 ± 4.0**[b]
Sub-lethal toxicity			
Hatching rate (%)	**90.3 ± 3.2**[a]	**87.7 ± 7.3**[a]	**63.3 ± 3.2**[b]
Hatching time (DD)	332.8 ± 3.3	334.9 ± 2.1	336.8 ± 1.2
Total length (mm)	**17.6 ± 0.3**[a]	**18.36 ± 0.02**[b]	**17.9 ± 0.3**[ab]
Head length (mm)	3.8 ± 0.1	4.1 ± 0.02	4.0 ± 0.1
Ratio of head/body length (%)	21.8 ± 0.4	22.5 ± 0.1	22.2 ± 0.3
Whole body copper concentration (µg/g d.w.)	**5.66 ± 0.08**[a]	**5.71 ± 0.02**[a]	**6.03 ± 0.004**[b]
Abnormalities (%)			
Total deformed larvae	**23.3 ± 3.3**[a]	**58.4 ± 2.7**[b]	**67.8 ± 13.2**[b]
Edemas	6.7 ± 6.7	7.8 ± 4.2	17.8 ± 9.0
Yolk-sac malabsorption	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Spinal	**20.0 ± 5.8**[a]	**52.4 ± 3.5**[b]	**65.1 ± 11.8**[b]
Craniofacial	6.7 ± 3.3	10.8 ± 1.7	14.7 ± 10.8
Cardio-vascular	3.3 ± 3.3	10.8 ± 5.5	15.5 ± 7.8
Haemorrhages	0.0 ± 0.0	3.0 ± 3.0	15.5 ± 7.8

943

944

945 **Table 4:** Transcription of genes involved in antioxidant defense (*cat*, *sod*), DNA repair (*ogg1* and *rad51*),
946 detoxification (gst, mt1 and mt2), mitochondria metabolism (cox, 12S), apoptosis (*bax*) and cell cycle arrest (p53)
947 in whole-body rainbow trout larvae. Results are expressed as fold changes relative to control condition (1/4 is
948 corresponded to a 4-fold decrease). Asterisk indicate differences with the control condition (N = 3, ANOVA/Kruskal-
949 Wallis, *p < 0.05; **p<0.01).

	cat	*sod*	*gst*	*mt1*	*mt2*	*cox*	*12s*	*ogg1*	*rad51*	*bax*	*p53*
2 μg/L Cu	-	1/6**	1/4*	1/4*	1/7*	-	-	-	-	-	1/7*
20 μg/L Cu	-	-	1/7**	1/7**	1/7*	1/3	-	-	-	1/3	1/8*

950