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1 Physiological performance of common carp (*Cyprinus carpio*, L., 1758) exposed to a sublethal
2 copper/zinc/cadmium mixture
3
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15 Abstract

16 In a natural ecosystem, fish are subjected to a multitude of variable environmental factors. It is
17 important to analyze the impact of combined factors to obtain a realistic understanding of the
18 mixed stress occuring in nature. In this study, the physiological performance of juvenile
19 common carp (*Cyprinus carpio*) exposed for one week to an environmentally relevant metal
20 mixture (4.8 µg/L of copper; 2.9 µg/L of cadmium and 206.8 µg/L of zinc) and to two
21 temperatures (10 °C and 20 °C), were evaluated. After 1, 3 and 7 days, standard (SMR) and
22 maximum metabolic rate (MMR) were measured and aerobic scope (AS) was calculated. In
23 addition, hematocrit, muscle lactate, histology of the gills and metal accumulation in gills were
24 measured. While SMR, MMR and AS were elevated at the higher temperature, the metal
25 mixture did not have a strong effect on these parameters. At 20 °C, SMR transiently increased,
26 but no significant changes were observed for MMR and AS. During metal exposure, hematocrit
27 levels were elevated in the 20 °C group. The bioaccumulation of Cd in the gills reflected the
28 increased metabolic rate at the higher temperature, with more accumulation at 20 °C than at 10
29 °C. Anaerobic metabolism was not increased, which corresponds with the lack of significant
30 histopathological damage in the gill tissue. These results show that common carp handled these
31 metal exposures well, although increased temperature led to higher Cd accumulation and
32 necessitated increased hematocrit levels to maintain aerobic performance.

33

34 Keywords: aerobic scope, mixture stress, metal, temperature, bioaccumulation, gill histology.

35

36 **I. Introduction**

37 The common carp (*Cyprinus carpio* L., 1758) is one of the most important freshwater
38 aquaculture species worlwide, with an annual global production of 4.6 million metric tons
39 (FAO, 2018). Additionally, this species is considered to be a good bioindicator for
40 ecotoxicological studies (Altun et al., 2017; Rajeshkumar et al., 2017) and is recommended in
41 Organization for Economic Co-operation and Development (OECD) guidelines, as one of six
42 fish species for regulatory testing (OECD, 2019). As a model species, common carp is used to
43 study the impact of metals both in the lab and in the field, on for example bioaccumulation
44 (Bervoets et al., 2009; Castaldo et al., 2020; Delahaut et al., 2019; Delahaut et al., 2020), energy
45 status (De Boeck et al., 1995a; Kunwar et al., 2009), swimming capacity (Delahaut et al., 2019)
46 or oxidative stress (Cortes-Diaz et al., 2017; Dugmonits et al., 2013; García-Medina et al., 2017;
47 Pillet et al., 2019). Metal is found in every aquatic ecosystem and anthropogenic activity
48 discharges metals directly into water from mines, industry, intensive agriculture, household
49 waste or traffic (Burger, 2008; Coufalík et al., 2019; Stohs and Bagchi, 1995). This kind of
50 pollution is a major concern because metals are persistent, do not degrade naturally and are able
51 to accumulate along the trophic food chain (Díaz-de-Alba et al., 2017; Feng et al., 2015).
52 Fish can accumulate pollutants *via* direct uptake from the water or through the ingestion of
53 suspended particules or contaminated food(Newman and Unger, 2002). Gills, as the first organ
54 in contact with pollutants and the main organ for gas and ion exchanges, are the first target of
55 metals (Niyogi and Wood, 2004). It has been demonstrated that metals can increase mucus
56 secretion, induce pathological changes in gill tissue or disturb hematological parameters, such
57 as the destruction of erythrocytes (Coello and Khan, 1996; Guardiola et al., 2015; Gwoździński
58 et al., 1992; Javed and Usmani, 2012). The concommitant reductions in the oxygen diffusion
59 and oxygen carrying capacity disrupt oxygen transport to other organs and may result in a

60 reduction of swimming speed (Mager and Grosell, 2011), as well as changes in energy budgets
61 (De Boeck et al., 1995b).
62 Knowing that temperature can affect tolerance to trace metals was one the first findings in
63 ecotoxicology (Cairns et al., 1975). Typically, elevated temperature tends to increase metal
64 effects on organisms (Cairns et al., 1975). The increased toxicity is partly explained by faster
65 ventilation of fish at higher temperatures and a concomittent higher uptake rate of metal (Cairns
66 et al., 1975). However, previous studies showed that the impact of metal depends of the duration
67 of the exposure and of the metal concentration used. In common carp, the effect of metals on
68 physiological performance has mainly been studied for copper (Cu) (De Boeck et al., 2006; De
69 Boeck et al., 2001; Malekpouri et al., 2016). Common carp exposed to Cu for 28 days showed
70 a transient reduction in oxygen consumption within the first day, and a longer lasting reduction
71 in swimming capacity (*U*crit), with only partial recovery at the end of the exposure (De Boeck
72 et al., 2006). This general reduction of the physiological performance of the carp was explained
73 by ammonia accumulation, which might depolarise muscle cells and impair the contraction of
74 muscle fibers (Beaumont et al., 2000). In contradiction with these results, another study did not
75 show any reduction in the standard (SMR, defined as the minimal metabolic demand required
76 to sustain life in fasting and resting animals (Fry, 1971)), and maximum metabolic rate (MMR)
77 of common carp exposed to lethal (immediate exposure to 100% of the Cu LC_{50}), sublethal
78 (24 h exposure to 50% of the Cu LC_{50}) and long (7 days exposure to 10% of the Cu LC_{50})
79 exposures (Malekpouri et al., 2016). In contrast, their SMR, MMR and aerobic scope (i.e. MMR
80 – SMR) increased in the lethal and long Cu exposures (Malekpouri et al., 2016). These results
81 could be attributed for the lethal exposure, to an initial behavioural stress response (Wilson and
82 Taylor, 1993) or for the long exposure, by an acceleration of aerobic metabolism to increase
83 oxygen uptake and fulfill the higher metabolic demands following pollutant-induced stress
84 (Suresh et al., 1993). However, even if testing the impact of a single metal is the first step to

85 assess the impact of metal in general, it does not represent what occurs in a natural environment.
86 It becomes important to consider pollution as a range of various substances and to take into
87 account other parameters (Niyogi and Wood, 2004), even in laboratory experiments.
88 Gills are the organ of choice for histology studies due to their vital functions, such as gas
89 exchange, osmoregulation, excretion, acid-base regulation and large surface area which is in
90 direct and permanent contact with the aquatic environment (Fonseca et al., 2016; van Dyk et
91 al., 2009). At the same time, gill structural alterations are neither species- nor stressor-specific,
92 but are affected by an irritant's intensity, revealing merely a general adaptation syndrome
93 (Baberschke et al., 2019). Still, structural changes of the gills represent a valuable complement
94 when studying related physiological processes such as gas exchange. Due to economic
95 importance and wide presence of the species, carp gill histology has been increasingly
96 investigated (Gupta et al., 2016; Rašković et al., 2016).
97
98 A recent study investigating the impact of a similar environmentally relevant Cu/Cd/Zn
99 mixtures on common carp, showed a number of effects on ion-regulation (reduced Na^+
100 transport) and detoxification functions (strong induction of metallothionein expression)
101 (Castaldo et al., 2020), as well as oxidative stress capacities (Pillet et al., 2019) reflecting
102 different toxicity mechanisms of the Cu/Cd/Zn mixture. The present study aimed to investigate
103 whether these mechanisms translate into changes in whole-animal physiological performance
104 linked to bioaccumulation of metal in tissues, and whether these are affected by temperature.
105 The impact of such a metal mixture on physiological performance and on bioaccumulation is
106 assumed to be reduced at a lower temperature. However, at the higher temperature, it is
107 hypothesized that gill damage might occur, leading to reduced oxygen uptake and aerobic
108 performance and increased anaerobic metabolism. This effect is expected to be more prominent
109 when measuring maximum metabolic rate compared to standard metabolic rate. However, at

110 both temperatures, complete acclimatisation of the carp is expected by the end of the
111 experiment. To facilitate direct comparisons with previous observations (Castaldo et al., 2020;
112 Pillet et al., 2019), the concentrations of metal used in the present study targeted the
113 concentrations previously used (10% of the LC_{50} for single metal, as defined by Delahaut et al.
114 (2020)), and also reflected ecologically-relevant concentrations.
115
116 **II. Material and methods**
117 **A. Experimental animals**
118 Common carp were obtained from the fish hatchery at the Agricultural University of
119 Wageningen (The Netherlands) and kept at 20 °C in 200 L aquaria supplied with medium hard
120 water (pH 8.2 ± 0.2). As defined by the US Environmental Protection Agency (USEPA, 2002),
121 medium hard water was reconstituted with deionized tap water (Aqualab, VWR International,
122 Leuven, Belgium) supplemented with 96 mg/L $NaHCO_3$, 60 mg/L $CaSO_4.2H_2O$, 123 mg/L
123 $MgSO_4.7H_2O$ and 4 mg/L KCl (VWR Chemicals). Each tank was equipped with a recirculating
124 water system and water quality was ensured through a biofilter containing wadding, glass stones
125 and plastic balls. Water quality was checked daily with Visicolor Test Kits (Macherey-Nagel,
126 Düren, Germany) to ensure that ammonia and nitrite were kept at undetectable levels and nitrate
127 levels never exceeded 20 mg/l. In each tank, oxygen was provided by an individual air stone
128 and the photoperiod was 12 h light and 12 h dark. Fish were fed *ad libitum* once a day with
129 commercial pellets (*Hikari*® *Staple*™, Klundert, The Netherlands) during this period. Fish were
130 then divided in two groups of 70 individuals: one group was kept at 20 °C while for the other
131 group, the temperature was progressively decreased (by 1 °C every three days) until 10 °C. For
132 acclimatisation, fish were moved into a climate chamber maintained at 20 °C or 10 °C under
133 the same conditions for at least two weeks.

134 Experimental methods complied with regulation of the Federation of European Laboratory
135 Animal Science Associations and were approved by the local ethics committee, University of
136 Antwerp (Permit Number: 2015-94 Project 32252).
137
138 **B. Exposure conditions**
139 For each exposure temperature (20 °C and 10 °C), control fish (length = 60.4 ± 5.8 mm; weight
140 = 2.6 ± 0.7 g) were kept in EPA water (pH = 7.75 ± 0.1; conductivity = 277.8 ± 44.8 µS/cm)
141 while the exposed fish (length = 60.5 ± 4.3 mm; weight = 2.6 ± 0.5 g) were exposed to a
142 Cu/Cd/Zn mixture (Cu: 4.8 µg/L; Cd: 2.9 µg/L and Zn: 206.8 µg/L) for 12 h, one day, three
143 days and one week. The used concentrations correspond to the 10% of the 96 h LC_{50} of the fish
144 at 20 °C (Delahaut et al., 2020). The experimental set up consisted of 10 L polypropylene tanks
145 (5 tanks for control and 5 for metal exposure), each containing 2 fish. Stock solutions of $CuSO_4$
146 (0.09 g/L, Sigma), $CdCl_2$(0.05 g/L, Merck) and $ZnCl_2$(3.7 g/L, Sigma) were prepared in MilliQ
147 water and added to the exposure water to reach the desired concentrations. In each tank, oxygen
148 was provided by an individual air stone and water was renewed daily to avoid accumulation of
149 waste products (such as ammonia). Before and after changing the water, 10 mL of water were
150 sampled from each tank to check the stability of the metal concentrations. Concentrations of
151 metals were measured in the water samples by inductively coupled plasma mass spectrometry
152 (7700x ICP-MS, Agilent Technologies, Santa Clara, CA, USA) after acidification of the sample
153 by adding 150 µL of nitric acid (67-69%, trace metal grade, Fisher Chemical). The recorded
154 water metals concentrations (n = 107) during the experiment were 0 ± 0.1 µg/L Cu, 0 µg/L Cd
155 and 0.6 ± 1.5 µg/L Zn for control tanks and 4.8 ± 0.8 µg/L Cu, 2.8 ± 0.2 µg/L Cd and 179.9 ±
156 30.4 µg/L Zn for exposure tanks at 20 °C and 0.6 ± 0.2 µg/L Cu, 0 µg/L Cd and 0 µg/L Zn for
157 control tanks and 5.4 ± 1.2 µg/L Cu, 2.5 ± 0.4 µg/L Cd and 154.5 ± 21.9 µg/L Zn for exposure
158 tanks at 10 °C. Fish were not fed during the experiment to avoid differences in appetite that

159 could have made difficult the comparisons among treatments and increase the inter-individuals
160 variability.
161
162 **C. Respirometry**
163 Sixty fish were used, at each temperature, for respirometry analyses: 10 for control at the
164 beginning of the experiment (12 h), 10 for each exposure (12 h, one day, three days and one
165 week) and 10 for the control at the end of the experiment (one week).
166 Static respirometers (dimension = 15 × 15 × 7 cm, volume = 600 mL) were submerged in 60 L
167 tanks where oxygenation was constant and temperature kept at 20 °C or 10 °C. The water
168 composition in the 60 L tank and in the respirometer was the same as the water composition of
169 the exposure tanks (EPA water for control and Cu/Cd/Zn mixture for exposed fish). A flush
170 pump allowed water exchange between the respirometer and the 60 L tank. Water (60 L) was
171 renewed in the tank before placing the fish into the respirometer. The oxygen concentration was
172 continuously recorded in the respirometers, using fiber optic mini sensors (optodes, Loligo®
173 Systems, Denmark) connected to Witrox 4 oxygen meter (Loligo® Systems, Denmark)
174 transferring data every second to a computer. The oxygen concentration was automatically
175 adjusted according to the real-time temperature and never fell below 85% of air saturation.
176
177 For standard metabolic rate (SMR) determination, each fish was placed in the respirometer
178 during the evening, and measurements occurred during the night (12 h measurement, in the
179 dark). Oxygen consumption ($\dot{M}O_2$) was measured by intermittent-flow respirometry consisting
180 of 15 min flushing phase and 30 min or 1 hour (at 20 °C and 10 °C, respectively) measuring
181 phase during which the pump was turned off. The linear decline of dissolved oxygen in the
182 respirometer during the measuring phase allowed the calculation of $\dot{M}O2$ according to the
183 equation:

184 $$\dot{M}O_2 = \frac{Vr \times \Delta Cwo_2}{\Delta t \times bw}$$

185 where Vr is the volume of the respirometer (volume of the fish substracted), ΔCwo_2 is the
186 concentration of oxygen, Δt the time period and bw the weight of the animal (Steffensen, 1989).
187
188 The $\dot{M}O_2$ ($mg.h^{-1}.kg^{-1}$) during SMR was calculated using RespR 1.0.5 R package (Harianto et
189 al., 2019). SMR was considered as the lowest 10% of the values, as recommended by Clark et
190 al. (2013).
191
192 For the determination of maximum metabolic rate (MMR), fish were removed from the
193 respirometer, transferred into a tank with 1.5 L of well aerated water (EPA water for control
194 and Cu/Cd/Zn mixture for exposed fish). Water in the tank was changed between each fish.
195 Then fish were chased by hand until exhaustion (during 10 min at 20 °C and 5 min at 10 °C)
196 and transferred back into the respirometer where oxygen concentration was measured, without
197 flushing phase, during 40 min (previous tests showing that fish were back at their SMR at the
198 end of this period). The $\dot{M}O_2$ ($mg.h^{-1}.kg^{-1}$) during MMR test was calculated using RespR 1.0.5
199 R package (Harianto et al., 2019). It was considered as the highest rate sustained over 5 min
200 during the MMR measurement period. Aerobic scope (AS) was calculated as the difference
201 between MMR and SMR. At the end of the MMR measurement, fish were removed from the
202 respirometer, ammonia level was measured (Visicolor Test Kits, Macherey-Nagel, Düren,
203 Germany) in the 60 L tank and background respiration was recorded for 2 hours to correct SMR
204 and MMR calculations. Then, fish were immediately euthanised with neutralized MS222 (pH
205 7.0, ethyl 3-aminobenzoate methane-sulfonic acid, 300 mg/L, Acros Organics, Geel, Belgium),
206 their weight and total length were measured, and blood, gill and muscle tissue were sampled
207 (see below).
208

209 **D. Hematocrit**

210 Blood samples from the caudal blood vessel (n = 10 per condition) were collected using 60 µL
211 Na-heparinized capillary tubes. They were immediately centrifuged for 3 min in micro-
212 hematocrit centrifuge Van der Heyden (Martin Christ Gefriertrocknungsanlagen GmbH,
213 Osterode am Harz, Germany). The hematocrit value was determined as the percentage of red
214 blood cells in whole blood.
215
216 **E. Lactate content**
217 White muscle (n = 10 per condition) were sampled for measuring lactate content. The samples
218 were snap frozen in liquid nitrogen after dissection and stored at -80 °C until further analyses.
219 Determination of lactate was carried out following manufacturer instructions, using L-Lactic
220 acid assay kit from R-Biopharm (Darmstadt, Germany).
221
222 **F. Gill histology**
223 For histological analysis, fish from both control and exposed groups kept at 20 °C were sampled
224 (n = 10 per condition). After euthanizing fish with neutralized MS222, the second gill arch from
225 the left side of each fish was sampled for histology analyses. Tissue samples were fixed in 4%
226 formaldehyde during 48 h and then transferred to 70% ethanol. Samples were further processed
227 according to the standard histological techniques: dehydrated in ethanol series, followed by
228 xylene treatment and paraffin embedding (automatic tissue processor Leica TP 1020, Nussloch,
229 Germany); serial sectioned at 5 µm nominal thickness (microtome Leica SM 2000R, Nussloch,
230 Germany); sections mounted on glass slides, deparaffinised, rehydrated, and haematoxylin and
231 eosin (HE) stained (slide stainer Leica ST 4040, Nussloch, Germany) (Humason, 1979).
232 Blinded slides were evaluated by two experienced pathohistologists independently (V.P. and
233 B.R.) using semi-quantitative scoring system: a score value, related to the extent and degree of

234 alteration, ranging from 0 (unchanged) to 6 (severe occurrence) is determined during slides
235 examination, as recommended by Bernet et al. (1999). Scores given by both pathohistologists
236 were averaged and subsequently used for statistical analysis. Micrographs of representative
237 alterations were made using Leica DM2000 microscope (Leica Microsystems, Wetzlar,
238 Germany) equipped with Leica DFC320 digital camera (Leica Microsystems, Wetzlar,
239 Germany).
240

241 **G. Metal accumulation**

242 A parrallel experiment (Castaldo et al. unpublished) using fish of similar size (control fish:
243 length = 57.3 ± 5.5 mm; weight = 2.6 ± 0.6 g and exposed fish: length = 56.4 ± 4.3 mm; weight
244 = 2.4 ± 0.6 g) and similar experimental setup (same fish size; one week metal mixture exposure
245 = Cu: 4.8 µg/L; Cd: 2.9 µg/L and Zn: 206.8 µg/L), accumulation of Cu, Cd and Zn were
246 measured in gills. Ten fish per condition (control and exposed to metal mixture) and per
247 sampling date (1 and 7 days) were euthanized with an overdose of MS222 as previoulsy
248 described, and gill samples were pooled per 2 fish (n = 5 per condition), weighed and frozen at
249 -80 °C until further analysis. The protocol for metal accumulation measurement was the same
250 as described in Blust et al. (1988) and Reynders et al. (2006). Briefly, samples were oven-dried
251 for 48 h at 60 °C. After cooling down, the dry weight was recorded and the samples were
252 digested in 69% nitric acid with several microwave heating steps. For the last digestion steps,
253 hydrogen peroxide (29%) was added to the samples. Finally, samples were diluted to reach a
254 2% concentration of acid and analyzed using a 7700x ICP-MS (Agilent Technologies). Mussel
255 tissue (mussel tissue SRM-2976, National Institute of Standards and Technology, Gaithersburg,
256 MD, USA) was used as reference material and processed following the same protocol to ensure
257 the quality of the analysis.
258

259 **H. Statistical analyses**

260 Normality and homogeneity of variances were verified by Shapiro-Wilks and Levene tests,
261 respectively. Data were log10 transformed to avoid heteroscedasticity when necessary. Outliers
262 were identified using Rosner test and removed. Two-way ANOVAs were performed to test the
263 effects of the metal exposures and temperature on SMR, MMR, AS, hematocrit, lactate content
264 and metal accumulation in *C. carpio*. When significant effects were found, *a posteriori* Tukey
265 tests were used to compare means ($\alpha = 0.05$). When normalization of the data was not possible,
266 non-parametric equivalent tests were used, namely Kruskal-Wallis H test and Mann-Whitney
267 U test, which were used for analyses of semi-quantitative histopathological scores. All
268 statistical analyses were performed with R 3.6.0 software.

269

III. Results

As expected, no mortality nor adverse behavior were observed during the whole course of the experiment.

A. Metabolic parameters

The SMR of carp was significantly affected by temperature ($P < 0.001$), metal exposure ($P < 0.001$) and by the interaction between these factors ($P < 0.001$). At both temperatures, SMR was not significantly different between initial and final controls. At 20 °C, the SMR of carp (fig. 1) increased progressively during the beginning of the experiment and became significantly different from the initial control group (12 h) after 3 days, where oxygen consumption peaked. At this sampling time, the SMR of fish exposed to the metal mixture was 3.5 times higher than fish in the initial control group. After one week of exposure, the SMR of the carp decreased to reach a level comparable to the SMR of the initial control group. At 10 °C, the SMR remained stable during the one-week exposure.

The MMR (fig. 2) and the AS (fig. 3) of carp were not impacted by the exposure to the metal mixture ($P <0.5$) but increased at 20 °C ($P < 0.001$). Carp had a MMR and an AS respectively 2.6- and 2.5- fold higher at 20 °C compared to the one at 10 °C.

B. Hematocrit

The hematocrit percentage of carp was impacted by temperature ($P < 0.001$), metal exposure ($P < 0.001$) and the interaction of both factors ($P < 0.01$). While the hematocrit of carp stayed stable during the one-week exposure to metal at 10 °C, it increased quickly at 20 °C. At 20 °C, carp exposed to Cu/Cd/Zn mixture for 12 h and 3 days had a significant higher hematocrit than

294 both initial (12 h) and final (7 days) control groups (fig. 4). At 1 day and one week this
295 difference was not significant for the comparison with the initial control group.
296
297 **C. Lactate content**
298 Lactate levels in muscle tissue were similar at both temperatures. During exposure, no
299 significant change due to metal mixture was observed in the lactate content, neither at 10 °C
300 (7.25 ± 1.47 nmol/g of tissue) nor at 20 °C (4.79 ± 2.22 nmol/g of tissue).
301
302 **D. Gills histology**
303 In general, gill histopathological alterations were mild to moderate (table 1). Moderate average
304 histopathological scores (above 2) were found for hyperemia, oedema of respiratory epithelium
305 and enlarged nuclei of respiratory epithelium (fig. 5a). Initial (at 12 h) and final (at 7 days)
306 control groups were sampled; if scores of histopathological alterations were compared among
307 control and exposure group at 12 h, significantly higher scores were noted in exposed group for
308 hypertrophy (fig. 5b) and hyperplasia of gill epithelium (fig. 5c, 5e; $P < 0.05$), while there were
309 no significant differences among control and exposure group at 7 days for any of alterations (P
310 > 0.05). As average scores for hyperplasia of epithelium were highest in fish sampled at 12 h
311 that time point was also higher compared to 1 and 3 days groups ($P < 0.05$). It is worth noting
312 that there was a difference between initial and final control groups of fish, as higher
313 histopathological scores for hypertrophy and hyperplasia of epithelium were calculated at the
314 end of exposure.
315
316 **E. Metal accumulation**
317 The accumulation of Cu in gills (fig. 6a) was only affected by metal exposure ($P < 0.001$) but
318 not by temperature ($P > 0.5$). After one day of exposure to metal mixture, the accumulation of

319 Cu was 1.5 times higher compared to the accumulation in the gills of the control group at both
320 temperatures. At 20 °C, further accumulation seemed to have leveled off after a week, while at
321 10 °C, the accumulation still tended to increase, even if the difference between groups was not
322 significant.

323 Temperature ($P < 0.5$) and metal exposure ($P < 0.001$) significantly affected the Cd
324 accumulation (fig. 6b), whereas the interaction of both factors was not significant ($P > 0.5$). At
325 20 °C, Cd increased in the gills by 10-fold after 1 day of exposure and by 65-fold after 7 days
326 of exposure. At 10 °C, the increase in Cd accumulation became significant only after one week
327 of exposure but is still significantly lower than the accumulation at 20 °C.

328 Despite a significant effect of the temperature on the Zn accumulation (fig. 6c) in gill tissue
329 ($P < 0.5$), no significant difference between the groups of fish was detected during the one-
330 week exposure.

331

332 **IV. Discussion**

333 The concentrations of metals used in this study were comparable to the ones found in the field.
334 In Flanders, the Flemish Environmental Agency (VMM) measured concentrations of the single
335 metals ranging from 1.27 to 34.32 µg/L for Cu, 0.05 to 3.37 µg/L for Cd and 7.84 to 330.17
336 µg/L for Zn (VMM, 2014). In the present study, as shown in other studies (Castaldo et al., 2020;
337 Pillet et al., 2019), carp cope well with this sublethal metal mixture for a week. Despite some
338 impacts on ionoregulation (Castaldo et al., 2020) and defensive mechanisms (Castaldo et al.,
339 2020; Pillet et al., 2019), at the whole-animal level, physiological performance is almost
340 unaffected. By evaluating the combined effect of metal mixture and temperature, it was
341 demonstrated that the temperature had a more important influence than the metal mixture on
342 the physiological performance of carp.
343
344 The impact of temperature on metabolic rate was obvious in the present study. The coefficient
345 of temperature Q_{10} values (rate of change as a consequence of a 10°C increase) for SMR was
346 3.7 for both controls groups (at 12 h and 7 days), and for MMR was respectively 2.9 and 2.4
347 for initial and final control groups. This temperatuer dependence of fish metabolic rate is long-
348 known (Fry, 1971) and usually, the Q_{10} values in ectothermic organisms undergoes
349 approximately a two-fold increase with every 10 °C rise (Cairns et al., 1975). Common carp
350 can survive a wide range of temperatures but they grow best between 23 and 30 °C (FAO,
351 2020). Logically, as metabolic rate increases with temperature, stronger responses to metal
352 mixture exposure were observed at the higher temperature in the present study. While at 10 °C,
353 no significant responses were observed in any of the measured parameters, some of them were
354 temporally affected by the metal mixture at 20 °C.
355

356 The change in metabolic rate due to temperature is directly linked, *via* the increase of
357 ventilation, to the uptake rate of pollutants. In fish, the accumulation of waterborne pollutants
358 happens mainly from the direct uptake through the gills (Newman and Unger, 2002). So there
359 is a continuous chain of reaction: water is filtered through the gills to meet oxygen demands,
360 and at the same time, metals can target the gill tissue and bioaccumulate. For Cu and Zn in gills,
361 temperature had no impact on the net accumulation, and Zn levels even stayed stable during the
362 one-week exposure to metal mixture. These two metals, defined as essential elements because
363 they are necessary for biological processes, help to maintain healthy cellular functioning
364 (Mayor et al., 2013) and act as an enzyme cofactor in several metabolic pathways (Ritter et al.,
365 2008). However, if the supply exceeds the demand, Cu and Zn can become toxic, and can have
366 detrimental effects on fatty acid and protein metabolism, or cellular respiration (Sibi et al.,
367 2014). As Cu and Zn are essential to the functioning of the organism, fish are able to regulate
368 the elements, by upregulating their excretion, and their concentrations cannot be reflected only
369 by metabolic rate or uptake rate (Newman and Unger, 2002; Reichle and Hook, 1970), as
370 observed in the present study. On the other side, Cd is not an essential metal and can show toxic
371 properties at very low concentrations (Bae and Lim, 2012). As Cd is not used for any cellular
372 or physiological process, its net accumulation can be explained to a larger extent by metabolic
373 rate and the concomittant water flow over the gills. This direct link between metabolic rate and
374 net accumulation for non-essential metal is well observed in the present study. This is especially
375 true when the temperature factor is considered: the net accumulation of Cd is much lower at 10
376 °C than at 20 °C, as is the SMR of carp.
377
378 The higher SMR in common carp exposed to metal mixture at 20 °C demonstrated in the present
379 study can have several explanations. Such an increase could be necessary to meet energy

380 demands following metal mixture induced stress (such as oxidative stress) or to maintain
381 homeostasis (Grosell and Wood, 2002), repairing or recovery processes (Peles et al., 2012).
382 Among other effects, metal can cause oxidative stress (Cortes-Diaz et al., 2017; Dugmonits et
383 al., 2013; Flora, 2014; García-Medina et al., 2017; Valavanidis et al., 2006), but in previous
384 studies under similar experimental conditions, no proof of oxidative stress (via
385 malondialdehyde measurements) was found in gills nor liver of common carp (Pillet et al.,
386 2019) despite a strong induction of gene coding for metallothionein (Castaldo et al., 2020). It
387 would be interesting to quantify other biomarkers of oxidative stress, such as 8-OHdG
388 (Valavanidis et al., 2009), to clarify the importance of oxidative stress after such metal mixture
389 exposure.
390 In a similar experiment (Castaldo et al., 2020), an impairment of ionoregulation was also
391 demonstrated by a drop of sodium in the gills in common carp exposed to a Cu/Cd/Zn metal
392 mixture (at 10% LC_{50}) at 20 °C. It is then probable that the increase of SMR noted in the present
393 study after three days was due to a higher energy demand to maintain ion homeostasis. The
394 disturbance of homeostasis could also be supported by the observed increase of hematocrit
395 percentage observed in fish exposed to metal mixture at 20 °C. Na^+ being the major cation in
396 the intracellular environment (Niyogi et al., 2015), ion loss is usually accompanied by
397 dehydration. In contrast, at 10°C, as ventilation decreased, the ion loss is less important and no
398 change in the hematocrit percentage was observed. However, the increase of hematocrit could
399 also simply reflect a release of red blood cells from the spleen, to fulfill a higher metabolic
400 demand and subsequent need for oxygen.
401
402 The intermittent increase of SMR at the beginning of the experiment could also be necessary to
403 fulfill an intermittent energy demand for repairing processes. Fast spikes in the first 6-12 h
404 followed by a gradual increment of histopathological alterations afterwards is already

405 documented in other metal exposure studies (Martinez et al., 2004). Here, characteristic rapid
406 increases in hyperthrophy of the respiratory epithelium and hyperplasia of the primary and
407 respiratory epithelium was visible 12 h after exposure in the present study. However, no
408 subsequent gradual increment was demonstrated, at least in case of hyperplastic changes of gills
409 epithelium, which could point to possible acclimatisation of common carp to mixture of three
410 metals in sublethal concentrations. Hyperplasia of epithelium is the histopathological alteration
411 that shows the highest correlation to various metal concentrations in fish gills, and is therefore
412 pointed as a key marker during histopathological assessment (Fonseca et al., 2017). If
413 alterations were compared to a previous conducted exposure (Delahaut et al. 2020), some gill
414 histopathological alterations that dominated during single metal exposure were not even present
415 in the present study. This may be due to antagonistic effects of metals or to difference in size
416 and/or genetics (due to adaptation and tolerance properties) of experimental fish (Klerks et al.,
417 2011), since the batch of fish were different.
418
419 Despite the small increase in SMR at the higher temperature, the metal mixture used in the
420 present experiment did not have a major impact on the aerobic metabolism of the carp. The
421 aerobic scope was not impacted at all during the one-week exposure, even if the individual
422 variation was important. These results are in accordance with some previous studies. For
423 example, Malekpouri et al. (2016) observed similar results in common carp exposed to Cu,
424 however, these authors observed changes in SMR and MMR that both increased when the carp
425 were exposed to 10% LC_{50} Cu for a week, resulting in a stability of the AS. The stability of the
426 aerobic scope in the present study is also corroborated by the absence of anaerobic metabolism
427 induction and the general lack of tissue damage in the gills. Fish gills are well-known for their
428 adaptation and rapid remodelling properties in the presence of various stressors (Nilsson et al.,
429 2012; Sales et al., 2017). The Cd/Cu/Zn metal mixture (10% LC_{50}) did not cause significant

430 effects on carp gills' microanatomy after 7 days, as all alterations were mild or moderate in
431 intensity. This is in line with one comparable study, when common carp were exposed to
432 environmentally relevant concentration mixture of Cd/Cr/Pb (Rajeshkumar et al., 2017) for 7
433 days and developed only mild gill alterations. *Channa punctata* exposed to environmentally
434 relevant concentration mixture of four metals (Cd/Cu/Fe/Ni) in 7 days period also resulted in
435 mild histopathological alterations in gills (Pandey et al., 2008).
436
437 The stability of physiological performance and even the increase of SMR of carp while exposed
438 to the metal mixture is in contradiction with the first hypothesis. A suppressive effect on carp
439 metabolism was expected, as already shown in field experiments on wild yellow perch, *Perca*
440 *flavescens,* captured in four lakes varying in Cu and Cd contaminations (Couture and Rajender
441 Kumar, 2003) and an experimental study on inanga *G. maculatus* exposed to Cd at 2.5 $\mu g/L^{-1}$
442 (McRae et al., 2018). But it is difficult to predict the change in metabolic rate in fish facing
443 metal exposure, especially because it can change over time. Peles et al. (2012) and Pistole et al.
444 (2008) showed that the metabolic rate of golden shiners, *Notemigonus crysoleucas,* exposed to
445 four Cd concentrations (500, 800, 1100, and 1400 $\mu g.L^{-1}$) and fathead minnows *Pimephales*
446 *promelas* exposed to Cd (1000 to 2000 $\mu g.L^{-1}$) and Cu (90 to 150 $\mu g.L^{-1}$), decreased after 24 h
447 but increased after 96 h of exposure.
448 Takeng together, these results demonstrated that responses to metal exposure differ as a
449 function of exposure duration (Peles et al., 2012; Pistole et al., 2008) and concentrations used
450 (Malekpouri et al., 2016).However, it was partly confirmed that increased temperature would
451 lead to increased metal accumulation and effects, with increased Cd accumulation and
452 transiently increased SMR and hematocrit levels. As expected, physiological parameters
453 returned to normal by the end of the exposure.
454

455 **V. Conclusion**

456 Common carp tolerated the sublethal metal mixture well, at least for one week, as no major
457 responses were observed at the whole-body level. However, temperature affected the carps'
458 response to the metal mixture. At 20 °C, the SMR and the hematocrit, reflecting oxygen
459 carrying capacity, increased. Histopathological alterations in fish, were mainly mild during the
460 course of the exposure, but with a small spike at the 12 h time point, were also noted. These
461 responses only induced few variations in aerobic metabolism and consequently, anaerobic
462 metabolism was not impacted, as shown by the stability of lactate content. At 10 °C, no
463 significant modification was observed, showing that carp were even more tolerant to metal
464 mixture at lower temperatures.

465

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472

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681 Table 1: Histopathological alterations observed in experimental fish exposed to Cu/Cd/Zn
682 mixture at 20 °C sampled at different time points (12 h, 1 day, 3 days, 7 days); control fish were
683 sampled at 12 h and 7 days (mean ± standard deviation, n = 9 - 10).

Group	Control		Exposure			
Sampling point	12 h	7 days	12 h	1 day	3 days	7 days
Hyperaemia	3.2 ± 1.0	3.6 ± 0.9	2.8 ± 1.0	2.6 ± 0.8	2.8 ± 1.0	3.8 ± 1.0
Hypertrophy of respiratory epithelium	0.4 ± 0.7*†	1.2 ± 0.7*	1.8 ± 1.4†	1.8 ± 0.9	1.1 ± 1.0	2.0 ± 1.1
Oedema of respiratory epithelium	2.6 ± 0.5	1.9 ± 1.3	2.4 ± 1.3	2.2 ± 1.1	2.6 ± 1.1	3.0 ± 1.7
Hyperplasia of primary and respiratory epithelium	1.1 ± 0.6†	1.2 ± 1.1	2.8 ± 1.2[a]†	0.8 ± 1.0[b]	0.6 ± 0.8[b]	1.2 ± 1.6[ab]
Architectural and structural alterations	0.4 ± 0.5	0.7 ± 0.7	0.8 ± 0.6	1.1 ± 0.7	1.3 ± 0.8	1.2 ± 1.0
Hyperplasia/hypertrophy of mucous cells	0.1 ± 0.3*	0.9 ± 0.8*	0.3 ± 0.5	0.9 ± 0.6	1.3 ± 1.1	1.2 ± 0.7
Hyperplasia of complete primary lamellae	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 1.3
Presence of EGCs/mucous cells in secondary lamellae	0.4 ± 0.9	1.3 ± 1.4	1.4 ± 1.6	2.0 ± 0.9	0.8 ± 1.0	2.4 ± 1.7
Infiltration of leukocytes	0.0 ± 0.0	0.1 ± 0.3	0.1 ± 0.3	0.2 ± 0.4	0.1 ± 0.3	0.2 ± 0.4
Nucleus enlargement in cells of respiratory epithelium	0.9 ± 1.1	2.0 ± 1.7	2.8 ± 2.1	3.0 ± 1.4	1.8 ± 1.8	2.4 ± 1.3

684 Tissue alterations were scored as following: 0 = none, 2 = mild, 4 = moderate and 6 = severe;
685 different superscript letters within the same row indicate statistical significance between time
686 points in exposure treatments ($P < 0.05$); asterisk (*) represents significant difference between
687 control fish sampled at 12 h and control fish sampled at the end of the exposure ($P < 0.05$);
688 obelisk (†) represents significant difference between control fish sampled at 12 h and exposure
689 fish sampled at 12 h ($P < 0.05$).
690
691

692 Figure captions:

693 Figure 1: Standard metabolic rate (mg O_2/h/kg) of *Cyprinus carpio* exposed to Cu/Cd/Zn
694 mixture for 12 h, 1 day, 3 days or 1 week, at 10 °C and 20 °C (median ± quartile, n = 8-10).
695 Asterisks indicate significant differences between temperatures; letters indicate significant
696 differences between treatments ($P < 0.05$).

697

698 Figure 2: Maximum metabolic rate (mg O_2/h/kg) of *Cyprinus carpio* exposed to Cu/Cd/Zn
699 mixture for 12 h, 1 day, 3 days or 1 week, at 10 °C and 20 °C (median ± quartile, n = 8-10).
700 Asterisks indicate significant differences between temperatures ($P < 0.001$).

701

702 Figure 3: Aerobic scope (mg O_2/h/kg) of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 12
703 h, 1 day, 3 days or 1 week, at 10 °C and 20 °C (median ± quartile, n = 7-10). Asterisks indicate
704 significant differences between temperatures ($P < 0.001$).

705

706 Figure 4: Hematocrit (% of red cells) of *Cyprinus carpio* exposed to Cu/Cd/Zn mixture for 12
707 h, 1 day, 3 days or 1 week, at 10 °C and 20 °C (median ± quartile, n = 9-10). Asterisks indicated
708 significant differences between temperatures; letters indicate significant differences between
709 treatments ($P < 0.05$).

710

711 Figure 5: Illustration of histopathological alterations of common carp gills from the study: a)
712 hyperaemia (double arrowheads), oedema of respiratory epithelium (arrowheads) and enlarged
713 nucleus of respiratory epithelium (arrow) (HE ×400); b) hypertrophy of respiratory epithelium
714 (arrows); note increased number of eosinophilic granulocytes in the lower left corner (HE
715 ×400); c) hyperplasia of primary epithelium (arrow) and mucous cells (arrowhead), hyperaemia

716 (double arrowheads) (HE ×400); d) Presence of mucous cell in the secondary lamellae (arrow)

717 and hyperplasia of mucous cells; note release of mucous to interlammelar space (HE ×400).

718

719 Figure 6: Metal accumulation (µg/g dry weight) in gills of *Cyprinus carpio* exposed to

720 Cu/Cd/Zn mixture for 1 day or 1 week, at 10 °C and 20 °C (mean ± standard deviation,). a)

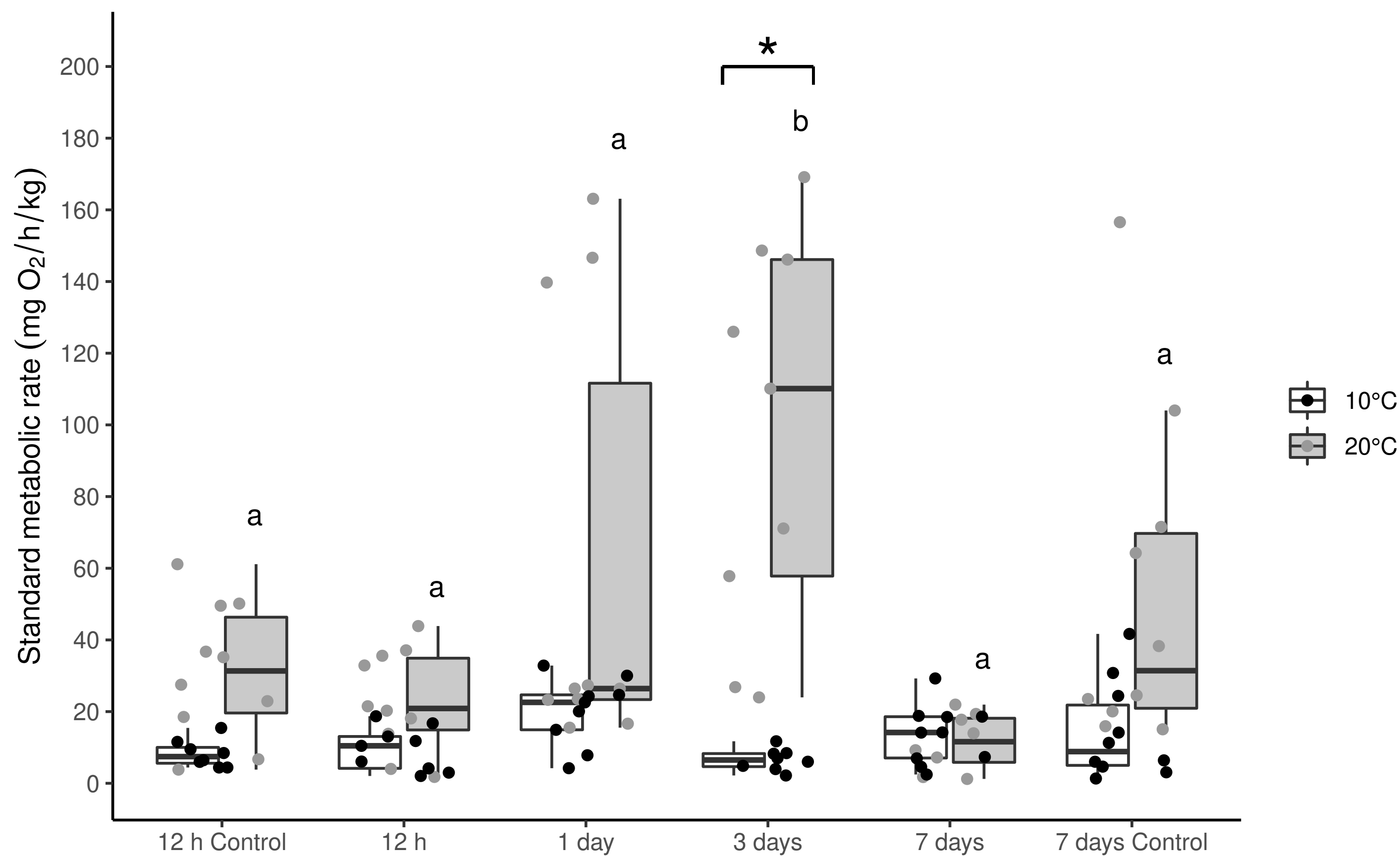
721 copper (n = 3-5); b) cadmium (n = 3-5) and c) zinc (n = 4-5). Asterisks indicated significant

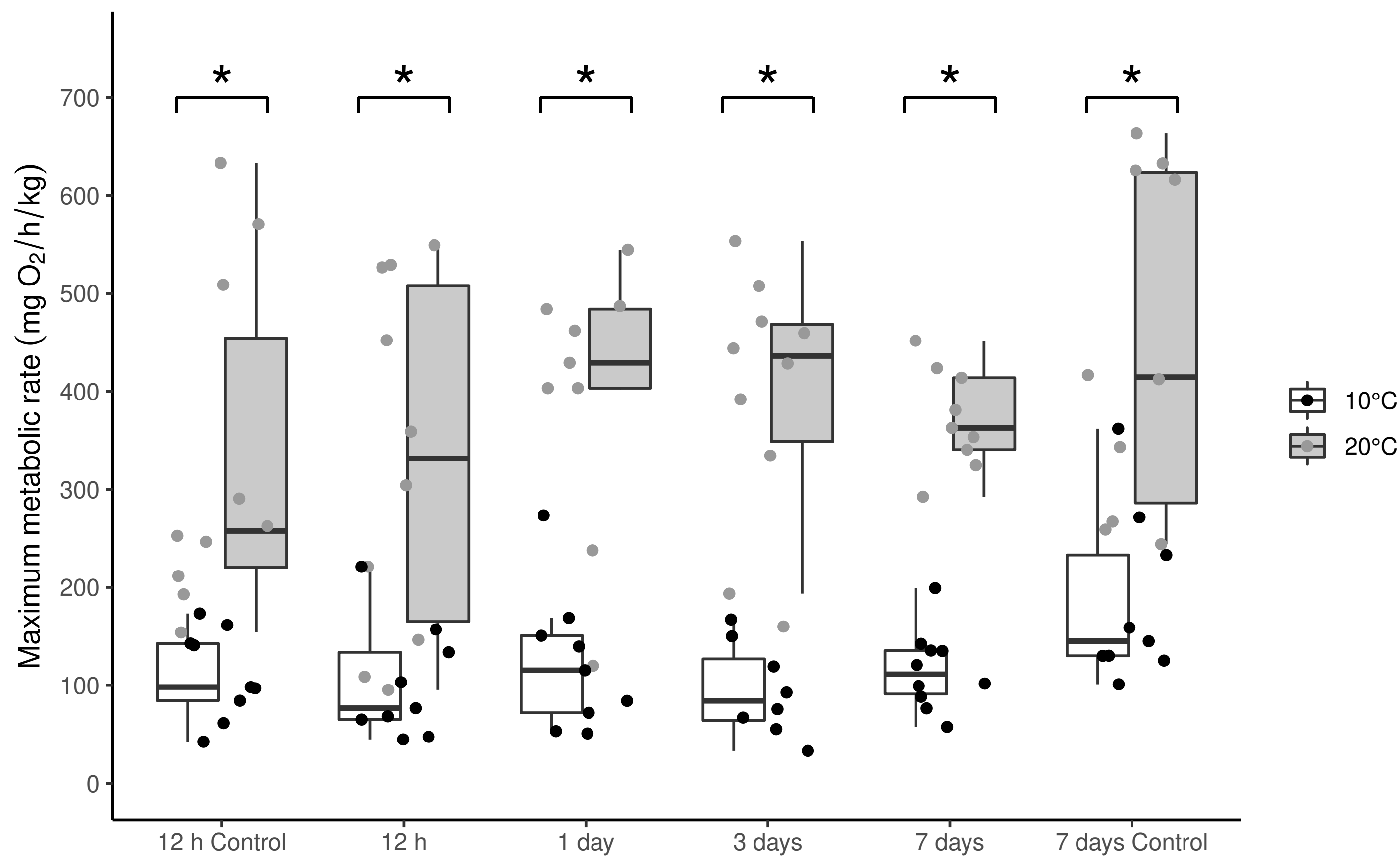
722 differences between temperatures; letters indicate significant difference between treatments (P

723 < 0.05).

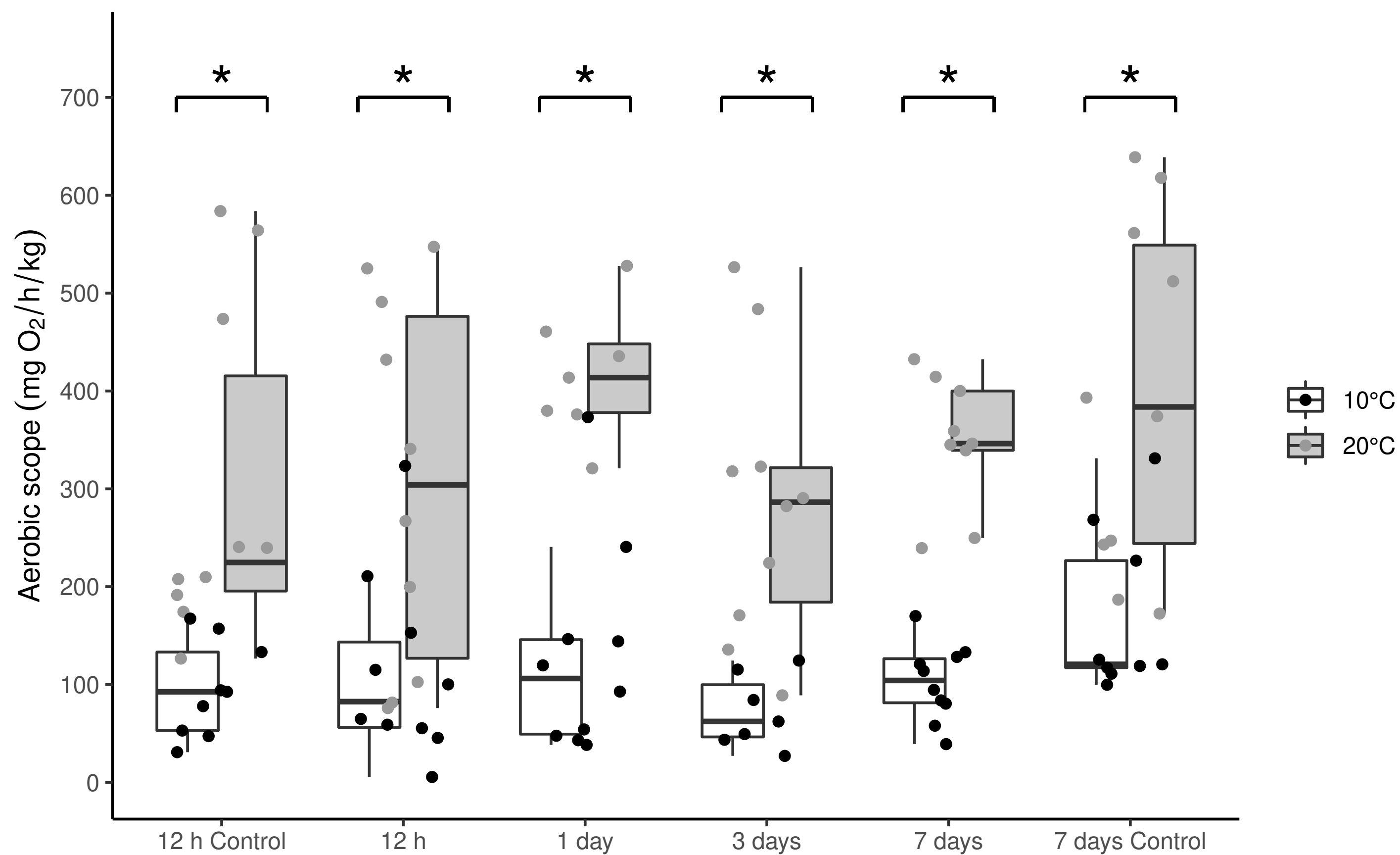
724

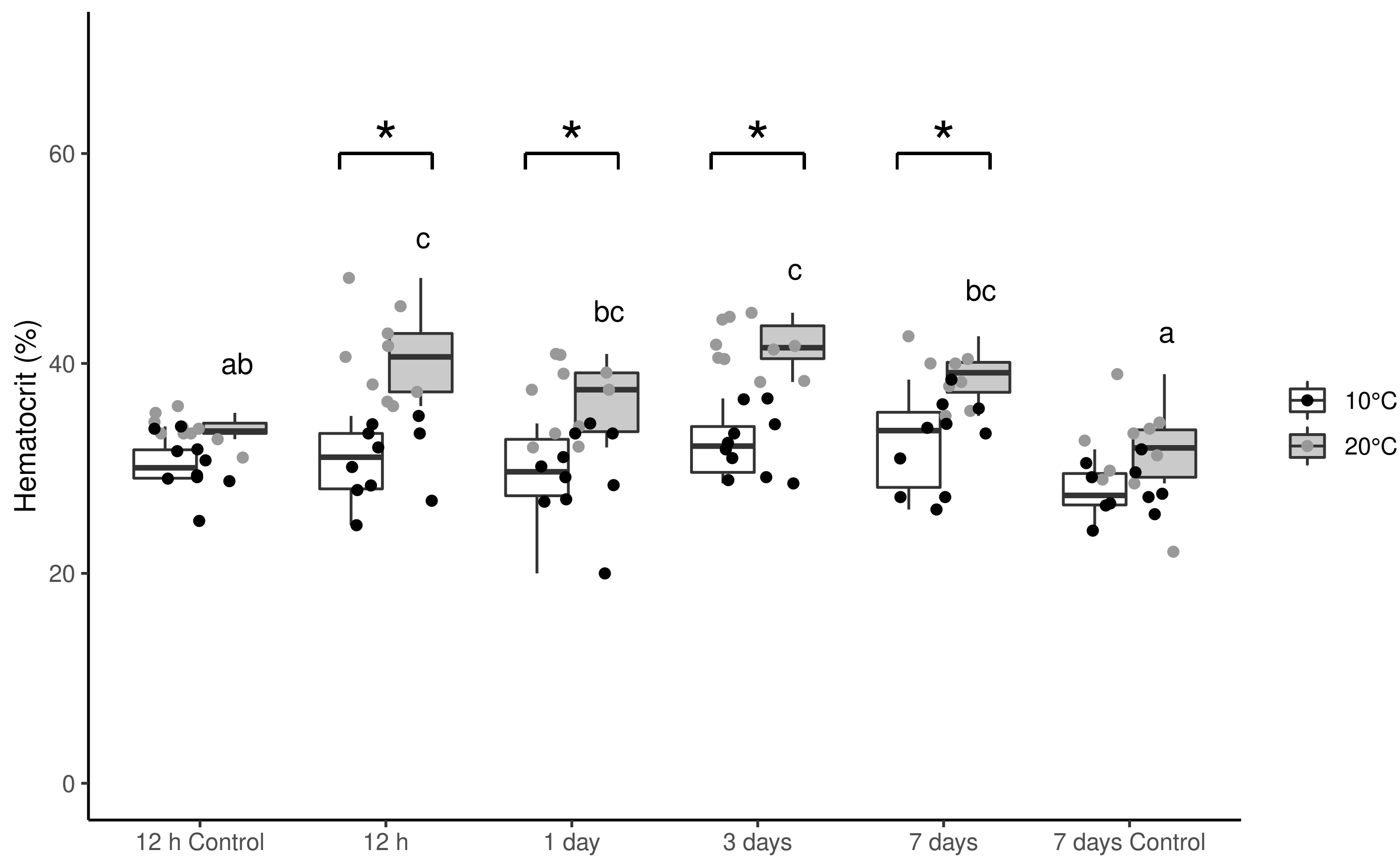
725

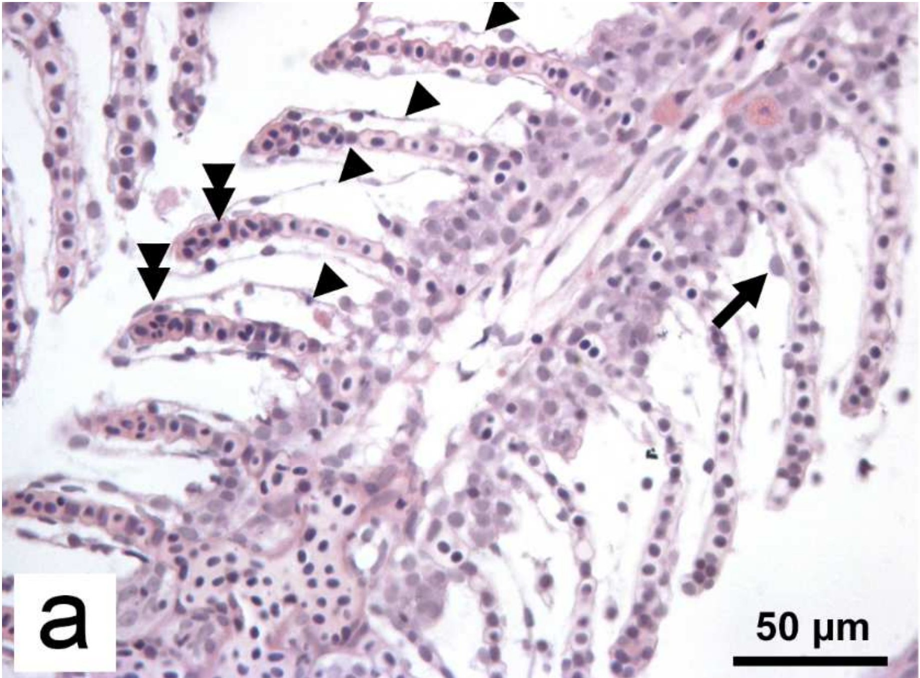

Standard metabolic rate (mg O_2/h/kg)
200
180
160
140
120
100
80
60
40
20
0
*
a
a
a
b
a
a
12 h Control
12 h
1 day
3 days
7 days
7 days Control
10°C
20°C

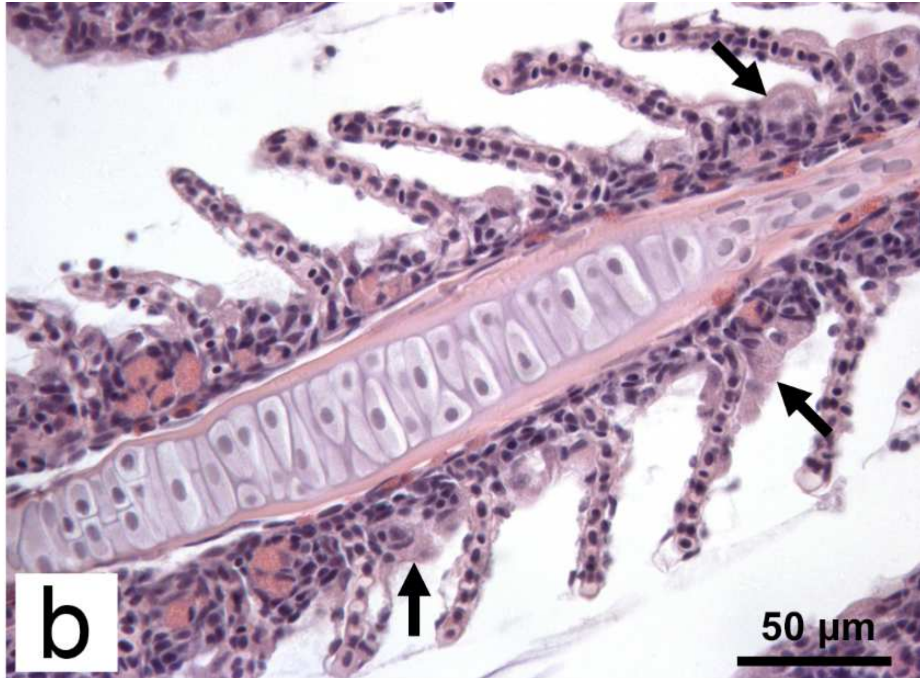


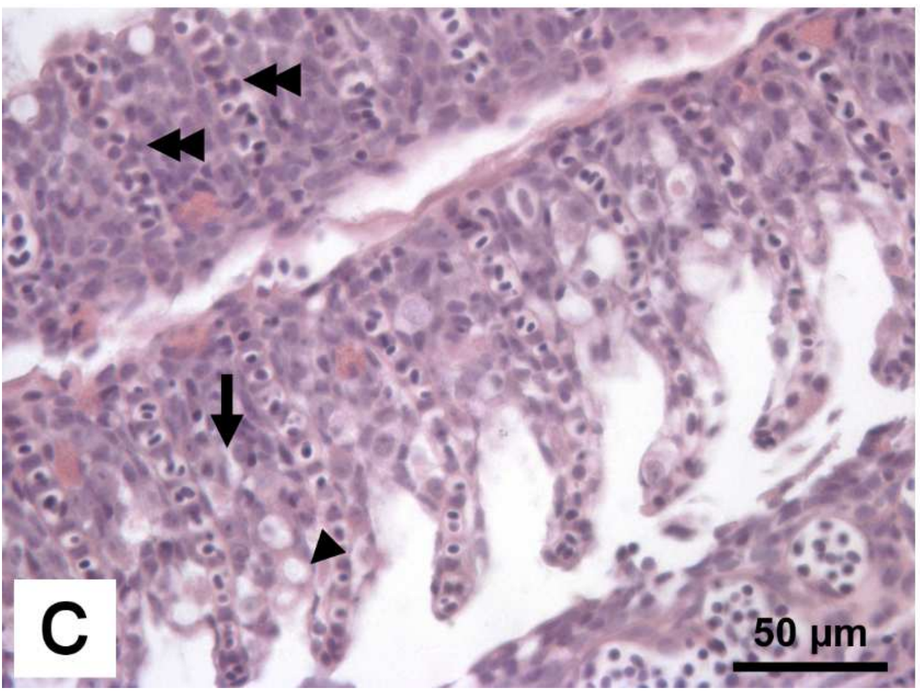
Maximum metabolic rate (mg O_2/h/kg)
0
100
200
300
400
500
600
700
*
*
*
*
*
*
12 h Control
12 h
1 day
3 days
7 days
7 days Control
10°C
20°C

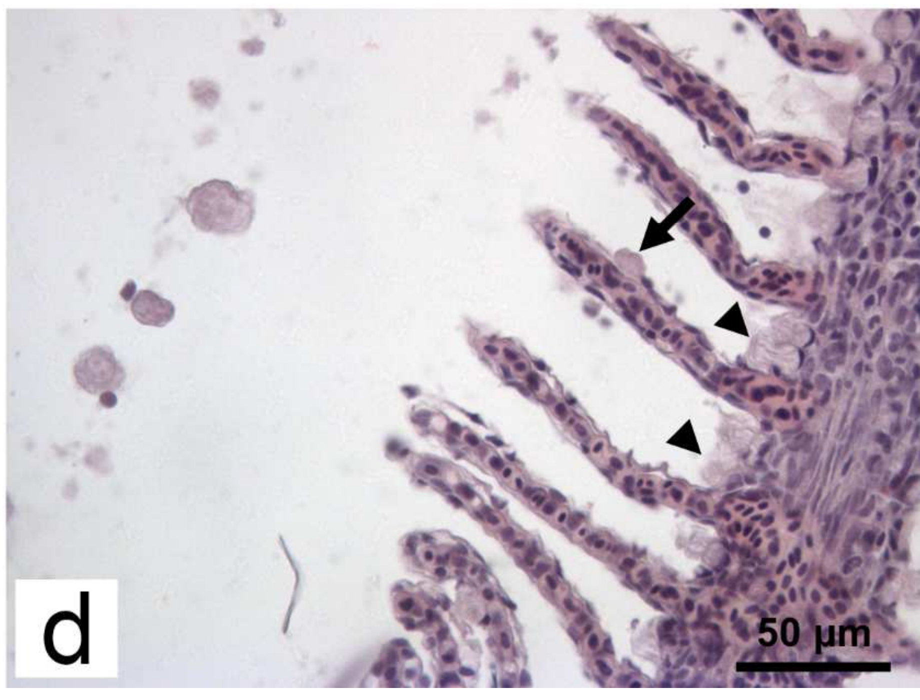

Aerobic scope (mg O_2/h/kg)
700
600
500
400
300
200
100
0
*
*
*
*
*
*
12 h Control
12 h
1 day
3 days
7 days
7 days Control
10°C
20°C

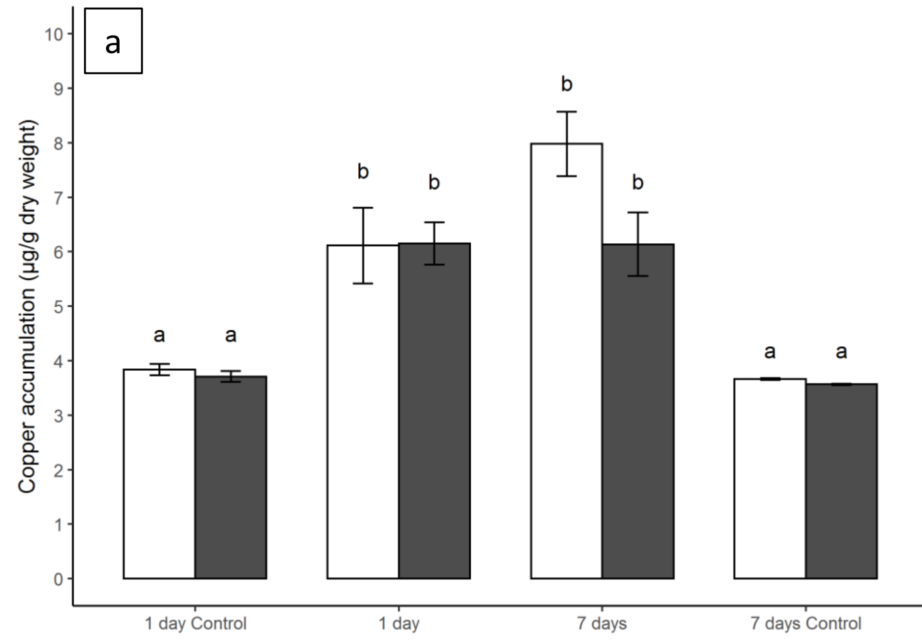

Hematocrit (%)
60
40
20
0
*
*
*
*
ab
c
bc
c
bc
a
12 h Control
12 h
1 day
3 days
7 days
7 days Control
10°C
20°C

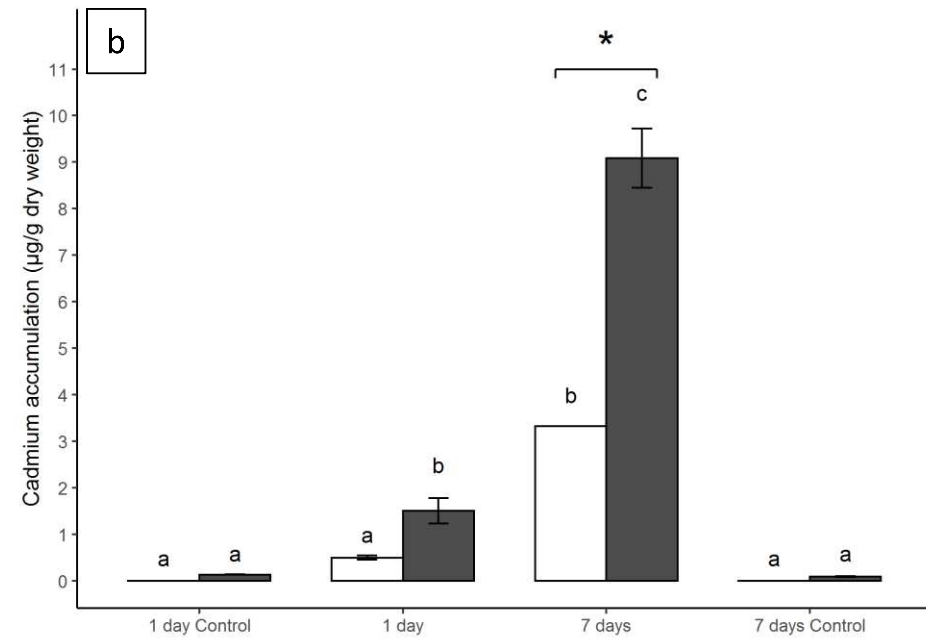

a
50 µm

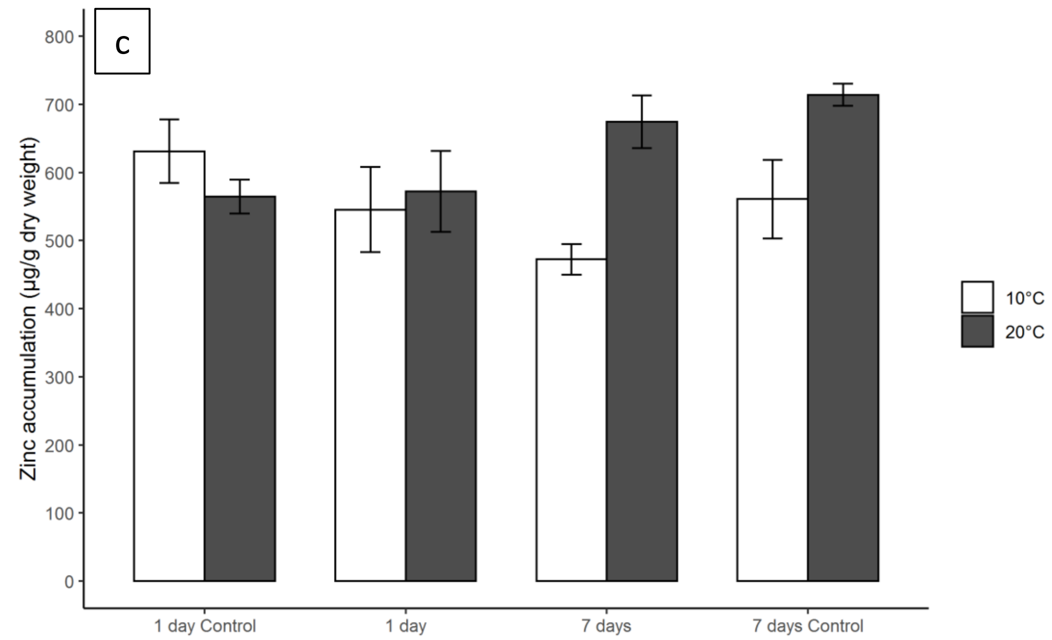

b
50 µm


c
50 µm


d
50 µm


a
Copper accumulation (µg/g dry weight)
0
1
2
3
4
5
6
7
8
9
10
a a
b b
b b
a a
1 day Control
1 day
7 days
7 days Control


b
Cadmium accumulation (µg/g dry weight)
0
1
2
3
4
5
6
7
8
9
10
11
*
a a
a b
b c
a a
1 day Control
1 day
7 days
7 days Control


c
Zinc accumulation (µg/g dry weight)
0
100
200
300
400
500
600
700
800
1 day Control
1 day
7 days
7 days Control
10°C
20°C