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1 **Water temperature and acid pH influence the cytotoxic and genotoxic effects of**
2 **aluminum in the freshwater teleost *Astyanax altiparanae* (Teleostei: Characidae)**

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24 **Abstract**

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25 The toxicity of metals, including aluminum (Al), can be potentiated by temperature and
26 acid pH, a concern in view of the current global warming scenario. The aim of this
27 study was to evaluate the bioconcentration of Al in the testes and semen of *Astyanax*
28 *altiparanae* and the potential of this metal, at different environmental temperatures and
29 acid pH, to cause cytotoxicity and genotocixity in erythrocytes and spermatozoa. *A.*
30 *altiparanae* males were divided into nine experimental groups: at each of three different
31 water temperatures (20, 25 and 30 ºC), the fish were exposed to a neutral pH, an acid
32 pH and acidic water containing Al (0.5 $mg.L^{-1}$). The fish were subjected to subacute,
33 semi-static exposure and sampled at 24 and 96 h. After each exposure period the comet
34 assay (blood and semen) and micronucleus test (blood) were performed.
35 Bioconcentration of Al was evaluated in the testes and semen. Exposure time and
36 temperature influenced the Al bioconcentration pattern in the testes. Al concentration in
37 the semen was higher in fish exposed at 20 and 25 ºC (24 h). The DNA fragmentation
38 score for the semen and blood was higher in fish exposed to Al at 20 (24 h) and 30 ºC
39 (96 h). The frequency of nuclear abnormalities in erythrocytes was higher in the group
40 exposed to Al at 30 ºC (96 h). It was concluded that Al bioconcentrates in the testes and
41 semen of *A. altiparanae* at different temperatures and is potentially cytotoxic and
42 genotoxic to erythrocytes and spermatozoa in this species.

43 **Keywords:** pH; Spermatozoa; Semen; Metal; Temperature

44

45 **Highlights**

46 *Increased temperature enhances the bioaccumulation of Al the testes after 96 h.

47 *A shorter exposure period (24 h) triggers Al bioaccumulation in the semen.

48 *Temperature and pH potentiate the genotoxic and cytotoxic effects of Al.

49 * The DNA damage caused by Al to the erythrocytes and spermatozoa is reversible.

50

51 ## 1. Introduction

52 Human activities, such as discharging domestic, agricultural and industrial
53 effluents into the environment, are the main drivers behind the environmental
54 degradation process, whether characterized by impaired river water quality or adverse
55 effects on animal physiological processes (Manzano *et al.*, 2015, Viana *et al.*, 2017).
56 Discharged waste can contain many toxic substances, including metals, pesticides,
57 drugs and solvents, which can bioconcentrate in different tissues, alter growth, behavior,
58 reproduction and metabolism, and damage DNA (Authman, 2011; Kida *et al.*, 2016;
59 Viana *et al.*, 2017).

60 Aluminum (Al) is one of the metals found in water bodies and is, in fact, one of
61 the most abundant metals in the earth's crust. It has many everyday applications (water
62 treatment, manufacturing of cans and cooking utensils, etc.), but to date no biological
63 function has been described for this metal (Nayak, 2002; Fernández-Dávila *et al.*, 2012).
64 Therefore, there are no specific binders or chaperones for Al transport, no transporters
65 or channels to selectively facilitate the passage of this metal across cell membranes, and
66 no intracellular storage proteins to aid in cellular homeostasis. Furthermore, there are no
67 pathways for Al metabolization and excretion (Exley and Mold, 2015). The presence of
68 Al in the organism can cause diseases and induce toxic effects, as reported by several
69 studies on different animal groups. Al can cause genotoxic effects in fish kidney cells
70 (Klingelfus *et al.*, 2015) and affect amphibian growth and larval and embryonic
71 development (Peles, 2013; Herkovits *et al.*, 2015). In humans, it can accumulate in the
72 brain and has been linked to the etiology of autism (Mold *et al.*, 2018) and Alzheimer's
73 disease (Tomljenovic, 2011).

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74 Kida *et al.* (2016) analyzed *A. altiparanae* males exposed to Al during the active
75 phase of the reproductive cycle and observed an increase in plasma levels of
76 testosterone and 11-ketotestosterone after exposure for 96 hours, suggesting alterations
77 in the reproductive physiology of males impairing sperm production and spermiation.
78 Genotoxic effects of Al were also observed in the erythrocytes of juveniles of the
79 tropical species *Prochilodus lineatus* (Galindo *et al.*, 2010), oxidative stress in *C. carpio*
80 (García-Medina *et al.*, 2013), ionic imbalance and inhibition of Na^{+}/K^{+}–ATPase
81 enzymatic activity in *P. lineatus* (Camargo *et al.*, 2009), and increased DNA damage
82 (time-dependent) and a higher level of oxidized DNA in *C. carpio* (García-Medina *et*
83 *al.*, 2011).

84 In addition to the known toxic effects of Al and other metals such as cadmium,
85 mercury, arsenic and selenium on aquatic organisms, it is also known that higher
86 temperatures can aggravate the effects of these metals on fish health (Muniz and
87 Oliveira-Filho, 2006). Poleó and Muniz (1993) observed an increase in ventilation
88 frequency to adjust the respiratory rate in *Salmo salar* exposed to Al at higher
89 temperatures. The occurrence of this synergistic effect is worrisome in light of the
90 current global warming scenario. Data from the Intergovernmental Panel on Climate
91 Change (IPCC, 2013/2014) state that the earth's temperature will increase by 0.3 to 1.7
92 °C from 2010 to 2100, potentially aggravating the toxicity of metals.

93 Teleosts inhabit different trophic levels in the food chain, and for this reason are
94 widely used in ecotoxicological trials and studies to evaluate the genotoxic potential of
95 various substances, including metals. They are also sensitive to environmental changes
96 and capable of bioconcentrating contaminants, even at low concentrations in water
97 bodies (Jesus *et al.*, 2014, 2016; Chua *et al.*, 2018). Furthermore, fish bioassay data
98 correlate well with genotoxicity in human cells exposed to environmental mutagenic

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99 agents (Marcon *et al.*, 2010), and some of the results obtained using teleosts can be
100 extrapolated to humans. *Astyanax altiparanae* has also been used in bioassay studies
101 (Gomes *et al.*, 2013; Vieira *et al.*, 2013; Chehade *et al.*, 2014; Bettim *et al.*, 2016; Kida
102 *et al.*, 2016; Abdalla *et al.*, 2019), mainly due to its high plasticity to different
103 environments, small size so that experiments can be conducted in tanks, and ease of
104 handling under laboratory conditions.

105 Micronucleus tests, nuclear abnormality tests and the comet assay (Osman,
106 2014; Lapuente *et al.*, 2015) are the most widely used methods for evaluating mutagenic
107 and genotoxic effects in fish. They can be combined with other biochemical methods
108 and biomarkers, as well as quantification of metals in animal tissues, to evaluate levels
109 of water pollution. The data available in the literature to date reveal the genotoxic effect
110 of Al in juvenile fish, but not in fish during the reproductive phase, combined with
111 variations in water temperature. Based on the current scenario of climate change and
112 human activities, our hypothesis is that changes in water temperature and acid pH can
113 bring about the accumulation of Al in fish testes and semen, triggering genotoxic effects
114 in erythrocytes and sperm cells. Therefore, the aim of this study was to evaluate the
115 bioconcentration of Al in fish testes and semen and the potential of this metal, combined
116 with temperature changes and acid pH, to cause cytotoxicity and genotoxicity in the
117 erythrocytes and spermatozoa of *A. altiparanae*.

118

119 **2. Material and Methods**

120 2.1. Experimental conditions

121 Sexually mature *A. altiparanae* (n=360) males were donated by the Paraibuna
122 Hydrobiology and Aquaculture Station (*Companhia Energética de São Paulo*, CESP,
123 Paraibuna, São Paulo, Brazil). Prior to the experiment, the fish were kept for seven days

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124 in the ectotherm facility at the Universidade de São Paulo, Institute of Biosciences,
125 Department of Physiology (IB/USP). They were then transferred to 18 glass tanks (132
126 L water/tank, 10 fish/tank) with water replenishment (90% every 24 h) and fed daily *ad*
127 *libitum* with extruded feed (32% crude protein). They were deprived of food for 24
128 hours before beginning the experiments and until the end of subacute exposure, in order
129 to obviate feces as a further experimental variable.

130 Separate studies were conducted at two exposure times: 1) 180 fish were
131 exposed to the experimental conditions for 24 hours; and 2) 180 fish were exposed to
132 the experimental conditions for 96 hours. These periods were chosen based on previous
133 studies (Correia *et al.*, 2010; Kida *et al.*, 2016; Abdalla et al., 2019) to verify responses
134 over different time intervals, since some physiological responses occur rapidly due to
135 the plasticity of the species studied.

136 For each experimental period (24 and 96 h), the animals were divided into nine
137 experimental groups (replicates) based on three different temperatures (20 ºC, 25 ºC and
138 30 ºC), at each of which the fish were exposed to neutral pH, acid pH (5.5) and acid pH
139 (5.5) with aluminum, resulting in the following experimental groups: T1) water at 20
140 ºC, no Al, neutral pH; T2) water at 20 °C, no Al, acid pH; T3) water at 20 ºC, with Al,
141 acid pH; T4) water at 25 °C no Al, neutral pH (control group); T5) water at 25 °C, no
142 Al, acid pH; T6) water at 25 °C, with Al, acid pH; T7) water at 30 °C, no Al, neutral
143 pH; T8) water at 30 °C, no Al, acid pH; T9) water at 30 °C, with Al, acid pH. Al was
144 added to the water for groups T3, T6 and T9 at a concentration of 0.5 $mg.L^{-1}$ (mg of
145 $Al.L^{-1}$ water).

146 The Al solution was prepared at a concentration of 0.5 $mg.L^{-1}$ (5.67 g
147 $Al_2(SO_4)_3.18H_2O$ - Sigma Aldrich - diluted in 700 ml of deionized water, adjusting the
148 pH to 2.5 with 65% HNO_3 - Suprapur, Merck). A quantity of 8.148 ml of this solution

149 was added to the 132-L tanks in order to obtain an initial concentration of 0.5 mg.L-1 of
150 Al and a pH of 5.5 (required for Al bioavailability), since Al is relatively insoluble at
151 pH 6 to 8 due to hydrolysis and the formation of $Al(OH)_3$ (Driscoll and Schecher, 1990;
152 Gensemer and Playle, 1999). This concentration of Al was chosen as representative of
153 contamination levels in some basins in the State of São Paulo, according to reports
154 published by *Companhia Ambiental do Estado de São Paulo* (CETESB, 2016), and is
155 below the LC_{50} value (1mg.L^{-1}) previously obtained (unpublished data).

156 Water temperatures were determined according to the known adequate survival
157 temperatures (Siqueira-Silva *et al.*, 2015). The 24- or 96-hour period started from
158 temperature stabilization for each experimental group, and temperature transitions were
159 controlled at a rate of 1 °C.h^{-1} (Trueman *et al.*, 2000). For each experimental group, the
160 water temperature was kept constant using the ectotherm facility cooling system, and
161 thermostat-controlled water heaters monitored periodically.

162 Thus, ten fish were collected from each experimental tank (at 24 h and 96 h),
163 giving a total of 20 fish per experimental group/time. The tests were semi-static, with
164 90% water renewal every 24 hours. The water in the tanks was filtered and the
165 physicochemical parameters (e.g. temperature and dissolved oxygen) analyzed daily
166 using an oximeter (YSI55) and a pH meter (Gehaka).

167 The experiment was approved by the Animal Use Ethics Committee (CEUA) at
168 IB/USP (265/2016; Process 16.1.417.41.3).

169

170 2.2. Water collection

171 Water samples (150 ml) from each tank were collected every 24 hours and
172 placed in amber flasks previously washed with nitric acid. The sample was always taken
173 with a 20 ml syringe at the same point inside the tank. It was aliquoted into two flasks

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174 (one for total Al - unfiltered, one for dissolved Al - filtered with a Valuprep PVDF 0.45,
175 Hexis filter syringe) and acidified with 65% nitric acid (Suprapur, Merck) at pH<2.5.
176 The vials were then kept refrigerated at 4 °C and Al quantified by Inductively Coupled
177 Plasma Mass Spectrometry (ICP-MS). To determine Al in the water samples, they were
178 diluted 10x in 2% HNO_3 and directly injected into the ICP-MS unit.

179

180 2.3. Collection of semen

181 The fish were always collected in the morning. Before collection, spermiation
182 was induced with pituitary extract of common carp, *C. carpio*, at a dose of 5 $mg.kg^{-1}$
183 body mass. Induction was carried out at different times during exposure, taking the
184 treatment temperature into account. Spermiation is faster at higher temperatures: for fish
185 kept at 20 ºC – hypophysation (induced breeding) was initiated 13 h before collection;
186 for fish kept at 25 ºC, 11 h before collection; and for fish kept at 30 ºC, 9 h before
187 collection. Fish from different experimental groups were anesthetized with eugenol-
188 based solution (clove oil) (1 ml eugenol: 10 ml ethanol: 10000 ml tank water). After
189 collecting the semen and blood, the anesthetized fish were euthanized by transection of
190 the spinal cord at the operculum (Schreck and Moyle, 1990).

191 To collect the semen, the urogenital papilla was carefully dried with paper
192 towels to avoid the contaminating the semen with water, urine, blood or feces, and the
193 abdomen massaged from the head toward the tail. Semen from each fish was collected
194 with an automatic pipette, aliquoted into polyethylene graduated tubes and kept at 4 ºC.

195

196 2.4. Quantification of aluminum in semen and testes

197 Al was quantified in an aliquot of 5 µL semen, diluted in 100 µL saline solution
198 (0.9% NaCl). Samples were frozen at -20 °C and Al quantified by ICP-MS following

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199 the protocol in Batista *et al.* (2009). The removed testes were frozen for Al
200 quantification by ICP-MS, following the protocol in Aguiar *et al.* (2012).
201
202 2.5. Comet assay on spermatozoa
203 A quantity of semen (10µL) was removed, diluted in 1000µL fetal bovine serum
204 (Gibco; Thermo Fisher Scientific) and the solution from each fish/treatment stored at 4
205 °C for three days before performing the comet assay (as described for blood samples).
206
207 2.6. Blood collection and blood tests
208 Blood was collected by caudal vasculature puncture using a heparin coated
209 syringe (5000 UI, Hepamax®). The blood (10 µL) was diluted in 1000 µL of
210 cryopreservation buffered solution (250 mM Saccharose, 40 mM Trisodic Citrate, 5%
211 DMSO, pH 7.6) (Evrard *et al.*, 2010; Jackson *et al.*, 2013). The samples were frozen for
212 one month and thawed in a polystyrene box at 4 ºC. Then, 10 µL of the cell suspension
213 was added to 120 µL of low melting agarose (0.8%) at 37 °C. To run the comet assay,
214 the slides were precoated with agarose (1% w/v in PBS buffer). Two slides were
215 prepared for each fish/treatment and incubated in the refrigerator in lysis buffer (2.5 M
216 NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% TRITON X-100, 0.01M
217 Sarcosine) at pH 10 for one hour. The slides were then transferred to a horizontal
218 electrophoresis cell and placed in denaturation buffer (300 mM NaOH, 100 mM EDTA,
219 pH 13) for 30 minutes in the dark. The samples were then electrophoresed (25V and
220 300mA) for 20 minutes, and the slides placed in neutralization buffer (0.4M Tris - 15
221 minutes), fixed with 100% ethanol (10 minutes) and stored in the refrigerator for further
222 analysis. For fluorescence microscopy analysis (200x), 50 µL of GelRed (Biotium) were
223 deposited on each slide and 100 nucleoids counted, according to fragment migration,

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224 and classified as: class 0 (no damage); class 1 (low damage); class 2 (average damage)
225 and class 3 (high damage), based on Kobayashi *et al.* (1995). The scores of each
226 experimental group were calculated by multiplying the number of nucleoids visualized
227 in each class by the value of the class (0, 1, 2 or 3). Finally, the scores of all the
228 individuals subjected to a given treatment were summed and divided by the number of
229 individuals to obtain the mean score. The DNA data produced by the comet assay were
230 ranked based on scores from zero (minimum degree of fragmentation) to 300
231 (maximum degree of fragmentation).

232 To run the micronuclei and nuclear abnormalities test, blood smears (5μL) were
233 prepared on clean glass slides (duplicate) and left to dry overnight. The slides were then
234 fixed in methanol PA (100%) for 10 minutes, washed with distilled water, stained with
235 10% Giemsa (25 minutes), washed with distilled water, dried under ambient conditions
236 (24 hours), coated with Permount coverslips (Fisher-SP15-500) for analysis with an
237 optical microscope (1000x) and the micronuclei and nuclear abnormalities (binucleate,
238 segmented, kidney, lobed and blebbed) counted and identified according to Carrasco *et*
239 *al.* (1990). A thousand cells (totaling 12000/group) were counted on each slide and the
240 results expressed as percentage frequency (%).

241

242 2.7. Statistical analysis

243 Data were expressed as mean ± standard error of the mean (SEM) and subjected to the
244 Kolmogorov-Smirnov normality test. The groups were compared by the ANOVA two-
245 way test (variables: temperature and treatment) followed by the Student Newman Keuls
246 post-hoc test. Values were considered significantly different at $P \leq 0.05$.

247

248 **3. Results**

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249 3.1. Water chemistry and animal survival

250 The physicochemical characteristics of the water used in the experiments are
251 detailed in Table 1.

252 The physicochemical parameters of the water supply used in all experimental
253 treatments were as follows: Calcium – 2.10 $mg.L^{-1}$; Phosphates - <L.Q. (limit of
254 quantification); Total Inorganic Carbon - 12.5 $mg.L^{-1}$; Total Organic Carbon - <L.Q.;
255 Organic matter - 5 $mg.L^{-1}$; Total Nitrogen - 0.02 $mg.L^{-1}$; Turbidity - 1.43 NTU; Total
256 Alkalinity - 57.54 mg $CaCO_3.L^{-1}$; Total Hardness - 51.65 mg $CaCO_3.L^{-1}$; Chlorides -
257 21.35 mg $Cl^-.L^{-1}$; Iron - <L.Q.; Conductivity - 71.30 $\mu S.cm^{-1}$ at 25°C; Sulfates - <L.Q .

258 Under these conditions, fish survival was 100% in all groups during the 24 h
259 bioassay. During the 96-h exposure, survival was also 100% in groups T1 to T8. Only
260 group T9 produced different results (24 h - 100%; 48 h - 60%; 72 h - 50%; 96 h - 50%).

261

262 Table 1. Physicochemical parameters of the water used in the experimental treatments
263 (24 h and 96 h) of males of *Astyanax altiparanae*.

Treatment	pH	Temperature	Dissolved Oxygen	Total Al	Dissolved Al
24 h		°C	$mg.L^{-1}$	$mg.L^{-1}$	$mg.L^{-1}$
1	7.40 ± 0.05	19.37 ± 0.06	8.63 ± 0.06	0.08 ± 0	-
2	5.65 ± 0.08	18.68 ± 0.17	8.63 ± 0.08	0.08 ± 0	-
3	5.49 ± 0.08	18.37 ± 0.13	8.71 ± 0.13	0.45 ± 0.05	0.36 ± 0.09
4	7.50 ± 0.10	24.52 ± 0.19	6.82 ± 0.19	0.08 ± 0	-
5	5.73 ± 0.04	24.62 ± 0.23	7.04 ± 0.10	0.06 ± 0	-
6	5.50 ± 0.07	24.97 ± 0.09	7.26 ± 0.11	0.42 ± 0.04	0.38 ± 0.08
7	7.72 ± 0.06	30.88 ± 0.25	5.87 ± 0.11	0.08 ± 0	-
8	5.65 ± 0.16	29.92 ± 0.10	5.77 ± 0.07	0.07 ± 0	-

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9	5.38 ± 0.09	31.27 ± 0.07	5.71 ± 0.05	0.47 ± 0.01	0.44 ± 0.02

Treatment	pH	Temperature	Dissolved Oxygen	Total Al	Dissolved Al
96 h		°C	$mg.L^{-1}$	$mg.L^{-1}$	$mg.L^{-1}$
1	7.62 ± 0.02	20.69 ± 0.07	7.72 ± 0.08	0.08 ± 0	-
2	5.38 ± 0.02	19.95 ± 0.17	8.06 ± 0.10	0.07 ± 0	-
3	5.37 ± 0.02	20.44 ± 0.15	8.05 ± 0.11	0.54 ± 0.01	0.48 ± 0.02
4	7.49 ± 0.06	24.56 ± 0.11	7.53 ± 0.06	0.08 ± 0	-
5	5.42 ± 0.07	24.48 ± 0.11	7.28 ± 0.12	0.09 ± 0	-
6	5.47 ± 0.05	24.90 ± 0.13	7.25 ± 0.10	0.53 ± 0.01	0.50 ± 0.02
7	7.66 ± 0.03	31.24 ± 0.07	6.15 ± 0.04	0.07 ± 0	-
8	5.31 ± 0.07	30.44 ± 0.20	6.19 ± 0.06	0.07 ± 0	-
9	5.39 ± 0.05	31.27 ± 0.20	6.29 ± 0.07	0.53 ± 0.01	0.49 ± 0.02

264 T1) water at 20 ºC, no Al, neutral pH; T2) water at 20 °C, no Al, acid pH; T3)
265 water at 20 ºC, with Al, acid pH; T4) water at 25 °C, no Al, neutral pH (control group);
266 T5) water at 25 °C, no Al, acid pH; T6) water at 25 °C, with Al, acid pH; T7) water at
267 30 °C, no Al, neutral pH; T8) water at 30 °C, no Al, acid pH; T9) water at 30 °C, with
268 Al, acid pH. Data are means ± SEM (n= 6 - 24 h / n=10 – 96 h).

269 3.2. Bioconcentration of Al in testes and semen

270 In the 24 h bioassay (Fig. 1A), there was no difference in Al concentration in the
271 testes for any of the treatments. In the fish exposed for 96 h, the effect of water pH on
272 Al levels in the testes was temperature-dependent (P=0.004). No significant difference
273 was observed in the bioconcentration of Al in the testes at different temperatures in the
274 fish exposed to neutral pH. In the fish exposed to acid pH (with or without Al) there
275 was an increase in Al concentration in the testes as the temperature increased (P<0.001).
276 On analyzing the experimental groups at different temperatures, there was no difference

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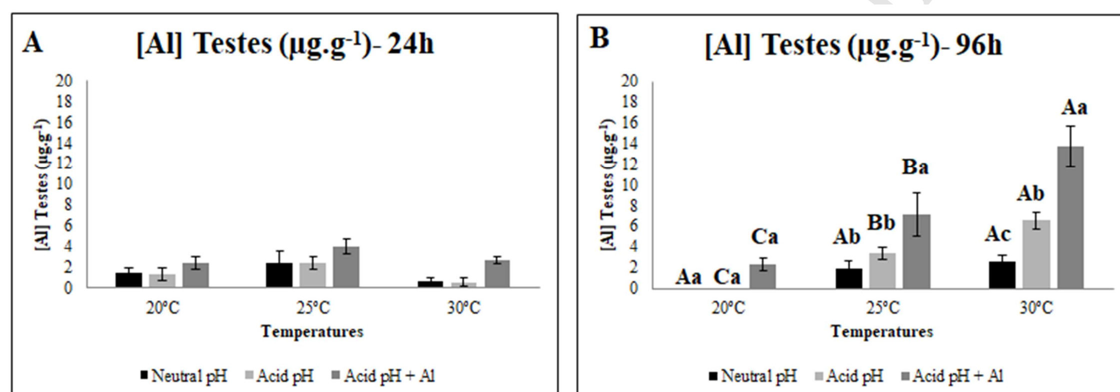
277 between groups at 20 ºC (P=0.30). At 25 ºC, the fish exposed to acid pH with Al
278 exhibited a higher bioconcentration of Al in the testes compared to the other groups
279 (neutral pH - P=0.004; acid pH- P=0.01). At 30 ºC, the fish kept at neutral pH exhibited
280 a lower concentration of Al in the testes than those in the other experimental groups,
281 and the fish exposed to acid pH with Al exhibited the highest concentration of Al (13.7
282 ± 1.98 $\mu g.g^{-1}$; P<0.001) (Fig. 1B).



283

284 Fig. 1. Concentration of aluminum in the testes ($\mu g.g^{-1}$) for each experimental group at
285 exposure times of 24 h (A) and 96 h (B) (n=6). T1) water at 20 ºC, no Al, neutral pH;
286 T2) water at 20 °C, no Al, acid pH; T3) water at 20 ºC, with Al, acid pH; T4) water at
287 25 °C, no Al, neutral pH (control group); T5) water at 25 °C, no Al, acid pH; T6) water
288 at 25 °C, with Al, acid pH; T7) water at 30 °C, no Al, neutral pH; T8) water at 30 °C, no
289 Al, acid pH; T9) water at 30 °C, with Al, acid pH. Different uppercase letters indicate
290 significant differences (P<0.05) for the same treatment at different temperatures;
291 different lowercase letters indicate significant differences (P <0.05) at the same
292 temperature for different treatments.

293

294 In the semen, Al was observed in the ejaculate of all experimental groups at both
295 24 (Fig. 2A) and 96 h (Fig. 2B). In the 24 h assay at acid pH, the concentration of Al
296 was higher at 25 ºC than at 30 ºC (P=0.02). In acid pH + Al, Al concentration was

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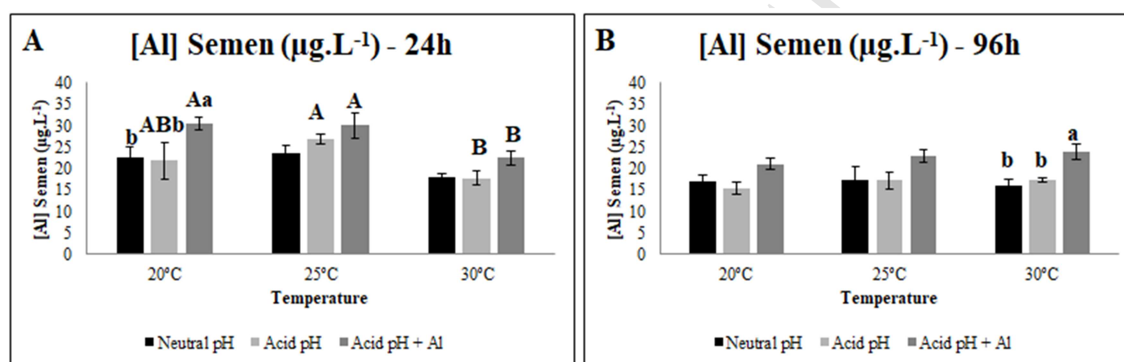
297 higher at 20 and 25 ºC compared to the fish exposed at 30 ºC (P=0.04, P=0.02). For
298 different treatments at the same temperature, a significant difference was observed only
299 at 20 ºC; fish exposed to acid pH + Al exhibited a higher concentration of Al in the
300 semen than those exposed to neutral and acid pH (Fig. 2A). In the fish exposed for 96 h
301 at 30 °C, the concentration of Al was higher in the semen of fish exposed to acid pH +
302 Al than in those in the other experimental groups (P=0.006) (Fig. 2B).
303

304


305 Fig. 2. Concentration of aluminum in the semen ($\mu g.L^{-1}$) for each experimental group at
306 exposure times of 24 h (A) and of 96 h (B) (n=6). T1) water at 20 ºC, no Al, neutral
307 pH; T2) water at 20 °C, no Al, acid pH; T3) water at 20 ºC, with Al, acid pH; T4) water
308 at 25 °C no Al, neutral pH (control group); T5) water at 25 °C, no Al, acid pH; T6)
309 water at 25 °C, with Al, acid pH; T7) water at 30 °C, no Al, neutral pH; T8) water at 30
310 °C, no Al, acid pH; T9) water at 30 °C, with Al, acid pH. Different uppercase letters
311 indicate significant differences (P<0.05) for the same treatment at different
312 temperatures; different lowercase letters indicate significant differences (P <0.05) at the
313 same temperature for different treatments.
314

315 3.3. Comet assay: blood and semen

316 Regardless of cell type and exposure time, the effect of pH and Al on the degree
317 of DNA fragmentation was temperature-dependent (P<0.001).

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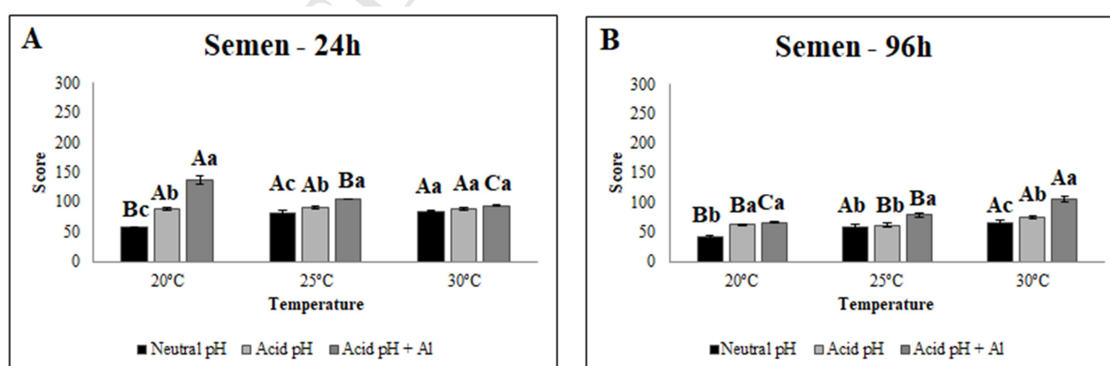
318 In the 24-h experimental groups, the DNA fragmentation score in the semen of
319 fish exposed to neutral pH was higher at 25 ºC and 30 ºC than at 20 ºC (P=0.02, P=0.01,
320 respectively). At acid pH + Al, as the temperature increased the degree of fragmentation
321 dropped (P=0.05). In terms of temperature-dependent effects, at 20 and 25 ºC the fish
322 exposed to acid pH + Al had a higher semen fragmentation score than those exposed to
323 a neutral or acid pH alone (Fig. 3A). After exposure for 96 h, the fish exposed to neutral
324 pH had a higher fragmentation score at 25 and 30 ºC compared to the fish exposed at 20
325 ºC (the same result was obtained after 24 h). In acid pH, fragmentation was higher at 30
326 ºC than at the other temperatures. In acid pH + Al, a higher temperature led to an
327 increase in the DNA fragmentation score (P=0.05). In terms of exposure at each
328 temperature, at 20 ºC the scores were higher in the semen of the fish exposed to acid pH
329 (with or without Al) compared to neutral pH. At 25 ºC, the score was higher in fish
330 exposed to acid pH + Al (P=0.02), and at 30 ºC, the score was higher at acid pH (this
331 score increased when Al was added) (Fig. 3B).
332



333

334 Fig. 3. DNA fragmentation score (0-300) for spermatozoa of *Astyanax altiparanae,*
335 obtained by the comet test. The fish were exposed in experimental groups for 24 h (A)
336 and 96 h (B) (n=6). T1) water at 20 ºC, no Al, neutral pH; T2) water at 20 °C, no Al,
337 acid pH; T3) water at 20 ºC, with Al, acid pH; T4) water at 25 °C no Al, neutral pH
338 (control group); T5) water at 25 °C, no Al, acid pH; T6) water at 25 °C, with Al, acid

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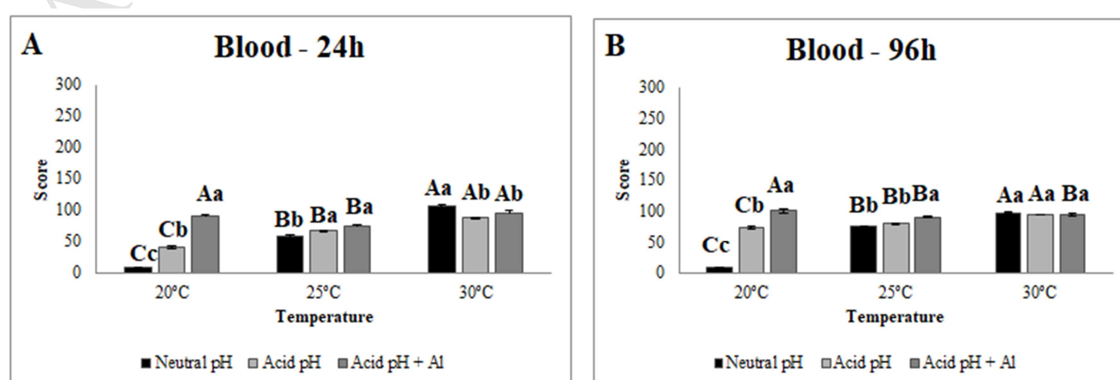
339 pH; T7) water at 30 °C, no Al, neutral pH; T8) water at 30 °C, no Al, acid pH; T9)
340 water at 30 °C, with Al, acid pH. Different uppercase letters indicate significant
341 differences (P<0.05) for the same treatment at different temperatures; different
342 lowercase letters indicate significant differences (P <0.05) at the same temperature for
343 different treatments.

344

345 In the erythrocytes (24 h, Fig. 4A), DNA fragmentation in fish exposed to
346 neutral and acid pH (no Al) increased with rising temperature. With the addition of Al,
347 fragmentation was higher at 20 and 30 ºC than at 25 ºC (P=0.02). An analysis of
348 erythrocyte fragmentation scores at each temperature showed that, at 20 ºC, fish
349 exposed to acid pH + Al exhibited a higher degree of fragmentation than fish in the
350 other experimental groups. At 25 ºC, fish exposed to acid pH (with and without Al)
351 exhibited higher fragmentation compared to fish exposed to neutral pH. At 30 ºC, the
352 fragmentation score was higher in fish exposed to neutral pH compared to the other
353 groups (P=0.02). At 96 h (Fig. 4B), fish exposed to neutral and acid pH (no Al)
354 exhibited a significant increase in the fragmentation score with increasing temperature.
355 When Al was added, the fragmentation score was higher at 20 ºC than at 25 and 30 ºC
356 (P=0.02). In overall terms, DNA fragmentation at 20 and 25 ºC was higher in fish
357 exposed to Al compared to the other groups.

358

359



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360 Fig. 4. DNA fragmentation score (0-300) for erythrocytes of *Astyanax altiparanae,*
361 obtained by the comet test. The animals were exposed in experimental groups for 24 h
362 (A) and 96 h (B) (n=6). T1) water at 20 ºC, no Al, neutral pH; T2) water at 20 °C, no
363 Al, acid pH; T3) water at 20 ºC, with Al, acid pH; T4) water at 25 °C no Al, neutral pH
364 (control group); T5) water at 25 °C, no Al, acid pH; T6) water at 25 °C, with Al, acid
365 pH; T7) water at 30 °C, no Al, neutral pH; T8) water at 30 °C, no Al, acid pH; T9)
366 water at 30 °C, with Al, acid pH. Different uppercase letters indicate significant
367 differences (P<0.05) for the same treatment at different temperatures; different
368 lowercase letters indicate significant differences (P <0.05) at the same temperature for
369 different treatments.

370

371 3.4. Micronucleus blood test

372 Kidney, lobed, segmented, blebbed and binucleate nuclear abnormalities were
373 found in erythrocytes at both exposure times. Micronuclei were found only at 96 h
374 (0.0001, 25 ºC, acid pH). The effects of pH and the presence of Al on the formation of
375 nuclear abnormalities was temperature-dependent at both 24 (P<0.001) and 96 h
376 (P=0.02).

377 Fish exposed for 24 h (Fig. 5A) to neutral and acid pH exhibited higher levels of
378 erythrocyte nuclear abnormalities at 20 ºC than at the other temperatures (P<0.001).
379 When Al was added, levels of nuclear abnormalities at 30 ºC were higher than at 25 ºC
380 (P=0.005). After 96 h (Fig. 5B), adding Al triggered a higher frequency of nuclear
381 abnormalities only at 30 ºC (P<0.001). In overall terms, adding Al at 25 and 30 ºC
382 increased the frequency of erythrocyte nuclear abnormalities compared to treatments at
383 neutral and acid pH without the addition of Al (P=0.007; P<0.001).

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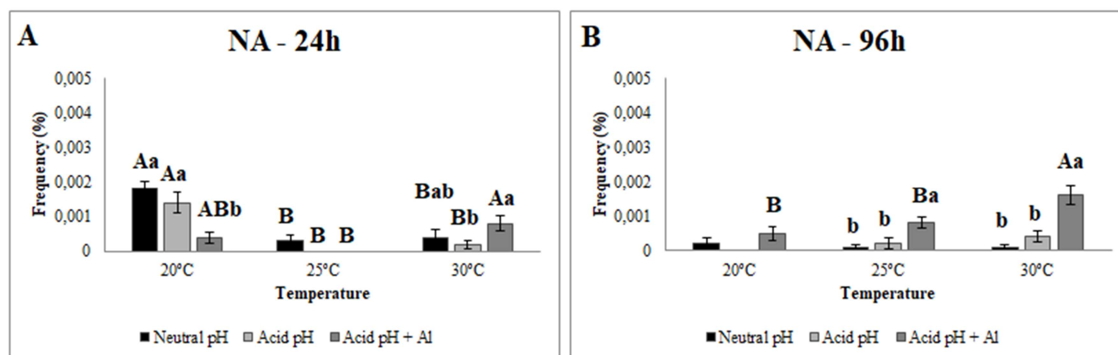
385



386 Fig. 5. Frequency of nuclear abnormalities (NA) in erythrocytes of *Astyanax*
387 *altiparanae*. The experimental groups of fish were exposed for 24 h (A) and 96 h (B)
388 (n=6). T1) water at 20 ºC, no Al, neutral pH; T2) water at 20 °C, no Al, acid pH; T3)
389 water at 20 ºC, with Al, acid pH; T4) water at 25 °C no Al, neutral pH (control group);
390 T5) water at 25 °C, no Al, acid pH; T6) water at 25 °C, with Al, acid pH; T7) water at
391 30 °C, no Al, neutral pH; T8) water at 30 °C, no Al, acid pH; T9) water at 30 °C, with
392 Al, acid pH. Different uppercase letters indicate significant differences (P<0.05) for the
393 same treatment at different temperatures; different lowercase letters indicate significant
394 differences (P <0.05) at the same temperature for different treatments.

395

396 **4. Discussion**

397 This study revealed that Al can bioconcentrate in both the testes and semen, and
398 this pattern is related to exposure time, acid pH and water temperature. The combination
399 of acidic water and lower temperature (20 ºC) had a cytotoxic and genotoxic effect on
400 the erythrocytes and spermatozoa of *A. altiparanae* at both 24 and 96 h, but this effect
401 was aggravated by adding Al to the water.

402

403 4.1. Water physicochemical properties and fish survival

404 The physicochemical properties of the water (e.g. temperature, pH and dissolved
405 oxygen) are of fundamental importance in ecotoxicological bioassays, since they can

406 influence biomarkers and fish survival. Moreover, the properties of the water directly
407 affect the solubility of metals and their bioavailability to aquatic organisms, since
408 changes in these properties promote the speciation of the metals, making them more or
409 less toxic (Namiesnik and Rabajczyk, 2010). Al is amphoteric, and therefore changes in
410 pH are followed by Al speciation. In addition to pH, other parameters that influence the
411 toxicity and bioavailability of Al are temperature, binder concentration and the ionic
412 strength of the solution (Matus and Kubova, 2005).

413 The Environmental Protection Agency (EPA) in the United States recommends
414 an acceptable limit on Al concentration of 0.2 $mg.L^{-1}$ and the National Environmental
415 Council (CONAMA) in Brazil sets the maximum value of dissolved Al at 0.1 $mg.L^{-1}$.
416 Therefore, the concentration used in this study, similar to that observed in some rivers
417 in the State of São Paulo (CETESB, 2016), is five times higher than the EPA permitted
418 limit and can affect aquatic life. The quality of the water used was compliant with the
419 recommendations of the EPA and CONAMA.

420 The survival of the fish in this study was influenced by high temperature (30 ºC)
421 combined with the presence of Al in the water (96 h). It is possible that the lower
422 survival rate at 30 ºC with the addition of Al was due to the Al potentiation effect in
423 combination with higher temperature and acid pH. Under these conditions,
424 polymerization of Al in the gills increases leading to functional hypoxia, a fact
425 corroborated by the behavior of the fish in this group (seeking oxygen on the surface of
426 the water when oxygen was available in the water). These findings are corroborated by
427 Poleó and Muniz (1993), who reported that Al toxicity in *Salmo salar* depends not only
428 on pH but also on temperature, emphasizing the importance of these variables when
429 evaluating the effects of Al on fish.

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431 4.2. Bioconcentration of Al in the testes and semen

432 In this study, the bioconcentration capacity of Al in the testes was found to be
433 related to temperature and exposure time, corroborating the findings of Poleó and
434 Muniz (1993), who showed that Al toxicity increases at higher temperatures.
435 Furthermore, Al bioconcentration in the testes after 96 h exposure was higher than after
436 only 24 h exposure. Al was also detected in testes and semen of fish that were not
437 deliberately exposed to this metal, revealing that even at the low concentration (0.07
438 mg.L^{-1}) found in water supply, Al can bioaccumulate in the tissues of aquatic
439 organisms, depending on the temperature and pH.

440 Correia (2012) exposed *Astyanax bimaculatus* to 0.5 mg.L^{-1} Al and found that
441 Al bioconcentrated in the liver, spleen, muscle, kidneys, ovaries, gall bladder,
442 encephalon and gills, and impaired the fecundity of females. Vuorinen *et al.* (2003)
443 exposed *Coregonus albula* to Al and observed Al bioconcentration in the gills, as well
444 as impacts on physiological reproductive parameters (delays in oocyte development and
445 gonadal maturation). Kida *et al.* (2016), exposing males of *A. altiparanae* to the same
446 concentration used in this study, did not test for Al bioconcentration in the testes, but
447 did observe increased plasma levels of androgens, suggesting that Al also acts as an
448 endocrine disruptor in male fish. This alteration in androgen levels may influence
449 spermatogenesis, spermiogenesis and sperm quality (Schulz *et al.*, 2010), since Al was
450 found in the testes and semen in our study, which was conducted at the same
451 concentration. These results show the plasticity of fish under Al stress, responding by
452 reproducing as fast as possible.

453 In teleosts, the effects of Al on male reproductive organs/cells have not been
454 investigated to date. In the nineties, it was been shown that Al affects mammals by
455 inhibiting testosterone synthesis (*in vitro)* in Leydig cells (Laskey and Phelps, 1991).

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456 Exposure of mammals to Al has also been shown to reduce testosterone levels, trigger
457 histopathological changes in the testes, reduce sperm concentration, motility and
458 viability, and increase sperm abnormalities in Wistar rats (Cheraghi *et al.*, 2017). Wistar
459 rats fed for 60 days on a diet containing 1.5 $mg.kg^{-1}$ of Al (mimicking human exposure
460 to this metal) exhibited Al in germ cells, and even at low concentration Al was found to
461 impair spermatogenesis and sperm quality (Martinez *et al.*, 2017). The same authors
462 reported an increase in oxidative stress and inflammation, highlighting the toxic action
463 pathways of Al in the male reproductive system (Martinez *et al.*, 2017). In human
464 semen, Klein *et al.* (2014) found high concentrations of Al in French patients and
465 discussed the implications of these levels for spermatogenesis and sperm concentration.
466 In regard to the effects of temperature, the data show that the pattern of Al
467 bioconcentration in the testes and semen differs according to exposure time. Exposure
468 to Al for 24 h was not sufficient to trigger Al bioconcentration in the testes, even at
469 different temperatures, but after 96 h exposure at acid pH, higher temperature boosted
470 the bioconcentration of Al in the testes. In the semen, the effects were observable after
471 only 24 h, with higher levels of Al at 20 and 25 ºC compared to 30 ºC. However, after
472 96 h exposure the pattern changed and the fish exposed to Al at 30 ºC exhibited higher
473 levels of Al in their semen. We suggest that the bioconcentration of Al in the semen
474 occurred faster than in testes due to its fluid consistency and the spermatozoa renewal
475 rate during spermatogenesis. However, no studies have been carried out so far
476 comparing the concentration of metals in the testes and semen.
477
478 4.3. Comet assay
479 One of the tools used to verify the effects of a compound on tissue is the comet
480 assay, which is a standard method for determining *in vivo/in vitro* genotoxicity, and is

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481 sensitive, robust, versatile and powerful enough to evaluate damage caused by
482 clastogenic agents by measuring DNA strand breaks in animal and plant cells (Lapuente
483 *et al.*, 2015). The comet assay has therefore been used in several ecotoxicological
484 studies to measure reversible DNA damage caused by caffeine (Santos-Silva *et al.*,
485 2018), pesticides and hydrocarbons (Bianchi *et al.*, 2017) and trace metals (D'Costa *et*
486 *al.*, 2017). The degree of fragmentation detected herein by the comet assay suggests that
487 Al binds to the DNA-histone complex causing conformational chromatin changes, as
488 found in the human nervous system (Lukiw *et al.*, 1987), and may cause cell DNA
489 fragmentation. Using the comet assay, Al genotoxicity has been found in the
490 erythrocytes of *Prochilodus lineatus* (Galindo *et al.*, 2010) and *Cyprinus carpio*
491 (Gómez-Oliván *et al.*, 2017), and in the liver of *Rhamdia quelen* (Klingelfus *et al.*,
492 2015).

493 Furthermore, the degree of DNA fragmentation triggered by Al is temperature-
494 dependent, suggesting that the fragmentation score is increased by the denaturation
495 process induced by the potentiated effect of Al, as observed in the semen after exposure
496 for 96 h. In erythrocytes, DNA fragmentation response patterns at different
497 temperatures were similar at 24 and 96 h for both neutral and acid pH, and the DNA
498 fragmentation score increased concomitantly with temperature (30 ºC>25 ºC>20 °C).
499 However, the addition of Al modified this pattern, suggesting that at 24 h, 25 ^{o}C is the
500 optimal temperature, but exposing the fish to a lower (20 ºC) or higher (30 ^{0}C)
501 temperature increases DNA fragmentation. At a longer exposure time (96 h), the lowest
502 fragmentation score was recorded at 20 ºC. Hassan *et al.* (2017) studied the effects of
503 ambient temperature on Nile tilapia (*O. niloticus*), a species whose optimum
504 temperature is 25-28 ºC, and observed that the thermal stress induced by cold (14 ºC)
505 and heat (36 ºC) affected the expression of heat shock proteins (HSP70), oxidative

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506 stress genes (metallothioneins, glutathione s-transferases) and immune response genes,
507 in addition to inducing micronucleus formation and DNA fragmentation. Buschini *et al.*
508 (2003) suggest that temperature induces damage to mussel (*Dreissena polymorpha*)
509 DNA, modifying cell sensitivity to environmental pollutants under *in vitro* conditions.
510 Therefore, elucidating the role of temperature is essential in determining the potential
511 genotoxicity of environmental pollutants.

512 Irrespective of the presence of Al, our data show that an acid pH is able to
513 induce genotoxic damage in the semen and erythrocytes of *A. altiparanae*, but this
514 effect is potentiated by the addition of Al, regardless of exposure time. There is no data
515 on the propensity of an acid pH to cause genotoxic damage in teleost cells, but
516 alterations in reproductive hormone levels in *Acipenser gueldenstaedtii* (Zelennikov *et*
517 *al.*, 1999) and in *A. altiparanae* (Kida *et al.*, 2016) have been shown, as well as impacts
518 on larval development in *Carassius auratus gibelio* (Taghizadeh *et al.*, 2013). Thus,
519 even during a period of subacute exposure, acidic water triggers physiological changes
520 and can impact fish populations. Therefore, this study corroborates the mutagenic
521 impact of Al on several cells. DNA fragmentation in both erythrocytes and sperm cells,
522 together with the propensity of water temperature to affect the degree of DNA
523 fragmentation, suggest that the reproduction of this species may be affected by the
524 variables studied herein.

525

526 4.4. Micronucleus test

527 In addition to the comet assay, the micronucleus test and the presence of nuclear
528 abnormalities are widely used as *in vivo/in vitro* biomarkers in molecular epidemiology
529 and the investigation of cytogenetic damage. A micronucleus consists of acentric
530 fragments expelled from the main nucleus in the late stages of anaphase (Kirsch-

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531 Volders *et al.*, 2003). These fragments can be formed by two mechanisms: clastogenesis
532 (chromosomal breaks) and aneugenesis (dysfunction of the mitotic apparatus). Nuclear
533 erythrocyte abnormalities may be indicative of errors during cell division (Carrasco *et*
534 *al.*, 1990; Udroiu, 2006). It is important to combine the results of the comet assay and
535 micronucleus test with mutagenic tests, since they are sensitive, powerful, simple,
536 versatile and cost-effective (Araldi *et al.*, 2015). In this study, a low frequency of
537 nuclear abnormalities was observed, possibly due to the short exposure time. Nuclear
538 abnormalities in fish erythrocytes are more frequent when the fish are exposed for long
539 periods to genotoxic agents (Osman *et al.*, 2010; Vicari *et al.*, 2012). This fact can be
540 observed in the natural environment, where a high number of nuclear abnormalities are
541 observed as a result of the impacts of human activities, providing a bioindicator for
542 water quality and organism survival potential (Gutiérrez *et al.*, 2015; Viana *et al.*, 2017;
543 Hussain *et al.*, 2018). According to Alink *et al.* (2007), prolonged exposure to low
544 concentrations of genotoxic agents aggravates these effects on fish cells, and in other
545 aquatic organisms. In this study, the micronucleus test, a biomarker that evaluates
546 irreversible erythrocyte DNA damage, was not useful, suggesting that Al causes
547 reversible DNA damage (evaluated by the comet assay) to the erythrocytes and
548 spermatozoa of *A. altiparanae*. The data suggest that semen quality parameters, such as
549 semen physicochemical characteristics, morphology and sperm kinetics, should also be
550 investigated to predict impacts on the reproduction of these organisms.
551
552 **5. Conclusion**

553 Under the experimental conditions described herein, it can be stated that Al,
554 even at environmental concentrations, bioconcentrates in the testes after 96 hours of
555 exposure and this accumulation increases at higher temperature. In the semen,

556 accumulation occurs sooner, just after 24 h exposure. In conjunction with water
557 temperature and acid pH, this accumulation triggers cytotoxic and genotoxic effects,
558 causing reversible DNA damage to the erythrocytes and spermatozoa of *A. altiparanae*.
559

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572

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1 **Highlights**

2 *Increased temperature enhances the bioaccumulation of Al the testes after 96 h.

3 *A shorter exposure period (24 h) triggers Al bioaccumulation in the semen.

4 *Temperature and pH potentiate the genotoxic and cytotoxic effects of Al.

5 * The DNA damage caused by Al to the erythrocytes and spermatozoa is reversible.

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