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Aluminum, at an environmental concentration, associated with acidic pH and high water temperature, causes impairment of sperm quality in the freshwater teleost *Astyanax altiparanae* (Teleostei: Characidae)



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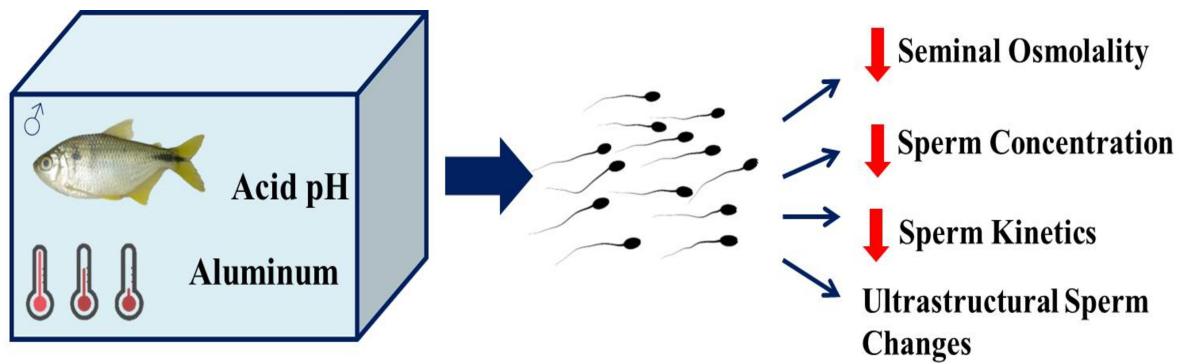
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1 **Aluminum, at an environmental concentration, associated with acidic**  
2 **pH and high water temperature, causes impairment of sperm quality**  
3 **in the freshwater teleost *Astyanax altiparanae* (Teleostei: Characidae)**

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19

20 **Abstract**

21 Given the toxicity of metals, including aluminum (Al), and the effects of water  
22 temperature on ectotherms, we investigated the individual or association effect of these  
23 variables (Al + acidic pH + temperature changes) on sperm quality of *Astyanax*  
24 *altiparanae*. Mature males were divided into nine experimental groups based on the  
25 combination of each of three water temperatures (20, 25, and 30 °C) with neutral and  
26 acidic pH values (7.0 and 5.5, respectively) with or without 0.5 mg L<sup>-1</sup> Al. The fish

27 were subjected to subacute, semi-static exposure and at 24 and 96 h were evaluated for  
28 seminal parameters: (1) pH; (2) osmolality; (3) sperm concentration; (4) sperm  
29 morphology; (5) sperm kinetics; and (6) sperm ultrastructure. At 30 °C, Al caused a  
30 reduction in osmolality (24 and 96 h) and sperm concentration (24 h). When analysing  
31 sperm kinetics (30 sec post-activation), Al caused a reduction in total motility at all  
32 temperatures (24 h), and when this exposure time was longer (96 h), both acidic pH and  
33 Al addition to the water caused sperm motility reduction. By analysing curvilinear  
34 velocity (VCL) 30 sec after sperm activation (24 and 96 h), the acidic pH caused a  
35 reduction in sperm movement at 20 and 30 °C, but at 25 °C Al triggered this reduction.  
36 Finally, Al in the water caused ultrastructural changes in the sperm head, midpiece, and  
37 flagella regardless of water temperature. Also, it was found that the combination of Al  
38 at 30 °C caused a reduction in sperm head area while at 20 °C, Al triggered a reduction  
39 in the midpiece area. Therefore, acidity influenced some *A. altiparanae* sperm  
40 parameters but Al in the water accentuated these effects on seminal quality, especially  
41 seminal osmolality and sperm concentration, kinetics, and ultrastructure. This toxicity  
42 was also influenced by changes in water temperature.

43 **Keywords:** Fish; Metal; Reproduction; Spermatozoa; Subacute exposure

44

45 Al negatively affects *A. altiparanae* sperm quality in a temperature-dependent manner.

46

## 47 **Introduction**

48 Aluminum (Al) is one of the most abundant elements on the Earth's crust;  
49 however, its bioavailability is limited due to low solubility at pH values between 6 and  
50 8. There are two major sources of Al in the aquatic environment: (1) indirect  
51 solubilization by the release of Al from rocks and soils (natural source) and (2) addition

52 of Al salts to freshwater by anthropogenic actions in order to decrease phosphate  
53 concentrations, reduce algal growth, or to clarify water through particulate precipitation.  
54 Additionally, as freshwater becomes progressively or episodically acidified by acid rain,  
55 Al bioavailability increases (Wilson, 2011).

56 In addition to pH, which acts on Al speciation, other environmental factors, such  
57 as temperature, may interfere with metal toxicity. The association of low temperatures  
58 with low pH values maximizes Al water solubility. High temperatures cause increase of  
59 animal metabolism, consequently promoting a higher respiratory rate and in turn,  
60 causing an increase in Al absorption by respiratory structures, such as gills, thus leading  
61 to death of aquatic animals (Poleo and Muniz, 1993; Wilson, 2011; Pinheiro *et al.*,  
62 2019). The United States Environmental Protection Agency (EPA) recommends an  
63 acceptable Al limit of  $0.2 \text{ mg L}^{-1}$  in the water, while the National Environmental  
64 Council (CONAMA) in Brazil sets the maximum dissolved Al value of  $0.1 \text{ mg L}^{-1}$ .  
65 However, it is possible to observe that the concentration found in many rivers exceeds  
66 these values, such as in the state of São Paulo, Brazil (e.g.  $0.1$  to  $1.0 \text{ mg L}^{-1}$  in the Mogi  
67 Guaçu River; CETESB, 2018).

68 Although Al has no apparent biological function in organisms (Nayak, 2002;  
69 Fernández-Dávila *et al.*, 2012), some studies have shown that Al can be found in  
70 different animal organs, such as brain (Mold *et al.*, 2018), liver, muscle, kidneys, gills,  
71 ovaries, and teleost testes (Correia, 2012; Pinheiro *et al.*, 2019), rat testes (Martinez *et*  
72 *al.*, 2017) and even in fluids from different animals, such as human (Klein *et al.*, 2014)  
73 and teleost semen samples (Pinheiro *et al.*, 2019). Furthermore, studies indicate that Al  
74 can interfere with several physiological processes, such as reproduction, by acting as an  
75 endocrine disruptor of the hypothalamic–pituitary–gonadal axis (Correia *et al.*, 2010;  
76 Correia, 2012; Kida *et al.*, 2016). Other studies with teleost have shown the capability

77 of different metals, such as mercury ([Hg] Dietrich et al., 2010; Hayati et al., 2019),  
78 cadmium ([Cd]; Dietrich et al., 2010), and copper ([Cu]; Bombardelli et al., 2016;  
79 Zbral et al., 2019) to negatively affect sperm quality by reducing motility rates,  
80 membrane integrity, normal morphology, mitochondrial functionality, DNA integrity,  
81 fertilization rates, and hatching.

82 The studies addressing the effects of Al in rats and humans have shown that this  
83 metal influences sperm concentration reduction, motility rates reduction, sperm  
84 abnormalities, and viability reduction (Klein et al., 2014; Cheraghi et al., 2017;  
85 Martinez et al., 2017); however, in teleost, this information is not available so far.  
86 However, Pinheiro et al. (2019) quantified Al in the semen of *Astyanax altiparanae* and  
87 observed that this metal bioaccumulates in this fluid and that there is an association of  
88 this bioaccumulation with temperature and acidic pH, thus triggering cytotoxic and  
89 genotoxic effects and generating reversible DNA damage in the sperm of this teleost  
90 species. This species has been used in several studies involving bioassays (Gomes et al.,  
91 2013; Vieira et al., 2013; Chehade et al., 2014; Bettim et al., 2016; Kida et al., 2016;  
92 Abdalla et al., 2019; Brambila-Souza et al., 2019; Pinheiro et al., 2019) due to its high  
93 plasticity and easy handling in the laboratory and thus, represents a good bioindicator  
94 for metal toxicity-related events.

95 Thus, in view of the above and after considering the current scenario of climate  
96 change, which includes the increase in temperature and anthropic action on water  
97 bodies, it was found that there are no available data about the effects of metals,  
98 including Al on the seminal quality of teleosts (*in vivo*) in association with  
99 environmental factors, such as temperature, during the reproductive period. Moreover,  
100 the reproductive capacity of animals is directly related to the environment in which they  
101 live and may be influenced by numerous physical and chemical factors in addition to

102 environmental pollutants. Therefore, given the abundance of Al on Earth, our main  
103 concern was the way in which this metal, at an environmental concentration (0.05 mg L<sup>-1</sup>)  
104 in combination with temperature changes (20, 25, and 30°C) in combination with  
105 acidic pH, which make do this metal soluble, can influence spermatic parameters in  
106 teleosts using *A. altiparanae* as a neotropical model. We hypothesized that Al would  
107 negatively affect *A. altiparanae* sperm quality in a temperature-dependent manner.  
108 Thus, the objective of this study was to evaluate the effects of subacute exposure of *A.*  
109 *altiparanae* males to Al at environmental concentrations in addition to the individual  
110 and/or synergistic actions of water temperature and acidic pH on the seminal quality of  
111 this species.

112

## 113 **Material and Methods**

### 114 *Animals*

115 Mature *A. altiparanae* males (n = 360, Lt = 8.40 ± 0.05 cm; Wt = 7.45 ± 0.16 g)  
116 were kindly donated by the *Companhia Energética de São Paulo* - CESP (Paraibuna -  
117 SP) and kept for seven days at the Ectothermic Facility in the Department of Physiology  
118 (IB/USP). The animals were divided into 18 glass aquariums (10 animals/aquarium; 132  
119 L water/aquarium), with water renewal every 24 h (90%) and daily feed *ad libitum* with  
120 extruded feed (32% crude protein). Furthermore, to avoid confounding factors due to  
121 faeces and other factors, the fish were deprived of food 24 h before the beginning of the  
122 experiment until the end of subacute exposure. The study was approved by the Animal  
123 Use Ethics Committee (CEUA) at IB/USP (265/2016; Process 16.1.417.41.3).

### 124 *Experimental Design*

125 Animal exposure (n= 360) to experimental treatments was carried out in two  
126 periods (each one started at the moment that temperature stabilization was achieved):

127 (1) 180 animals exposed to experimental conditions for 24 h and (2) 180 animals  
128 exposed to experimental conditions for 96 h.

129 Each exposure period consisted of nine experimental groups (duplicates), which  
130 were chosen based on previous ecotoxicological bioassays with *A. altiparanae* (Correia  
131 et al., 2010; Kida et al., 2016; Abdalla et al., 2019; Pinheiro et al., 2019) and the  
132 plasticity of the species studied in undergoing rapid physiological responses to stressors.  
133 The experimental groups consisted of combining each of the three temperatures (20, 25,  
134 and 30 °C) *versus* neutral pH (7.0), acidic pH (5.5), and acidic pH (5.5) with Al,  
135 resulting in nine experimental groups: (1) water at 20 °C, no Al, neutral pH; (2) water at  
136 20 °C, no Al, acidic pH; (3) water at 20 °C, with Al, acid pH; (4) water at 25 °C no Al,  
137 neutral pH (control group); (5) water at 25 °C, no Al, acidic pH; (6) water at 25 °C, with  
138 Al, acid pH; (7) water at 30 °C, no Al, neutral pH; (8) water at 30 °C, no Al, acidic pH;  
139 (9) water at 30 °C, with Al, acidic pH. Al was added to the water for groups T3, T6, and  
140 T9 at a concentration of 0.5 mg L<sup>-1</sup> water (Pinheiro et al., 2019). The Al group was  
141 carried out only in acid pH because of its bioavailability.

142 Temperature adjustment in each experimental group was conducted at a rate of 1  
143 °C h<sup>-1</sup> (Trueman et al., 2000). This Al concentration has been previously used in studies  
144 by our group (Correia et al., 2010; Kida et al., 2016; Abdalla et al., 2019; Pinheiro et al.,  
145 2019) and also represents actual contamination values of some basins in the state of São  
146 Paulo according to reports issued by the *Companhia Ambiental do Estado de São Paulo*  
147 (CETESB). Solutions were prepared with Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>.18H<sub>2</sub>O (Sigma Aldrich) and 65%  
148 HNO<sub>3</sub> (Suprapur, Merck; Pinheiro et al., 2019).

149 Aquarium water was filtered and analysed daily for physicochemical parameters  
150 (temperature, dissolved oxygen, and pH) with the aid of an oximeter (YSI 55) and pH  
151 meter (Gehaka). Al concentration was measured using inductively coupled plasma mass

152 spectrometry (ICP-MS) method and was within the expected range ( $0.36 \pm 0.09$  to  
153  $0.50 \pm 0.02$  mg L<sup>-1</sup>; Pinheiro et al., 2019).

154 *Seminal Collection*

155 Before each collection, the animals were induced with crude carp pituitary  
156 extract obtained commercially (Danúbio Aquacultura) to release sperm at a  
157 concentration of 5 mg kg<sup>-1</sup> of body mass. Since spermiation is faster at higher  
158 temperatures, the time of each injection was established according to the treatment  
159 temperature so that the values of accumulated thermal units (ATU) were between 260  
160 and 275 (13 h before collection for the animals kept at 20 °C; 11 h before collection for  
161 animals kept at 25 °C; 9 h before collection for animals kept at 30 °C). For seminal  
162 collection, the animals were sedated with eugenol-based solution (clove oil at 100 mg L<sup>-1</sup>  
163 ) in a 10 L aquarium until they presented loss of equilibrium.

164 After sedation, an animal's urogenital papilla was dried, and cranial-caudal  
165 abdominal massage was performed. The semen of each animal was collected with an  
166 automatic pipette, aliquoted in graduated polyethylene tubes, and kept in a polystyrene  
167 thermal box (4 °C). After these steps, samples contaminated with water, blood, faeces,  
168 and/or urine were discarded, and semen kinetics were immediately evaluated with an  
169 optical microscope to identify if the samples had been activated during collection. Also,  
170 semen samples that showed this activation, those activated with distilled water, and  
171 those that were immobile were discarded. Only the viable samples were kept for the  
172 seminal analyses described below.

173 The seminal volume was measured with graduated polyethylene tubes and  
174 automatic pipette. The volume of semen collected was  $31.24 \pm 2.78$  µL (24 h) and  $36.14$   
175  $\pm 3.19$  µL (96 h). The seminal pH was evaluated with pH reagent strips (Merck).

176 To measure the seminal osmolality aliquots of 20  $\mu$ L of semen from each animal  
177 were mixed with 30  $\mu$ L of distilled water in a graduated polystyrene tube and deposited  
178 on a digital osmometer (5004 MICRO-OSMETTE™ Automatic High Sensitivity -  
179 Precision Systems Inc.).

180 A 4  $\mu$ L semen sample from each animal was fixed in 400  $\mu$ L of formalized  
181 citrate solution for analysing sperm morphology. Ten microliters of this solution were  
182 then mixed with 3  $\mu$ L of Rose Bengal dye. From this mixture, 4  $\mu$ L were removed and  
183 dripped onto a glass slide (two slides per animal). After drying, 100 sperm cells per  
184 slide were analysed using an optical microscope according to the following criteria: (1)  
185 macrocephaly; (2) microcephaly; (3) normal tail; (4) curled tail; (5) folded tail; (6)  
186 corrugated tail; and (7) midpiece evaluation (adapted from Galo et al., 2011).

187 Semen samples from each animal were fixed in 4% formaldehyde citrate  
188 solution (4  $\mu$ L semen:4 mL fixative for a ratio of 1:1,000) in order to evaluate sperm  
189 concentration. From each diluted sample, 20  $\mu$ L were deposited on a Neubauer chamber  
190 and the number of sperm cells were counted under an optical microscope (400 x)  
191 (Pinheiro et al., 2016). The calculation of sperm concentration was based on a method  
192 by Wirtz and Steinmann (2006).

193 For sperm kinetics analysis, an aliquot of 1  $\mu$ L semen (in triplicate) was  
194 activated with 1000  $\mu$ L of distilled water (pH 6.9; 25°C; it was monitored in each  
195 motility evaluation and it was renewed when necessary) to evaluate motility duration,  
196 total motility (MOT), sperm velocities (curvilinear velocity [VCL], straight-line  
197 velocity [VSL], average path velocity [VAP]) and rectilinearity (STR). The images  
198 were obtained with a trinocular light microscope (BEL) coupled to a Basler camera  
199 (AcA640: 120 uc) and connected to a computer. The videos were captured with AVT  
200 Universal Package software at 100 fps (640 x 480 pixels) in \*.avi format, edited with

201 VirtualDub-1.9.0 software (virtualdub.org) and exported as \*.jpg image sequences. Thus  
202 100 images (1 sec) of 10 and 30 sec post-activation were edited by ImageJ (National  
203 Institutes of Health, USA, <http://rsbweb.nih.gov/ij/>) and analysed using the CASA  
204 plugin (University of California and Howard Hughes Medical Institute, USA,  
205 <http://rsbweb.nih.gov/ij/plugins/casa.html>). The videos were processed based on the  
206 description made for CASA free software (Wilson-Leedy and Ingemann, 2007) and  
207 adjusted settings according to Sanches et al. (2013) with minimum mobile speeds of  
208  $VCL = 15 \mu\text{m s}^{-1}$ ,  $VAP = 6 \mu\text{m s}^{-1}$  and  $VSL = 1 \mu\text{m s}^{-1}$ .

209 In order to carry out the analyses after semen collection, the animals were  
210 sacrificed through spinal cord section at the operculum level (Schreck and Moyle,  
211 1990). A ventral opening was performed to remove the testes, which were fixed in  
212 Karnovsky's solution (Karnovsky, 1965) for spermatozoa ultrastructural analysis. The  
213 animal samples were selected according to the results obtained by Pinheiro et al. (2019)  
214 regarding the absence of Al in the testes of the control groups for each temperature and  
215 the presence of Al in the gonads of males exposed to this metal after 96 h (n= 6 animals  
216 per treatment). This time was selected because after 24 h, there were no differences in  
217 Al concentration in the testes in any treatment described by these authors. Subsequently,  
218 after fixation of the samples, the testes were washed in phosphate buffer (0.1M; pH 7.3)  
219 and immersed in osmium tetroxide and 0.5% uranyl acetate. An increasing dehydration  
220 acetone series was used, the material was placed in the 1:1 mixture of 100% Araldite<sup>TM</sup>  
221 resin, and then immersed in pure resin. Finally, the ultrathin sections were stained with  
222 a saturated solution of uranyl acetate in 50% ethanol and lead citrate. The samples were  
223 processed at the *Centro de Microscopia Eletrônica* of the *Universidade Estadual Júlio*  
224 *de Mesquita Filho* (Botucatu Campus) and analysed under the EM900 Transmission

225 Electron Microscope Carl Zeiss (7,000 and 12,000 x) at the *Centro de Aquisição de*  
226 *Imagens e Microscopia* from the Institute of Biosciences (Caimi, IB/USP).

227 *Statistical analyses*

228 The data obtained were expressed as mean  $\pm$  standard error of the mean and  
229 subject to the Kolmogorov-Smirnov normality test and Spearman test for  
230 homoscedasticity testing. When necessary, data were normalized (log10). Comparisons  
231 between groups were made by the two-way analysis of variance (ANOVA) test  
232 (temperature and treatment as variables) followed by the Holm-Sidak post-test. In all  
233 cases, a significance level of 0.05 was considered statistically significant. Statistical  
234 analyses were performed using SigmaStat 3.5 for Windows software.

235

## 236 **Results and Discussion**

237 This is the first study that investigated the seminal quality of teleosts after  
238 exposure to Al and associated with physicochemical water factors. Sperm quantity  
239 (such as sperm volume and concentration) and quality (such as kinetics, seminal plasma  
240 pH, membrane composition and stability, and DNA integrity) can determine  
241 fertilization capacity and hence reproductive success (Fauvel et al., 2010). Some of  
242 these indicators, such as pH, osmolality, and seminal plasma composition, are specific  
243 biomarkers that directly influence sperm maturation and sperm capability to fertilize  
244 oocytes as sperm are immobile in the testes and seminal plasma (Kowalski and Cejko,  
245 2019).

246 *Seminal pH*

247 Seminal pH values generally vary from 6 to 9 (Alavi and Cossion, 2005; Sanches  
248 et al., 2011) between different species of teleosts. The seminal pH of *A. altiparanae* was  
249  $8.63 \pm 0.07$  in the control group (25 °C and neutral pH). After 24 h of exposure, within

250 each temperature setting, there was no difference in seminal pH considering the  
251 different experimental groups ( $P = 0.137$ ) and the same in neutral pH at different  
252 temperatures ( $P = 0.62$  – 20 °C *versus* 25 °C;  $P = 0.14$  – 25 °C *versus* 30 °C;  $P = 0.05$  –  
253 20 °C *versus* 30 °C). However, at acidic pH and acidic pH + Al, seminal pH decreased  
254 in animals kept at 30 °C (Fig. 1A). Thus, acid pH with or without the presence of Al, in  
255 the aquatic environment, did not influence seminal pH. However, the temperature  
256 variation interfered with the results in which a higher temperature yielded a lower pH at  
257 30 °C.

258 Despite this variation in semen pH, this indicator is still within the range that  
259 facilitates sperm mobility (slightly alkaline) when in contact with water for possible  
260 fertilization, and these differences found within 24 h of exposure along with other  
261 factors possibly influenced sperm kinetics among the different experimental treatments.  
262 After 96 h of exposure (Fig. 1B), seminal pH was not affected by either treatment or  
263 temperature. Probably, the exposure time allowed for a readjustment of the animals, and  
264 the seminal pH returned to the default value while maintaining its buffering capability.  
265 Although the influence of water quality on this sperm parameter has previously been  
266 recognized, studies evaluating the effect of teleost exposure to pollutants on seminal pH  
267 and seminal osmolality were not found.

#### 268 *Seminal Osmolality*

269 Osmolality is one of the main signals for the initiation of sperm motility in  
270 teleosts since sperm is immobile in the testes and activated when they come into contact  
271 with water (osmotic shock), which in freshwater teleosts, occurs at a low osmolality of  
272 up to 50 mOsmol kg<sup>-1</sup> (Cosson, 2004; Alavi and Cosson, 2006). Seminal osmolality  
273 varies among fish species, ranging from  $230 \pm 82$  to  $346 \pm 18.26$  mOsmol kg<sup>-1</sup> in  
274 cyprinids, from  $232 \pm 13$  to  $332 \pm 5.1$  mOsmol.kg<sup>-1</sup> in salmonids, and from  $38 \pm 3$  to

275  $93.6 \pm 7.3$  mOsmol.kg<sup>-1</sup> in acipenserids (Alavi and Cosson, 2006). The seminal  
276 osmolality of *A. altiparanae* was  $224.83 \pm 4.97$  mOsmol.kg<sup>-1</sup> (at neutral pH), which is  
277 within the range reported for teleosts. In the present study, after 24 h exposure (Fig.  
278 1C), animals maintained at 20 and 25 °C displayed a reduction in seminal osmolality  
279 when exposed to acidic pH and acidic pH with Al compared to neutral pH ( $P < 0.001$ ).  
280 Already at 30 °C, all groups differed from each other ( $P < 0.001$ ). The most significant  
281 reduction occurred when animals were exposed to acidic pH with Al. Besides, there was  
282 no significant difference ( $P = 0.101$ ) within each experimental group at different  
283 temperatures.

284 When the exposure period was prolonged (96 h), seminal osmolality (Fig. 1D)  
285 varied according to different pH values with a dependence on temperature; thus, there  
286 was an interaction between treatment and temperature ( $P \leq 0.001$ ). At 20 °C, there was a  
287 decrease in seminal osmolality in the animals maintained in acidic pH and acidic pH  
288 with Al compared to neutral pH ( $P < 0.001$ ), while at 25 °C, this decrease was only  
289 observed in the group exposed to acid pH and Al ( $P < 0.001$ ). Already at 30 °C, all  
290 groups differed from each other ( $P < 0.001$ ) since at 24 h, the sharpest reduction in the  
291 males exposed to pH acid with Al was noted. Additionally, in animals maintained in  
292 acidic pH, there was an increase in the seminal osmolality at 25 °C ( $P < 0.001$ ) and 30  
293 °C ( $P < 0.001$ ) compared to 20 °C. Thus, it can be emphasized that the association of  
294 higher temperatures (25 and 30 °C), acidic pH, and the presence of Al caused a  
295 significant reduction in seminal osmolality which consequently influences sperm  
296 kinetics as shown below. These alterations in seminal osmolality may have occurred  
297 because environmental pH and temperature impose changes on membrane permeability,  
298 enzymatic activity, and energy metabolism (Dadras et al., 2016). It was also described  
299 that heavy metals, such as mercury, can affect and block water channels or aquaporins,

300 which are responsible for osmotic regulation and activation of cell motility. Occlusion  
301 of water channels by heavy metals can block water transport across the plasma  
302 membrane, and therefore, osmotic rebalancing after osmotic shock does not occur. One  
303 side effect after this process is sperm swelling (for freshwater teleosts) that undoubtedly  
304 affects sperm movement (Preston et al., 1993; Kuwahara et al., 1997; Dietrich et al.,  
305 2010). Although Al is not a heavy metal, this process could explain the effects observed  
306 in seminal osmolality and sperm kinetics.

307 *Sperm Concentration*

308 In teleosts, sperm concentration varies among species according to reproductive  
309 stage and age, and between seasons, due to variations in photoperiod, temperature, and  
310 precipitation. Among abiotic factors, temperature is an important regulating factor in  
311 teleost life that modulates reproductive processes, gamete development, maturation,  
312 ovulation and spermiation, spawning, embryogenesis and hatching, and larval and  
313 juvenile development, in addition to survival (Pankhurst and Porter, 2003). With  
314 climate change, all of these processes will be or are already being affected, for example,  
315 interference with the hypothalamus-pituitary-gonads axis as low temperatures can  
316 inhibit and reduce steroid (such as testosterone) production, and high temperatures can  
317 also cause inhibitory effects such as protein conformational changes (such as follicle  
318 stimulation and luteinizing hormones [FSH and LH, respectively] receptors, and  
319 enzymes; Pankhurst and Munday, 2011). In the present study, when comparing the  
320 different experimental groups after 24 h of exposure to the solution with and without Al  
321 at the same temperature (Fig. 1E), it was found that at 25 °C the animals maintained at  
322 acidic pH with Al ( $2.72 \pm 0.27 \times 10^9$  sptz mL<sup>-1</sup>) had lower sperm concentration than the  
323 animals at neutral pH ( $4.17 \pm 0.16 \times 10^9$  sptz mL<sup>-1</sup>: control group;  $P = 0.002$ ). When  
324 analysing different temperatures within the same treatment, males at neutral and acidic

325 pH values presented higher sperm concentrations at 25 °C than at 20 and 30 °C, while in  
 326 animals maintained in acidic pH and Al, the sperm concentration was higher at 25  
 327 compared to 30 °C (P = 0.001).

328 The results clearly demonstrate that the extreme temperatures of the experiment  
 329 (20 and 30 °C) caused a reduction in the amount of sperm, suggesting once again that 25  
 330 °C seems to be the closest temperature to which this species is in homeostasis. Besides,  
 331 some anthropogenic factors, such as the presence of metals in water, can directly affect  
 332 spermatogenesis, sperm count, cause sperm DNA damage, and reduce sperm motility  
 333 (Rana, 2014; Jenardhanan et al., 2016). This fact was corroborated when *A. altiparanae*  
 334 at the accepted homeostatic temperature (25 °C) was exposed to acidic pH with Al and  
 335 caused a reduction in sperm quantity. This reduction in sperm count was also observed  
 336 by Cheraghi et al. (2017) in Wistar rats and by Yousef et al. (2005) in rabbits exposed to  
 337 Al. One effect of Al is a decrease in activities of various plasma membrane enzymes,  
 338 such as adenosine triphosphatase, alkaline phosphatase, and gamma-glutamyl  
 339 transferase in the testes, which impose indirect effects on spermatogenesis (Jenardhanan  
 340 et al., 2016; Kaizer et al., 2010). Abiotic factors can potentiate the effect of xenobiotics,  
 341 which are noticeable after 24 h of exposure when there was a reduction of more than  
 342 50% in sperm concentration at the higher temperature (30 °C). However, when the  
 343 exposure time was prolonged (96 h, Fig. 1F), there was a recovery in this parameter,  
 344 suggesting plasticity in this species in readjusting to adverse conditions.

345 *Sperm Morphology*

346 Another variable used to evaluate seminal quality and which directly influences  
 347 the fertilization rate is sperm morphology. In the present study, sperm were classified as  
 348 normal or abnormal with the presence of the following tail anomalies: (1) curled (a part  
 349 of the tail is above itself); (2) folded (a part of the tail shows curvature to one of the

350 sides); and (3) corrugated (the tail has wrinkles in its structure). When males were  
351 exposed to different experimental treatments for 24 h, there was no effect on sperm  
352 morphology (Table 1). Some studies demonstrate that xenobiotic compounds, such as  
353 metals, are capable of generating sperm pathologies that consequently affect the  
354 fertilization potential of gametes. Among these studies, we can highlight the one by  
355 Vergilio et al. (2015) in which alterations in the sperm head of carapó (*Gymnotus*  
356 *carapo*) exposed to cadmium chloride ( $CdCl_2$ ) and also sperm morphopathologies in  
357 rabbits (Yousef et al., 2005) and Wistar rats exposed to Cd (Cheraghi et al., 2017) were  
358 found.

359 In addition to xenobiotics, environmental factors, such as temperature, may also  
360 influence in the occurrence of anomalies in sperm as changes in these abiotic factors  
361 alter membrane permeability and enzyme activity in addition to modifying membrane  
362 proteins (Dadras et al. al., 2016). In *A. altiparanae* exposed to 30 °C for 96 h at neutral  
363 pH, there was a decrease in the percentage of morphologically normal sperm compared  
364 to those exposed to 20 and 25 °C ( $P = 0.002$  and  $P = 0.004$ , respectively) as shown in  
365 Table 1, suggesting that the increase in temperature caused protein denaturation that led  
366 to pathologies in the sperm tail. Also, an interaction of the variables on the sperm  
367 morphology after 96 h of exposure ( $P = 0.045$ ) was observed.

368 *Sperm kinetics*

369 Sperm kinetics is an important parameter for assessing seminal quality since  
370 sperm motility and velocities are directly related to fertilization rate (Rurangwa et al.,  
371 2004; Gage et al., 2004). Previous studies have shown that sperm kinetics may be  
372 influenced by physicochemical characteristics of the environment, such as temperature,  
373 (Dadras et al., 2016) and the presence of pollutants, such as copper (Zebral et al., 2019)  
374 in the water. In the present study, after 24 h exposure (Fig. 2A and 2B), when

375 considering the same treatment between different temperatures, there was a reduction in  
376 the motility in the sperm of animals in acidic pH with Al at 30 °C compared to 25 °C (P  
377 = 0.006). Also, by analysing the different groups within the same temperature, it was  
378 observed that in acid pH with Al at 20 °C and 30 °C there was decrease in sperm  
379 motility (10 sec after activation) compared to the animals in neutral and acidic pH (P =  
380 0.004; P = 0.006; P < 0.001; P < 0.001; Fig. 2A). In the group exposed 30 sec after  
381 sperm activation, an interaction between temperature and treatment (P = 0.002) on  
382 sperm motility was observed. When analysing the same treatment between different  
383 temperatures (30 sec), it was found that at the highest temperature, the sperm motility  
384 remained higher regardless of pH and the presence and/or absence of Al. When  
385 comparing the experimental treatments within the same temperature, at 20 °C, sperm  
386 motility was reduced when the animals were exposed to acidic pH (30.53% ± 4.59%; P  
387 < 0.001) and acidic pH with Al (19.16% ± 3.26%, P < 0.001) compared to neutral pH  
388 (56.20% ± 4.42%). Already at both 25 and 30 °C, sperm motility was reduced by more  
389 than 20% only when Al was added.

390 After 96 h of exposure (Fig. 2C and D), the same trend observed at 24 h was  
391 observed in sperm motility after 10 sec of activation. When comparing the same  
392 treatment between different temperatures, animals maintained in the acid pH group with  
393 Al presented lower sperm motility at 30 °C than at 20 and 25 °C. After comparing the  
394 different treatments at the same temperature, males at 30 °C and acidic pH with Al  
395 presented the lowest sperm motility (77.28% ± 3.37%). After 30 sec of activation (Fig.  
396 2D), in the same treatment at different temperatures, at neutral pH, sperm motility was  
397 higher at 25 °C (P < 0.001) and 30 °C (P < 0.001) compared to 20 °C. At acidic pH,  
398 sperm motility was also higher at 25 °C and reduced at 30 °C (P < 0.001) and 20 °C (P <  
399 0.001). At acidic pH with Al, the percentage of mobile sperm was higher at 25 and 30

400 °C ( $P < 0.001$  in both cases) than at 20 °C. After comparing the different groups within  
401 the same temperature, the same pattern was observed at 20, 25, and 30 °C: neutral pH >  
402 acidic pH > acidic pH + Al. High water temperature caused a reduction in the duration  
403 of sperm motility; however, this reduction was compensated for by a higher swimming  
404 speed compared to sperm activated at low temperatures and longer motility duration  
405 (Fig. 2D). This decrease in motility duration may have been due to limited energy  
406 resources and/or the effect of temperature on metabolic processes (Dadras et al., 2016).

407 Adriaenssens et al. (2012) studied long-term exposure (five weeks) of  
408 mosquitofish males (*Gambusia holbrooki*) at different temperatures (cold acclimation:  
409 18 °C and warm acclimation: 30 °C) and observed that the higher temperature favoured  
410 the increase of sperm motility, a finding that was corroborated in the present study, both  
411 at 24 and 96 h of exposure, implying that future climate changes could have an impact  
412 on species reproduction.

413 Besides temperature, the presence of Al in the water negatively interfered with  
414 sperm motility. At both exposure times and throughout the sperm motility period (10  
415 and 30 sec post-activation analyses), Al triggered a decrease in sperm motility of > 30%  
416 over conditions of neutral pH without this metal. Also, other studies with rats  
417 demonstrated this reduction in sperm motility when exposed to Al (Cheraghi et al.,  
418 2017; Martinez et al., 2017). Martinez et al. (2017) observed that this functional  
419 impairment appears along with a redox imbalance and with an increase in production of  
420 reactive oxygen species, lipid peroxidation, and altered antioxidant capacity in  
421 reproductive organs. Also, suppression of spermatogenesis and sperm impairments in  
422 addition to histopathological changes could be partially attributed to polyunsaturated  
423 fatty acid peroxidation in the sperm membrane (Martinez et al., 2017).

424 In addition to motility, the study of sperm velocities is of paramount importance  
425 for the evaluation of semen quality as some studies have shown a strong correlation of  
426 these variables, especially VCL, with fertilization rate (Viveiros et al., 2010; Gallego et  
427 al., 2017). When *A. altiparanae* males were exposed to experimental treatments for 24 h  
428 (Fig. 3A), in VCL after 10 sec of sperm activation, when comparing the same treatment  
429 between the different temperatures, no influence of temperature in each group ( $P =$   
430 0.958) was seen. However, when analysing the different treatments within the same  
431 temperature group, it was possible to observe that animals exposed to acidic pH + Al  
432 presented lower VCL for all temperatures. Moreover, after 30 sec of sperm activation,  
433 there was an interaction between treatment and temperature ( $P = 0.047$ ) in VCL. When  
434 comparing the same experimental group between different temperatures and neutral pH  
435 it was observed that as the temperature increased so did the VCL ( $20\text{ }^{\circ}\text{C} < 25\text{ }^{\circ}\text{C} < 30$   
436  $^{\circ}\text{C}$ ). Already at acidic pH at  $20\text{ }^{\circ}\text{C}$  and acid pH + Al at  $20$  and  $25\text{ }^{\circ}\text{C}$ , the lowest VCL  
437 values were observed. Besides, when the different treatments are compared at the same  
438 temperature, acidic pH + Al produced lower VCL values at all temperatures.

439 After 96 h of animal exposure (Fig. 3B), no differences ( $P = 0.062$ ) were found  
440 after comparing the same treatment between the different temperatures in VCL after 10  
441 sec of activation of male gametes. Regarding the 30 sec post-activation, with the same  
442 treatment, animals maintained at acidic pH at extreme temperatures of the study ( $20\text{ }^{\circ}\text{C}$   
443 and  $30\text{ }^{\circ}\text{C}$ ) presented lower VCL values ( $P < 0.001$  and  $P = 0.03$ , respectively).  
444 Similarly at 24 h exposure at 30 sec post-activation, acidic pH + Al demonstrated lower  
445 VCL values at all temperatures.

446 Some metals, such as Hg, can bind to flagellar proteins and affect sperm kinetics  
447 or enzymes and consequently sperm metabolism (such as inhibition of protein activity,  
448 denaturation, or conformational protein changes). Consequently, the structure of sperm

449 flagella may be altered, and the sliding process of the dynein-driven microtubules may  
450 be impaired (Dietrich et al., 2010). This fact can suggest that Al can attach to the sperm  
451 and reduce both motility and VCL at both exposure times.

452 VAP is another sperm parameter that was investigated in the present study. After  
453 24 h of exposure (Fig. 4A) at 10 sec and 20 °C, sperm from animals maintained at acidic  
454 pH + Al, presented the lowest VAP value. After analysing the different treatments at the  
455 same temperature at 30 sec post-activation, it was possible to verify that Al caused a  
456 reduction in the VAP values compared to the other groups at 25 °C ( $P = 0.002$ ;  $P =$   
457 0.016).

458 After 96 h (Fig. 4B), when comparing different treatments within the same  
459 temperature, animals at acidic pH + Al presented the lowest VAP values at 25 °C at 10  
460 sec post-activation. At 30 sec post-activation, animals at acidic pH presented lower VAP  
461 at the extreme study temperatures ( $P < 0.001$  and  $P = 0.035$ , respectively). Within each  
462 temperature group, 20 °C and acidity triggered a decrease in VAP, but at 25 °C, only the  
463 presence of Al caused this reduction (similar to 24 h).

464 Regarding VSL, after 24 h of exposure (Fig. 5A) was observed that the presence  
465 of Al at 20 °C caused a reduction in this velocity (neutral and acidic pH values;  $P = 0.01$   
466 and  $P = 0.005$ , respectively). Already after 30 sec of sperm activation, for the same  
467 treatment at different temperatures and both at neutral and acidic pH values, a decrease  
468 in VSL at 20 °C compared to 25 and 30 °C was seen. However, at acidic pH + Al, the  
469 lowest values were observed at 20 and 25 °C.

470 After 96 h (Fig. 5B), at 30 sec post-activation, analysing the same group at  
471 different temperatures at acidic pH values, it was possible to observe a lower VSL value  
472 at 20 °C. Also, when comparing the different groups at the same temperature, it was  
473 verified that acidity was responsible for the reduction in VSL at 20 °C. However, at 25

474     °C, this decrease occurred only due to the presence of Al. This decrease was also found  
475     in sperm from *Danio rerio* (Acosta et al., 2016) and *Salmo trutta* (Kowalska-Górska  
476     et al., 2019), which had been exposed to Cd and Cu and presented reductions in VCL,  
477     VAP, and VSL.

478           Regarding STR, at 24 h of exposure (Fig. 6A) and 30 sec after sperm activation,  
479     interaction between treatment and temperature ( $P = 0.024$ ) on this sperm parameter was  
480     observed. Moreover, at both times, it was found that animals exposed to neutral pH  
481     presented lower STR at 30 °C. After 96 h (Fig. 6B) and at 10 and 30 sec post-activation,  
482     temperature and treatment variables did not interact with STR ( $P = 0.207$  and  $P = 0.420$ ,  
483     respectively).

484     *Sperm Ultrastructure*

485           Another important parameter to evaluate in order to understand the action of a  
486     pollutant on gametes is cell ultrastructure since it may be possible to associate changes  
487     in morphological characteristics with the functions/mechanisms performed by each  
488     structure. As stated previously, for this analysis, the exposure time of 96 h was selected,  
489     because after 24 h, Al does not concentrate in *A. altiparanae* testes (Pinheiro et al.,  
490     2019). *A. altiparanae* sperm consists of a spherical nucleus containing granular  
491     chromatin with a mean diameter of  $1.73 \pm 0.02 \mu\text{m}$ , surrounded by a plasma membrane,  
492     totalling a nuclear area of  $0.49 \pm 0.01 \mu\text{m}^2$ . Below the nucleus and involving the  
493     insertion of the flagella, the midpiece is located with a mean diameter (measured above  
494     the insertion of the flagellum and the cytoplasmic canal) of  $1.58 \pm 0.04 \mu\text{m}$  and an area  
495     of  $0.28 \pm 0.01 \mu\text{m}^2$ , which is composed of mitochondria unevenly arranged throughout  
496     the region.

497           A qualitative analysis (Fig. 7) showed that the males that underwent treatment  
498     without Al regardless of temperature had similar sperm ultrastructural characteristics.

499 However, when Al was added and animals were exposed to different temperatures, a  
500 change in the ultrastructure was observed with the most pronounced changes at 30 °C. It  
501 was observed that Al favoured the disruption of the sperm nuclear membrane (Fig. 7B  
502 and 7D), conformational changes of chromatin (Fig. 7B and 7C), the clutter of the  
503 midpiece (Fig. 7C, 7D and 7F), presence of greater number of vesicles/vacuoles in the  
504 midpiece (Fig. 7E), and damage to the structure of the flagella (Fig. 7E).

505 It was possible to observe the qualitative effects of isolated temperature in  
506 addition to the interaction of this physical parameter with the presence of Al in the  
507 testes after 96 h (Table 2). The influence of the interaction between the temperature and  
508 the presence/absence of the metal in the diameter and the nuclear area ( $P = 0.002$  and  $P$   
509  $= 0.037$ , respectively). Thus, in the absence of Al, the animals maintained at 30 °C  
510 presented sperm head with the largest nuclear diameter and area compared to the other  
511 temperatures. Also, within each temperature group, the presence of Al caused a  
512 reduction in nuclear diameter and area of sperm head at 30 °C ( $P=0.002$  and  $P=0.036$ ,  
513 respectively). Regarding the midpiece temperature alone did not influence the diameter  
514 and area of the midpiece; however, when associated with Al the smallest diameter and  
515 smallest area of the midpiece were found when the animals were exposed to 20 °C.

516 Morphological changes were observed in sperm when animals or gametes were  
517 exposed to certain pollutants, such as different metals in rabbits (Castellini et al., 2009),  
518 Cd in sea urchins and mussels (Au et al., 2000), mercury in fish (Hatef et al., 2011), and  
519 insecticides in fish (Xu et al., 2005) and mammals (Sánchez et al, 2017). However, no  
520 studies of sperm ultrastructure under the influence of Al have been evaluated so far. The  
521 results suggest that Al favours nuclear membrane disruption and causes chromatin  
522 conformational changes, leading to higher DNA fragmentation scores (Pinheiro et al.,  
523 2019). Besides, Al modified the structure of the midpiece in addition to the

524 mitochondria inserted in it, which may have caused changes in enzymatic activities  
525 leading to reductions in sperm motility and VCL. Additionally, changes in head and the  
526 midpiece may affect fecundity at the micropile level. These changes could decrease  
527 fertilization and hatching rates and also influence the embryonic development pattern of  
528 *A. altiparanae*. With that, more studies are needed to clarify the way in which Al could  
529 enter the cell and whether it would affect generation and development of progenies.

530 **Conclusion**

531 Under the experimental conditions described in this study, acidity influences  
532 sperm parameters in *A. altiparanae*, but the presence of Al in the water at ambient  
533 concentrations accentuates the effects on seminal quality, especially sperm osmolality,  
534 concentration, kinetics, and ultrastructure. Also, this toxicity may be influenced by  
535 temperature. It is suggested that both water acidity and the non-optimal temperature,  
536 influence fertilization and hatching rates, which could trigger a reduction in *A.*  
537 *altiparanae* populations.

538

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550

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Table 1. Sperm morphology (% of normal) of *Astyanax altiparanae* after exposure (24 and 96 h) at different temperatures, pHs, and the presence or absence of aluminum (Al) shown as mean  $\pm$  standard error of the mean.

<b>Sperm Morphology - % Normal</b>			
<b>Treatment</b>	<b>24 h Exposure</b>		
	<b>20°C</b>	<b>25°C</b>	<b>30°C</b>
<b>Neutral pH</b>	99.92 $\pm$ 0.08	99.83 $\pm$ 0.17	99.92 $\pm$ 0.08
<b>Acid pH</b>	99.80 $\pm$ 0.11	100 $\pm$ 0	99.83 $\pm$ 0.11
<b>Acid pH + Al</b>	99.83 $\pm$ 0.11	99.58 $\pm$ 0.20	99.42 $\pm$ 0.30
<b>96 h Exposure</b>			
<b>Treatment</b>	<b>20°C</b>	<b>25°C</b>	<b>30°C</b>
	100 $\pm$ 0 <sup>A</sup>	99.92 $\pm$ 0.08 <sup>A</sup>	98.92 $\pm$ 0.35 <sup>B</sup>
<b>Neutral pH</b>	99.92 $\pm$ 0.08	99.33 $\pm$ 0.31	99.25 $\pm$ 0.11
<b>Acid pH</b>	99.67 $\pm$ 0.33	99.25 $\pm$ 0.31	99.67 $\pm$ 0.21

Uppercase letters indicate differences within the same treatment at different temperatures; n = 6; \*P <0.05

Table 2. Sperm Ultrastructure (head and midpiece diameters and areas) of male *A. altiparanae* after exposure (96 h) at different temperatures, pHs, and the presence or absence of Al shown as mean  $\pm$  standard error of the mean.

Sperm Ultrastructure			
Treatment	Head Diameter ( $\mu\text{m}$ )		
	20°C	25°C	30°C
<b>Neutral pH</b>	1.66 $\pm$ 0.02 <sup>B</sup>	1.73 $\pm$ 0.02 <sup>B</sup>	1.84 $\pm$ 0.02 <sup>Aa</sup>
<b>Acid pH + Al</b>	1.72 $\pm$ 0.02	1.74 $\pm$ 0.03	1.73 $\pm$ 0.03 <sup>b</sup>
Midpiece Diameter ( $\mu\text{m}$ )			
Treatment	20°C	25°C	30°C
	1.48 $\pm$ 0.04	1.58 $\pm$ 0.04	1.55 $\pm$ 0.03
<b>Neutral pH</b>	1.43 $\pm$ 0.03 <sup>B</sup>	1.57 $\pm$ 0.04 <sup>A</sup>	1.48 $\pm$ 0.04 <sup>AB</sup>
Head Area ( $\mu\text{m}^2$ )			
Treatment	20°C	25°C	30°C
	0.46 $\pm$ 0.01 <sup>B</sup>	0.49 $\pm$ 0.01 <sup>B</sup>	0.54 $\pm$ 0.02 <sup>Aa</sup>
<b>Neutral pH</b>	0.48 $\pm$ 0.01	0.52 $\pm$ 0.02	0.50 $\pm$ 0.01 <sup>b</sup>
Midpiece Area ( $\mu\text{m}^2$ )			
Treatment	20°C	25°C	30°C
	0.28 $\pm$ 0.01 <sup>a</sup>	0.28 $\pm$ 0.01	0.30 $\pm$ 0.01
<b>Neutral pH</b>	0.24 $\pm$ 0.02 <sup>Bb</sup>	0.27 $\pm$ 0.01 <sup>AB</sup>	0.30 $\pm$ 0.02 <sup>A</sup>
<b>Acid pH + Al</b>			

Uppercase letters indicate differences within the same treatment at different temperatures; lowercase letters indicate differences within the same temperature at different treatments. n = 6; \*P < 0.05

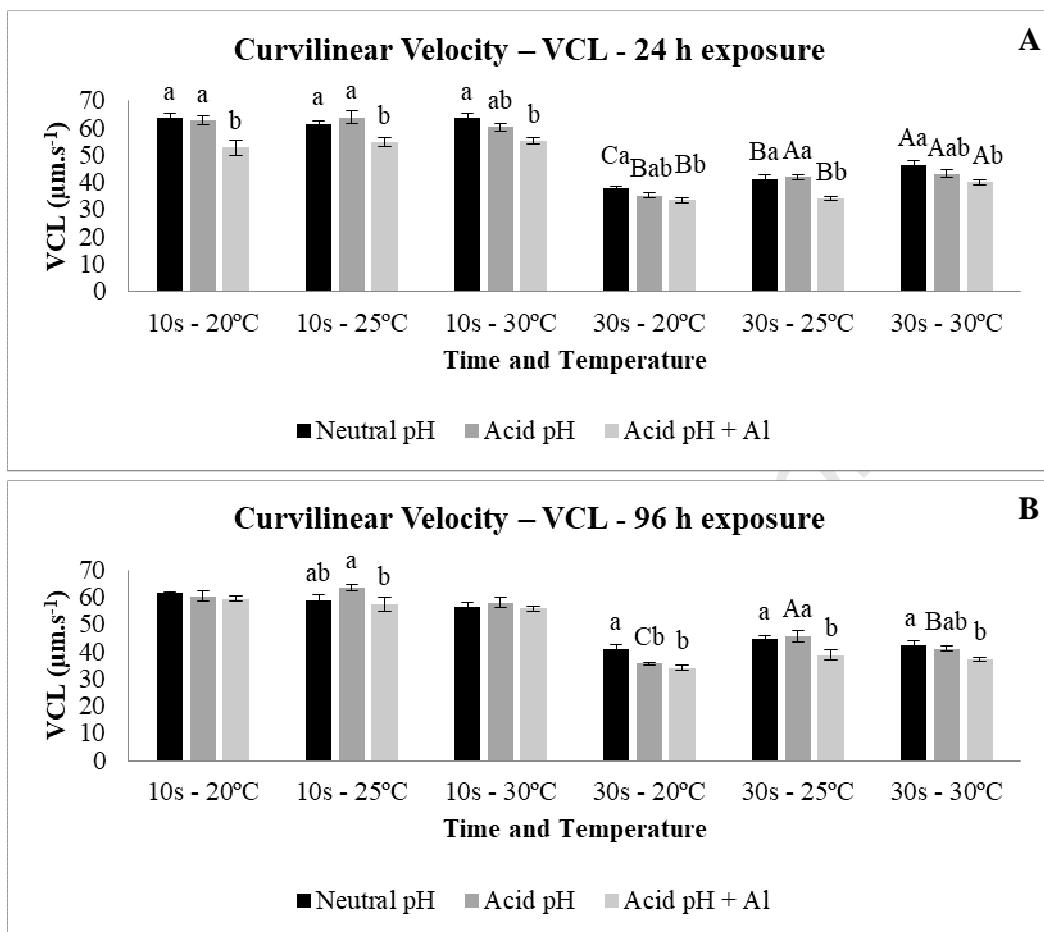


Fig. 3. Curvilinear velocity (VCL) of *A. altiparanae* sperm after 24 h (A) and 96 h (B) exposure at different temperatures, pHs, and the presence or absence of Al. Uppercase letters indicate differences within the same treatment at different temperatures; Lowercase letters indicate differences within the same temperature under different treatments. n = 6; \*P < 0.05

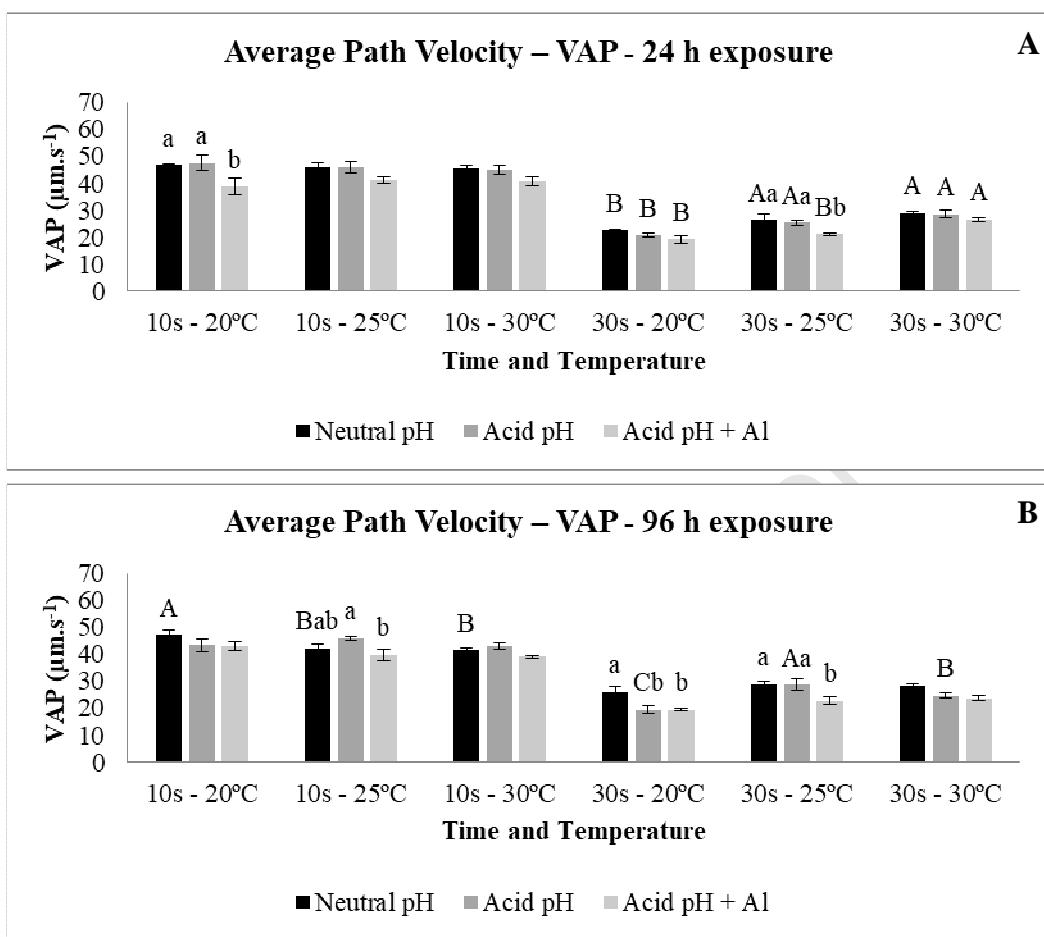


Fig. 4. Average path velocity (VAP) of *A. altiparanae* sperm after 24 h (A) and 96 h (B) exposure at different temperatures, pHs, and the presence or absence of Al. Uppercase letters indicate differences within the same treatment at different temperatures; lowercase letters indicate differences within the same temperature under different treatments. n = 6; \*P < 0.05

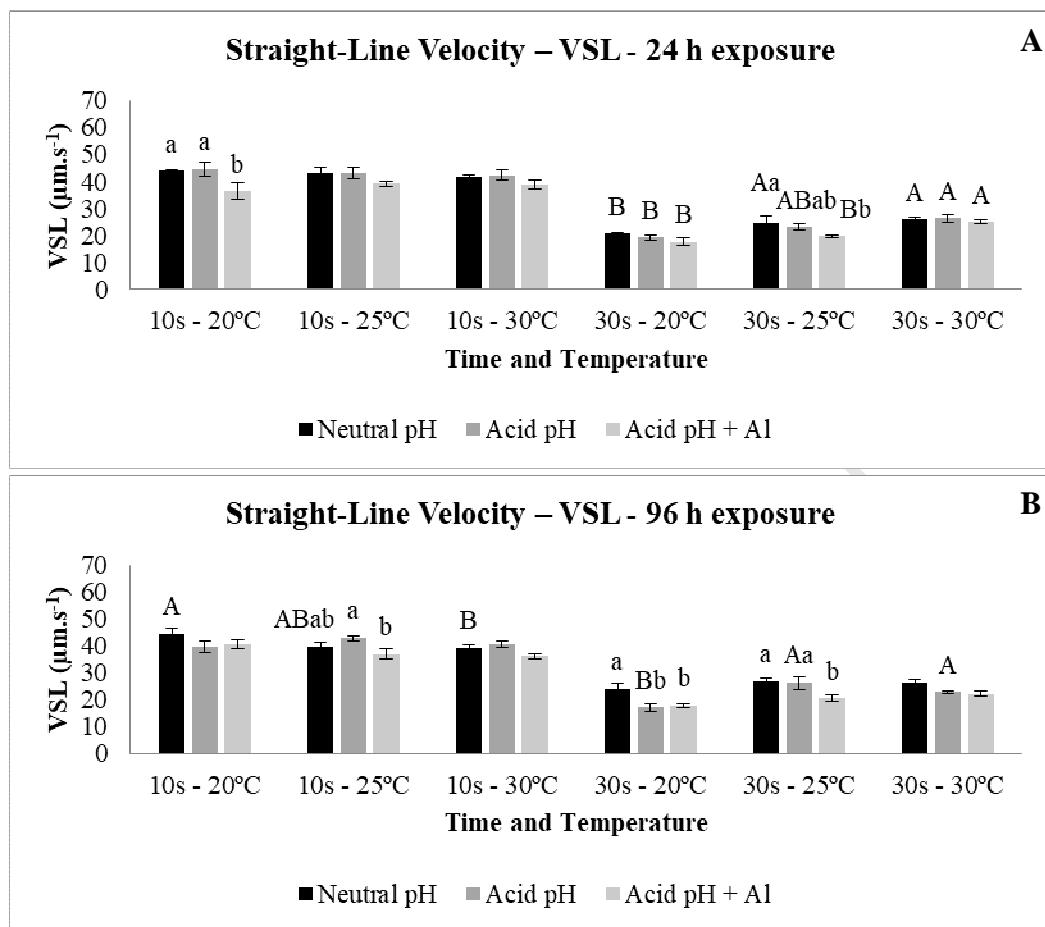


Fig. 5. Straight line velocities (VSL) of *A. altiparanae* sperm after 24 h (A) and 96 h (B) exposure at different temperatures, pHs, and the presence or absence of Al. Uppercase letters indicate differences within the same treatment at different temperatures; lowercase letters indicate differences within the same temperature under different treatments.  $n = 6$ ;  $*P < 0.05$

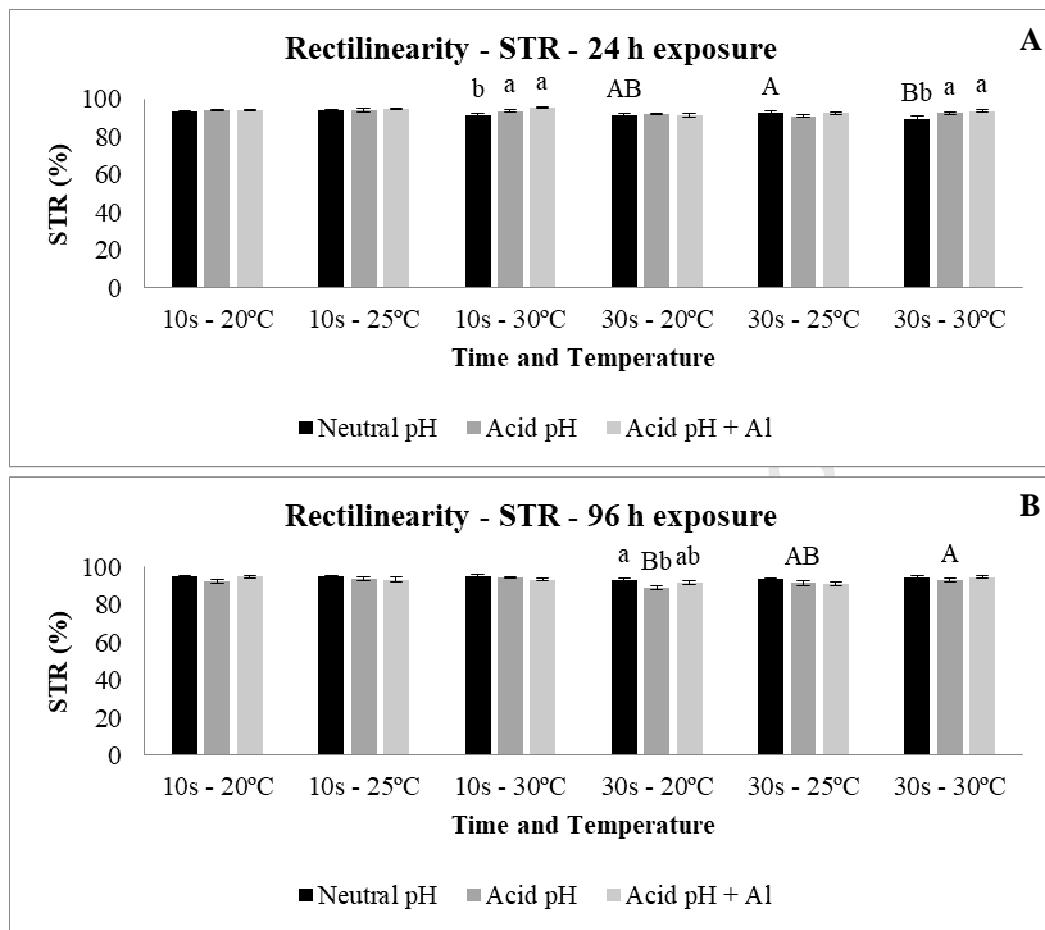


Fig. 6. Rectilinearity (STR) of *A. altiparanae* sperm after 24 h (A) and 96 h (B) exposure at different temperatures, pHs, and the presence or absence of Al. Uppercase letters indicate differences within the same treatment at different temperatures; lowercase letters indicate differences within the same temperature under different treatments. n = 6; \*P < 0.05

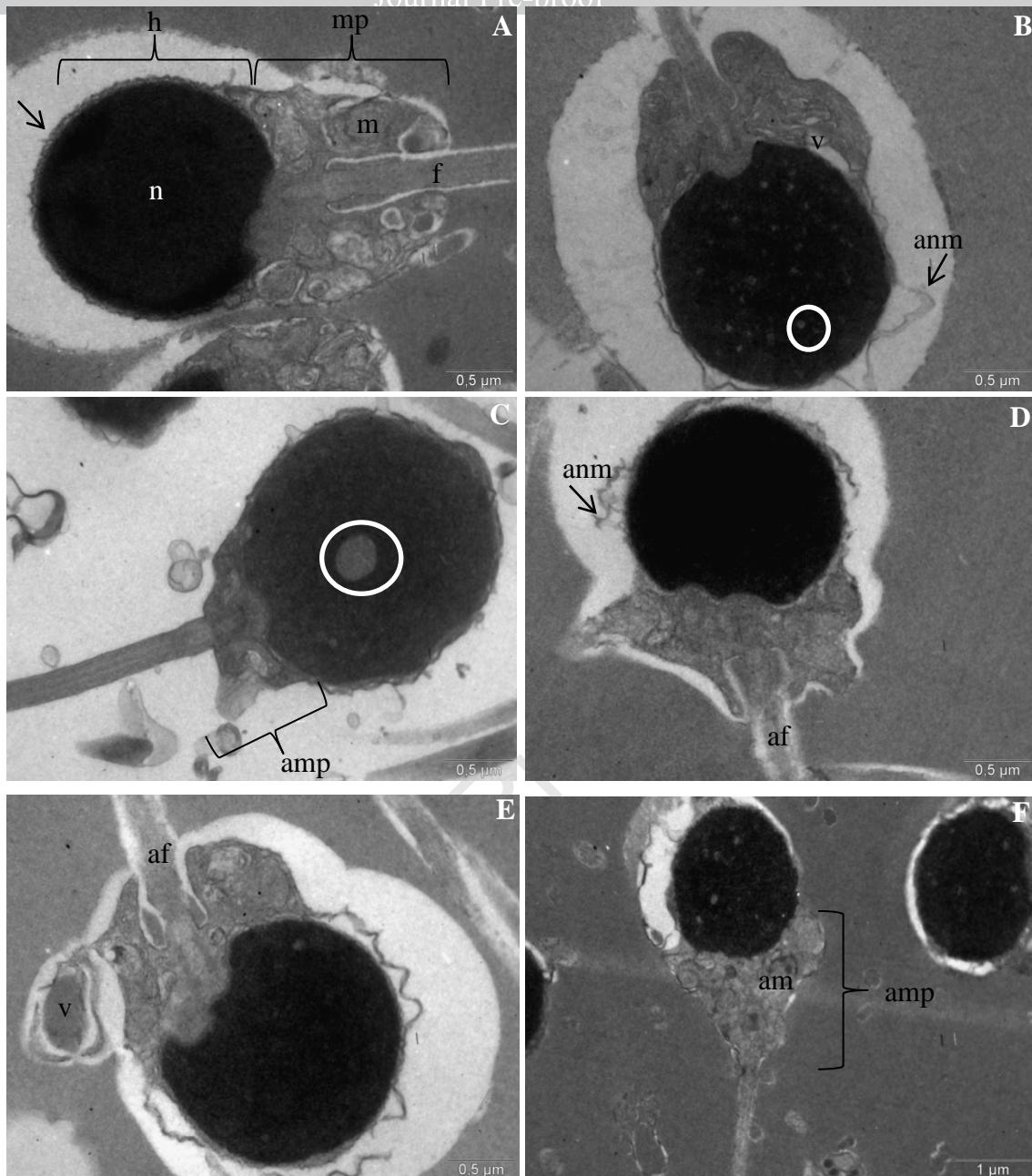


Fig. 7. Sperm ultrastructure of *A. altiparanae* after exposure to different temperatures and the presence or absence of Al. A. Normal spermatozoa (12.000 x; arrow: nuclear membrane; n: nucleus; h: head; mp: midpiece; m: mitochondria; f: flagellum; treatment: 25°C, and neutral pH). B–F. Abnormal spermatozoa (B–E: 12.000 x; F–7.000 x; anm: abnormal nuclear membrane; v: vesicle; circle: electro lucid areas; amp: abnormal midpiece; af: abnormal flagellum; am: abnormal mitochondria; B–D: treatment 20°C, acid pH, and Al; E–F: treatment 30°C, acid pH, and Al).

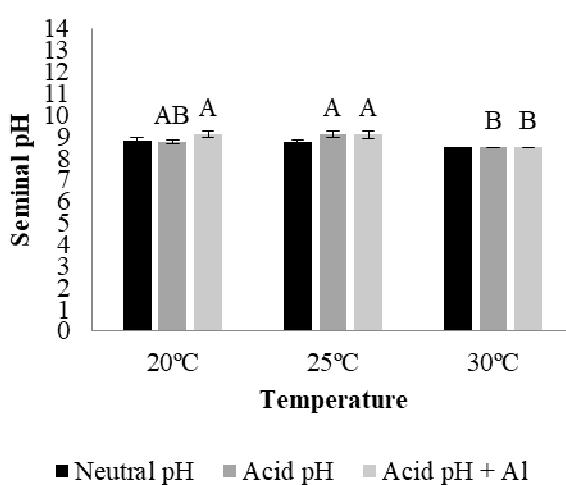
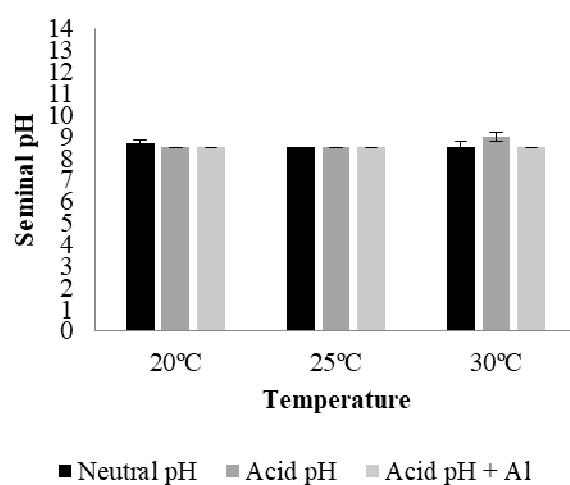
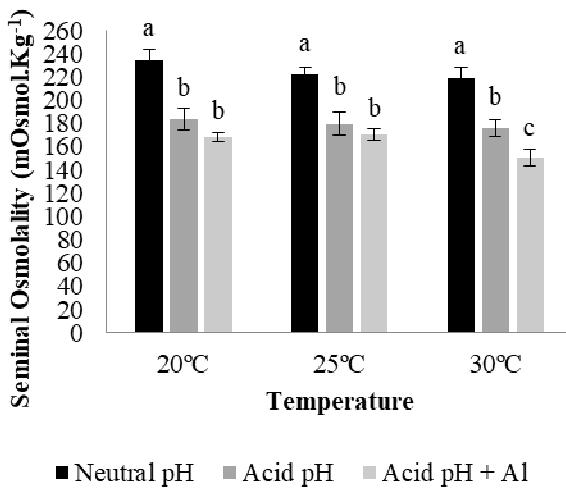
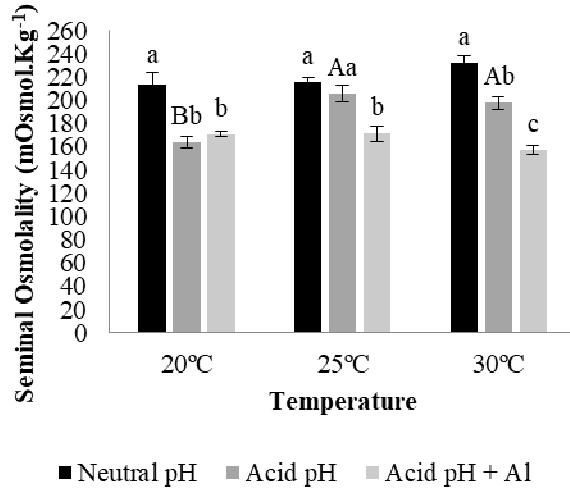
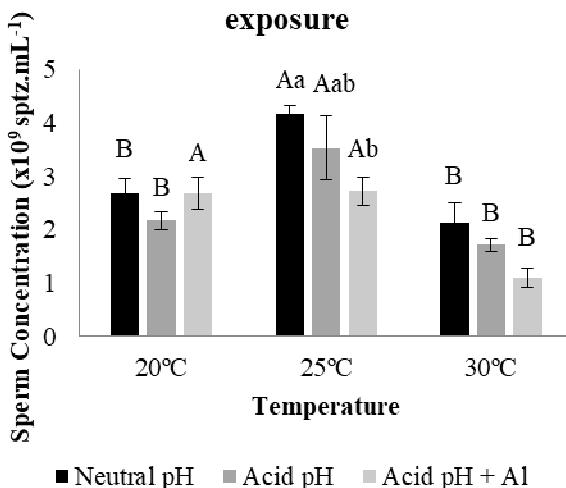
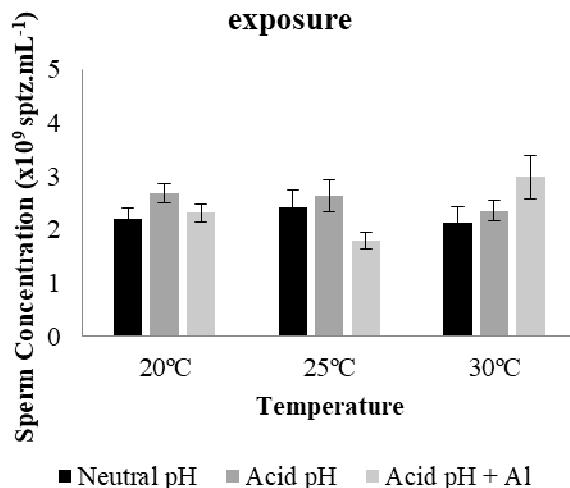
**Seminal pH - 24 h exposure****A****Seminal pH - 96 h exposure****B****Seminal Osmolality - 24h exposure****C****Seminal Osmolality - 96h exposure****D****Sperm Concentration - 24h exposure****E****Sperm Concentration - 96h exposure****F**

Fig. 1. Physicochemical characteristics of *Astyanax altiparanae* semen after exposure to different temperatures, pH values, and presence or absence of aluminum (mean  $\pm$  standard error of the mean). A. Seminal pH (exposure for 24 h); B. Seminal pH (exposure for 96 h); C. Seminal Osmolality (exposure for 24 h); D. Seminal Osmolality (exposure for 96 h); E. Sperm Concentration (exposure for 24 h); F. Sperm Concentration (exposure for 96 h). Uppercase letters indicate differences within the same treatment at different temperatures; Lowercase letters indicate differences within the same temperature in different treatment. n = 6/group; \*P < 0.05.

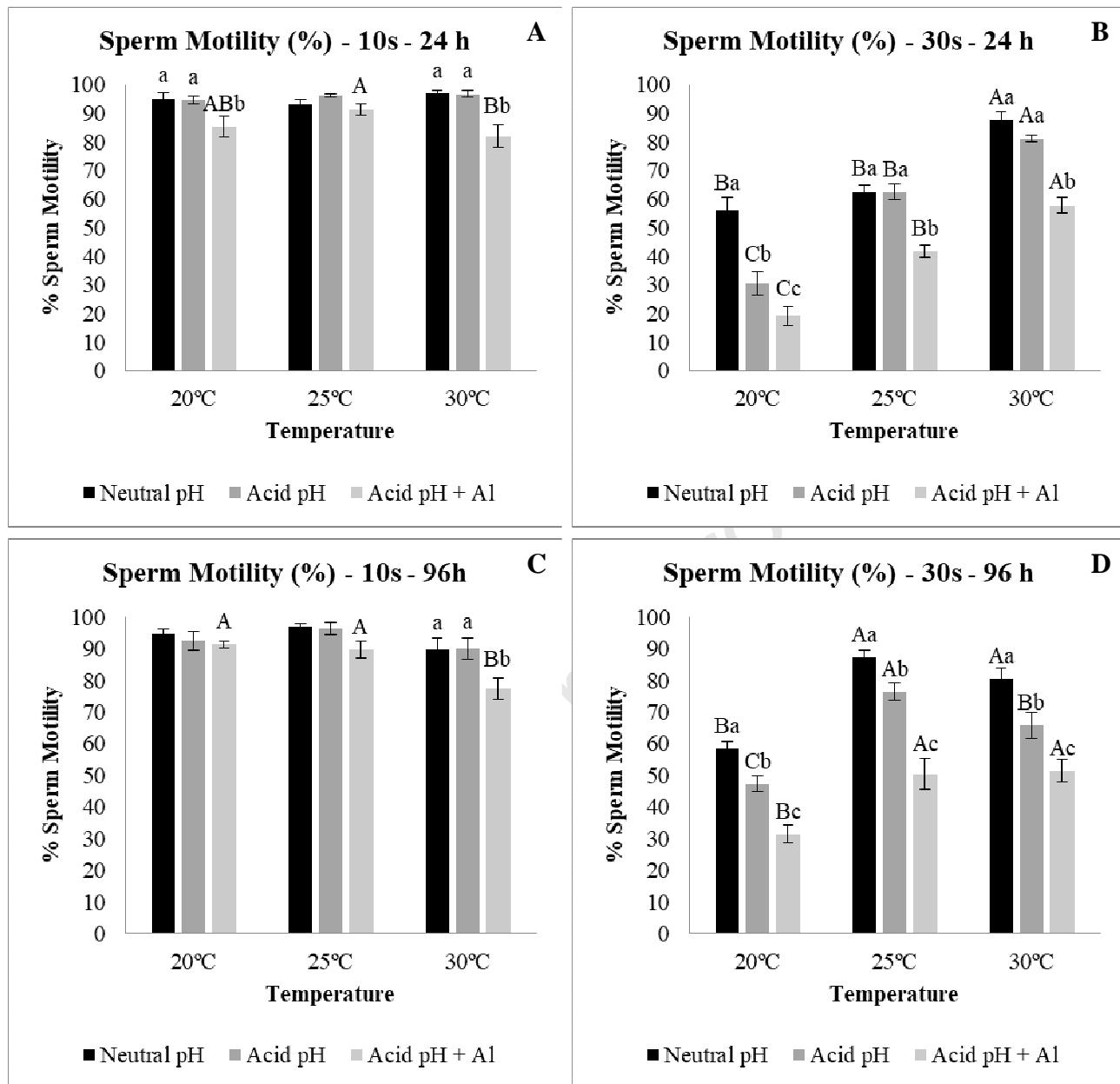


Fig. 2. Sperm motility (%) of male *A. altiparanae* after exposure at different temperatures, pHs and the presence or absence of Al. A. Sperm motility after 10 sec post-activation (animal exposure for 24 h); B. Sperm motility after 30 sec post-activation (animal exposure for 24 h); C. Sperm motility after 10 sec post-activation (animal exposure for 96 h); D. Sperm motility after 30 sec post-activation (animal exposure for 96 h). Uppercase letters indicate difference within the same treatment at different temperatures; Lowercase letters indicate differences within the same temperature in different treatments (n = 6; \*P <0.05).

1 **Highlights**

2 - Al at high water temperature reduces seminal osmolality at 24 h and 96 h.

3 - Al and a high water temperature reduce sperm concentration after 24 h.

4 - Acidic water induces changes in sperm kinetics after 24 h and 96 h.

5 - Al triggers reduction in sperm motility and curvilinear speed after 24 h and 96 h.

6 - Al generates ultrastructural changes in sperm after 96h.

7

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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