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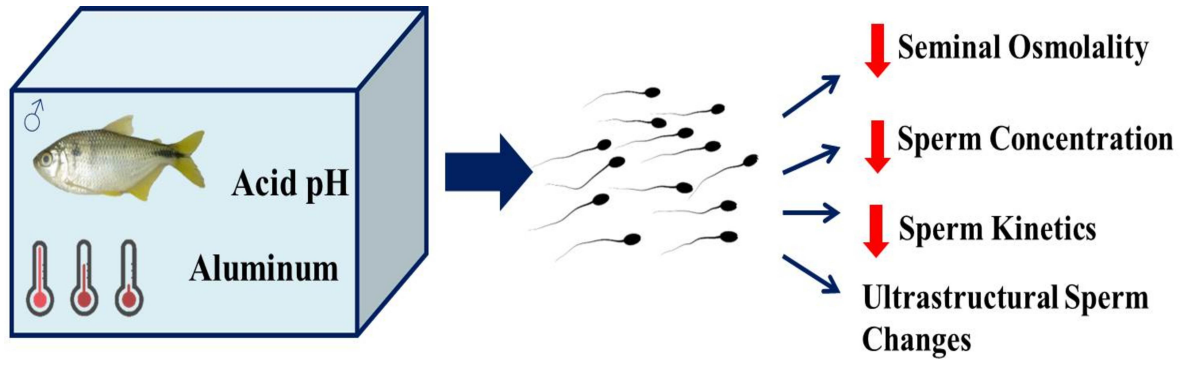
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Aluminum, at an environmental concentration, associated with acidic pH and high water temperature, causes impairment of sperm quality in the freshwater teleost *Astyanax altiparanae* (Teleostei: Characidae)

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Abstract

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

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

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

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

Introduction

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

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

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

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

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

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

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

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

Material and Methods

Animals

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

Experimental Design

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

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

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

Temperature adjustment in each experimental group was conducted at a rate of 1 °C h⁻¹ (Trueman et al., 2000). This Al concentration has been previously used in studies by our group (Correia et al., 2010; Kida et al., 2016; Abdalla et al., 2019; Pinheiro et al., 2019) and also represents actual contamination values of some basins in the state of São Paulo according to reports issued by the *Companhia Ambiental do Estado de São Paulo* (CETESB). Solutions were prepared with Al₂(SO₄)₃.18H₂O (Sigma Aldrich) and 65% HNO₃ (Suprapur, Merck; Pinheiro et al., 2019).

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

spectrometry (ICP–MS) method and was within the expected range (0.36 ± 0.09 to 0.50 ± 0.02 mg L⁻¹; Pinheiro et al., 2019).

Seminal Collection

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

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

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

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

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

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

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

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

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

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

Statistical analyses

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

Results and Discussion

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

Seminal pH

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

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

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

Seminal Osmolality

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

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

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

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

Sperm Concentration

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

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

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

Sperm Morphology

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

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

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

Sperm kinetics

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Sperm Ultrastructure

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

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

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

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

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

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

Conclusion

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

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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.

Sperm Morphology - % Normal			
Treatment	24 h Exposure		
	20°C	25°C	30°C
Neutral pH	99.92 \pm 0.08	99.83 \pm 0.17	99.92 \pm 0.08
Acid pH	99.80 \pm 0.11	100 \pm 0	99.83 \pm 0.11
Acid pH + Al	99.83 \pm 0.11	99.58 \pm 0.20	99.42 \pm 0.30
Treatment	96 h Exposure		
	20°C	25°C	30°C
Neutral pH	100 \pm 0 ^A	99.92 \pm 0.08 ^A	98.92 \pm 0.35 ^B
Acid pH	99.92 \pm 0.08	99.33 \pm 0.31	99.25 \pm 0.11
Acid pH + Al	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 AI shown as mean \pm standard error of the mean.

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

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

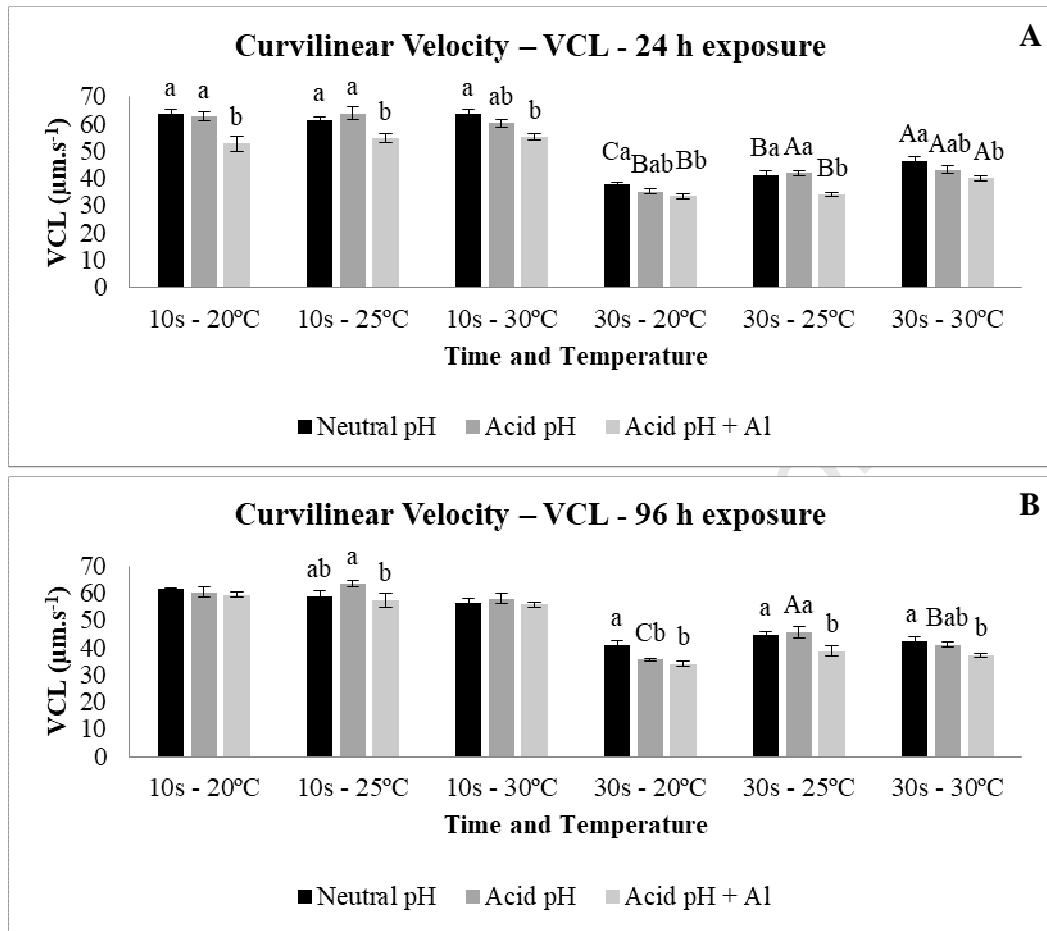


Fig. 3. Curvilinear velocity (VCL) of *A. altiparanae* sperm after 24 h (A) and 96 h (B) exposure at different temperatures, pHs, and the presence or absence of Al. Uppercase letters indicate differences within the same treatment at different temperatures; Lowercase letters indicate differences within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

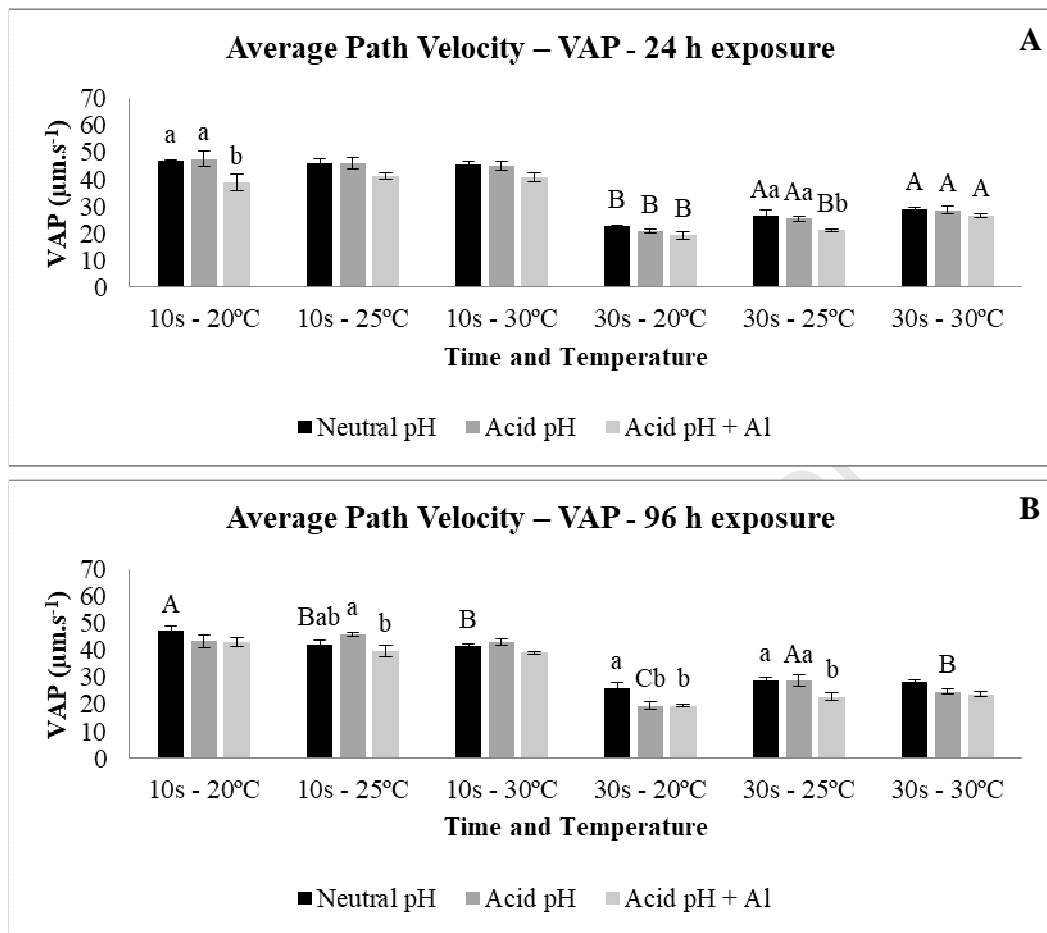


Fig. 4. Average path velocity (VAP) of *A. altiparanae* sperm after 24 h (A) and 96 h (B) exposure at different temperatures, pHs, and the presence or absence of Al. Uppercase letters indicate differences within the same treatment at different temperatures; lowercase letters indicate differences within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

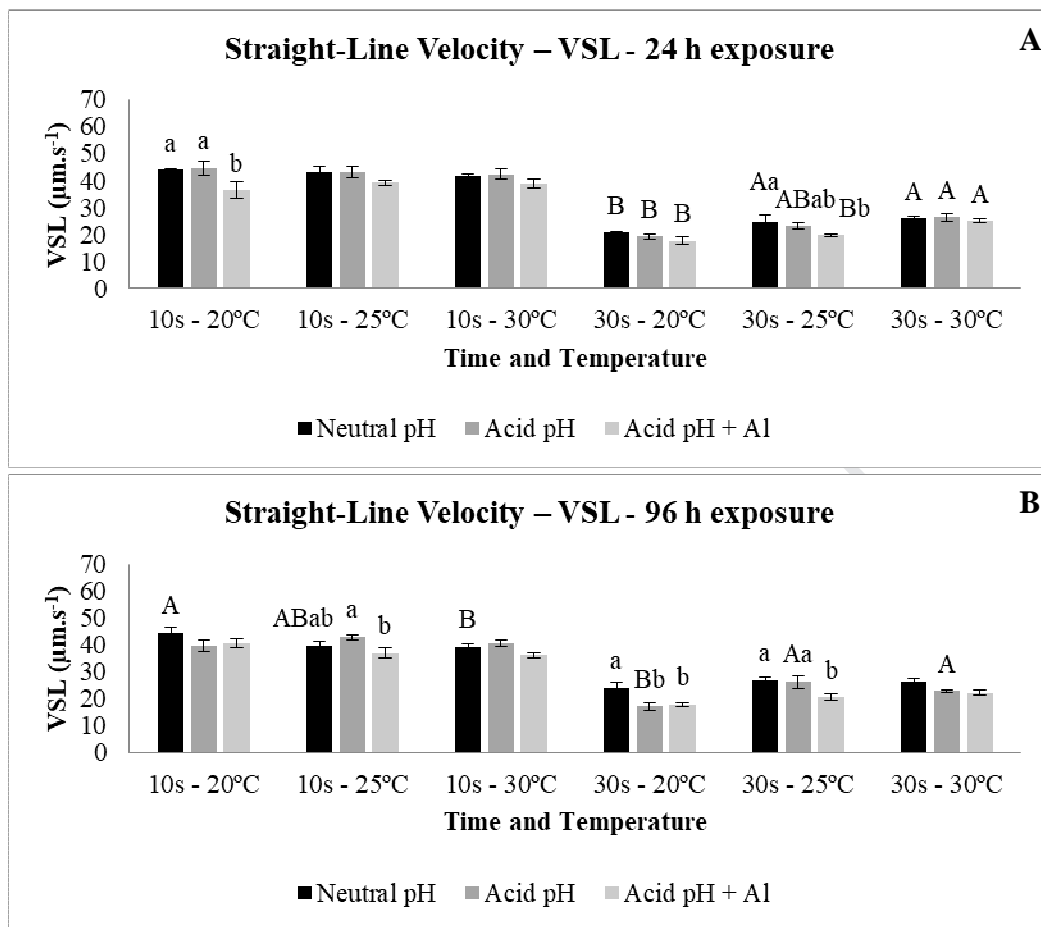


Fig. 5. Straight line velocities (VSL) of *A. altiparanae* sperm after 24 h (A) and 96 h (B) exposure at different temperatures, pHs, and the presence or absence of Al. Uppercase letters indicate differences within the same treatment at different temperatures; lowercase letters indicate differences within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

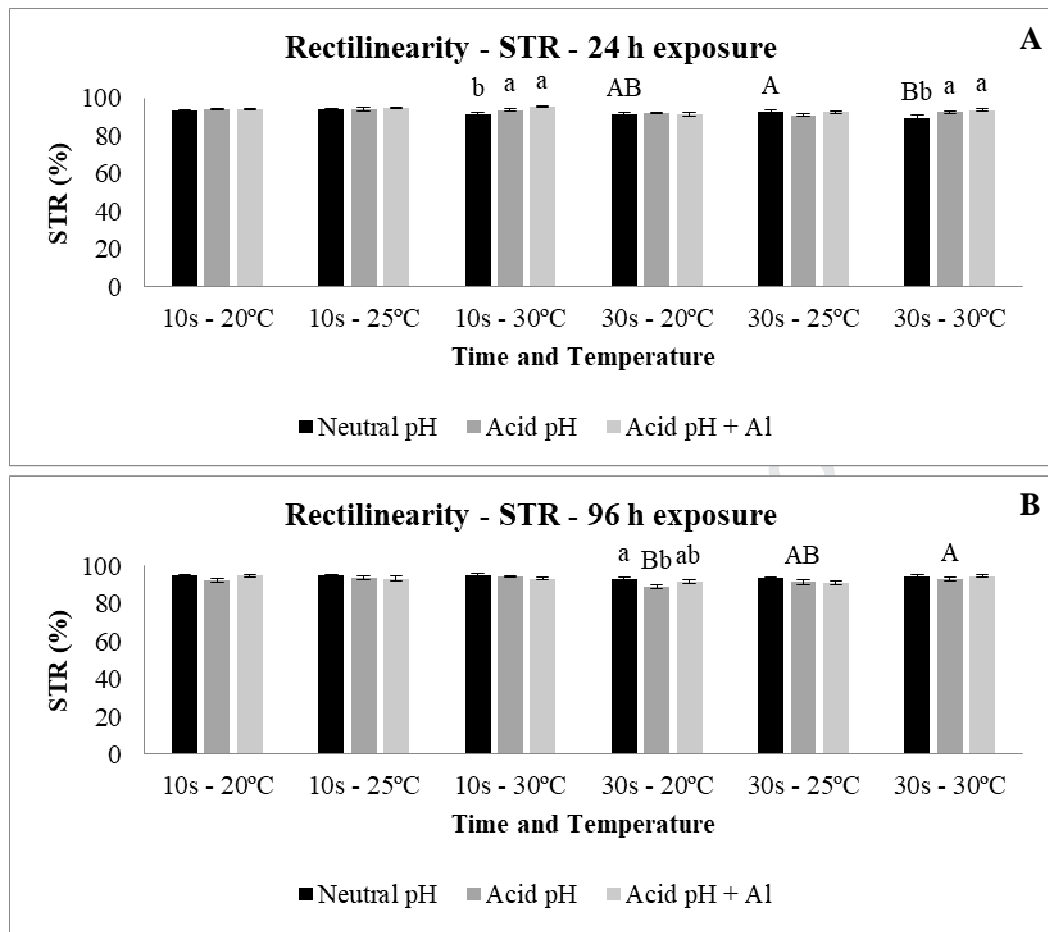


Fig. 6. Rectilinearity (STR) of *A. altiparanae* sperm after 24 h (A) and 96 h (B) exposure at different temperatures, pHs, and the presence or absence of Al. Uppercase letters indicate differences within the same treatment at different temperatures; lowercase letters indicate differences within the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

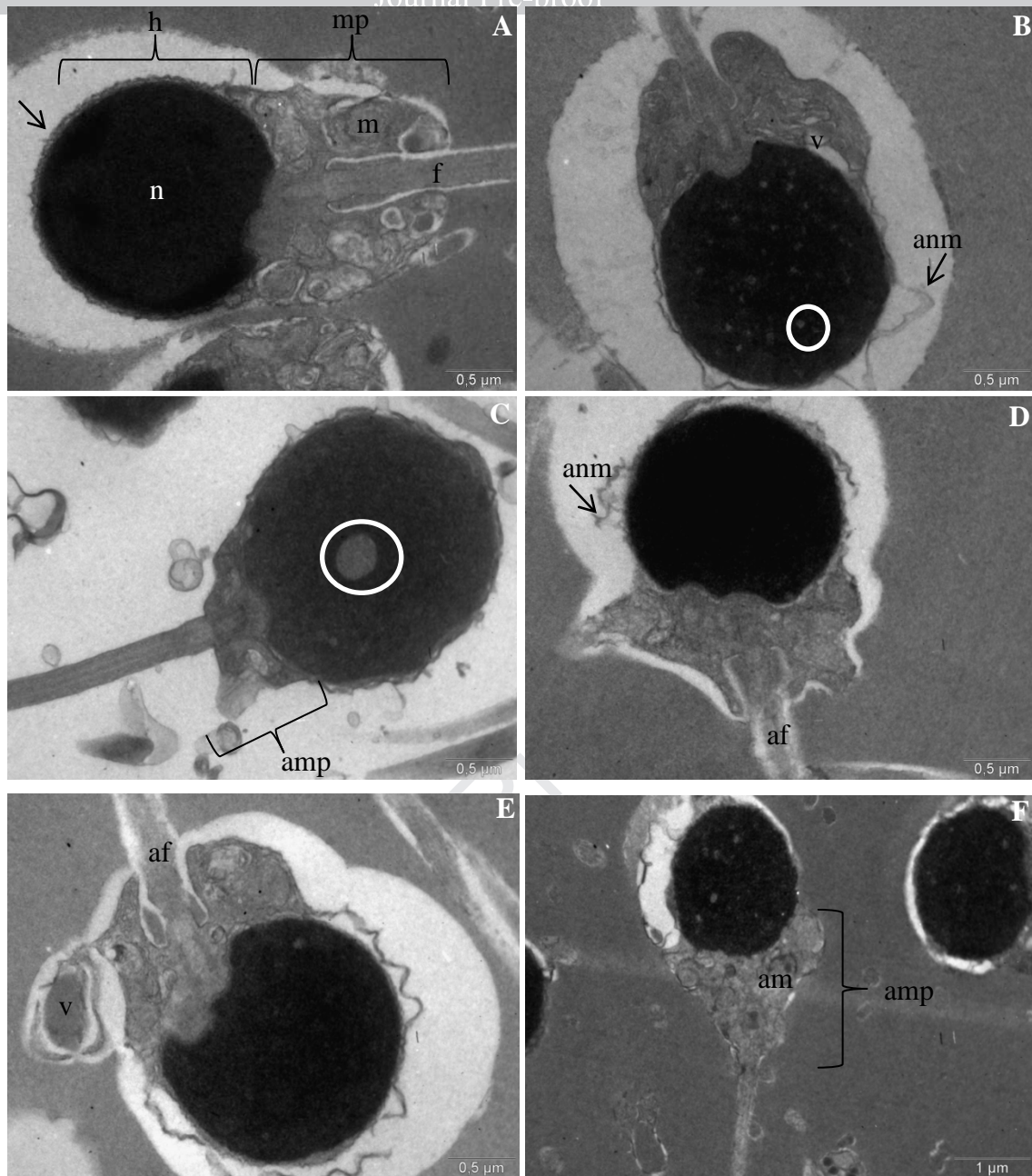


Fig. 7. Sperm ultrastructure of *A. altiparanae* after exposure to different temperatures and the presence or absence of AI. A. Normal spermatozoa (12.000 x; arrow: nuclear membrane; n: nucleus; h: head; mp: midpiece; m: mitochondria; f: flagellum; treatment: 25°C, and neutral pH). B–F. Abnormal spermatozoa (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 AI; E–F: treatment 30°C, acid pH, and AI).

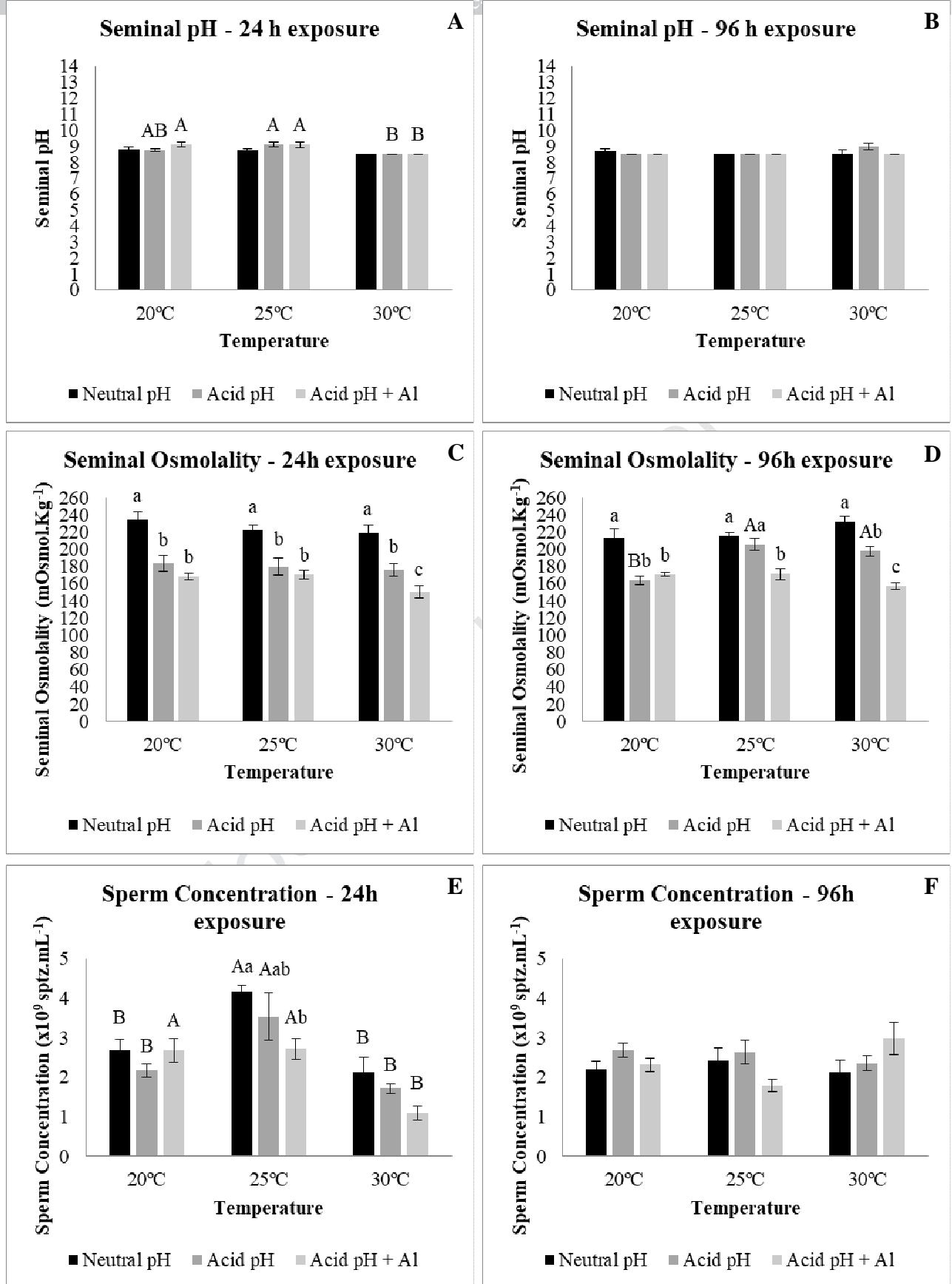


Fig. 1. Physicochemical characteristics of *Astyanax altiparanae* semen after exposure to different temperatures, pH values, and presence or absence of aluminum (mean \pm standard error of the mean). A. Seminal pH (exposure for 24 h); B. Seminal pH (exposure for 96 h); C. Seminal Osmolality (exposure 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.

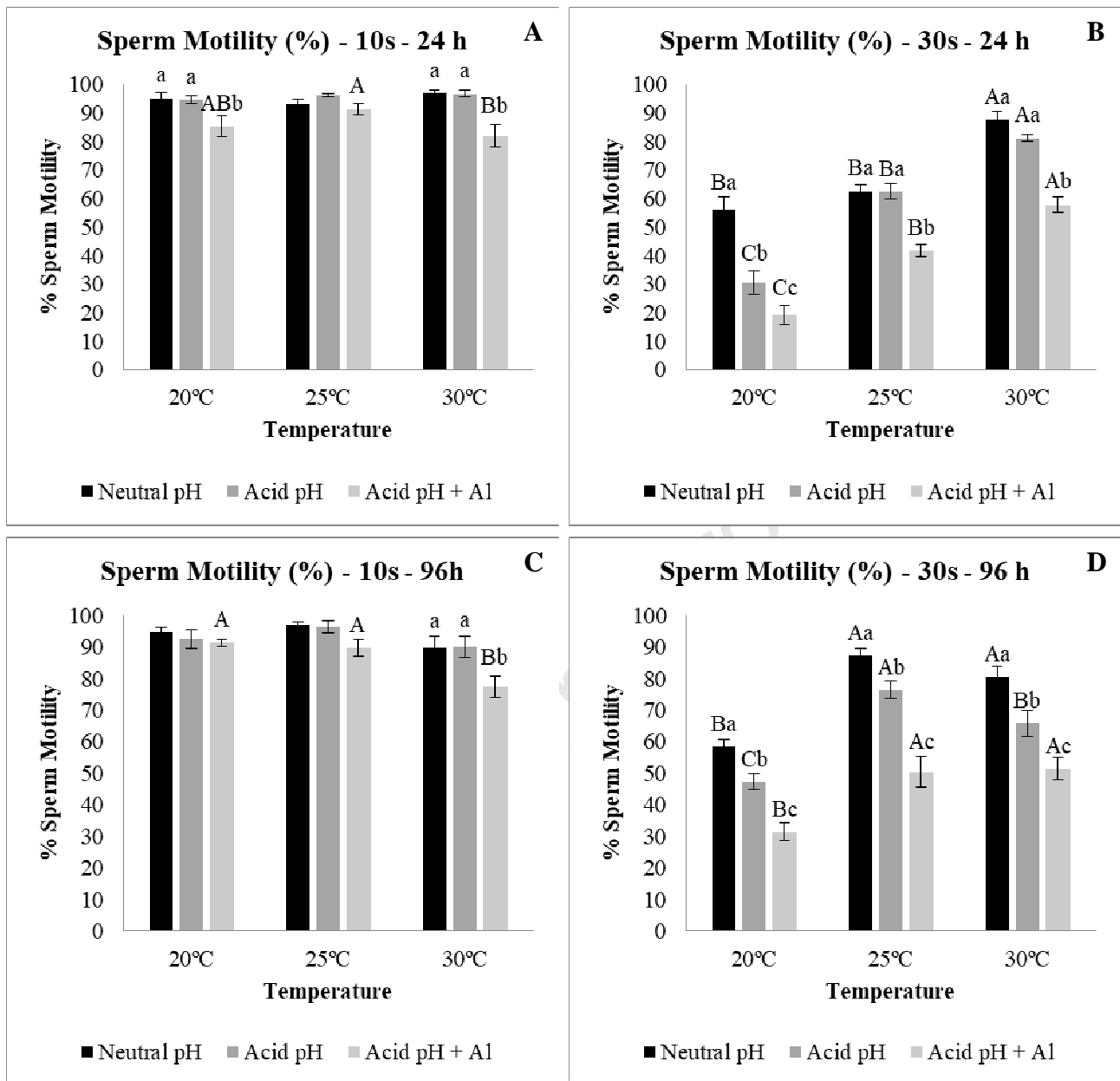


Fig. 2. Sperm motility (%) of male *A. altiparanae* after exposure at different temperatures, pHs and the presence or absence of Al. A. Sperm motility after 10 sec post-activation (animal exposure for 24 h); B. Sperm motility after 30 sec post-activation (animal exposure for 24 h); C. Sperm motility 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 - AI at high water temperature reduces seminal osmolality at 24 h and 96 h.
- 3 - AI 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 - AI triggers reduction in sperm motility and curvilinear speed after 24 h and 96 h.
- 6 - AI 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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