João Paulo Silva Pinheiro

Influência do alumínio e do pH ácido, associados às variações de temperatura, na qualidade seminal e variáveis reprodutivas de *Astyanax altiparanae* (Teleostei: Characidae)

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São Paulo

2020

VERSÃO CORRIGIDA

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Tese apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Doutor em Ciências, na Área de Fisiologia Geral.

Orientadora: Profa. Dra. Renata Guimarães Moreira Whitton

São Paulo

2020

Ficha catalográfica elaborada pelo Serviço de Biblioteca do Instituto de Biociências da USP,
com os dados fornecidos pelo (a) autor (a) no formulário:
'https://biblioteca.ib.usp.br/ficha-catalografica/src/ficha.php'

Pinheiro, João Paulo Silva
Influência do alumínio e do pH ácido, associados às variações de temperatura, na qualidade seminal e variáveis reprodutivas de Astyanax altiparanae (Teleostei: Characidae) / João Paulo Silva Pinheiro ; orientadora Profa. Dra. Renata Guimarães Moreira Whitton -- São Paulo, 2020.
161 p. + anexo

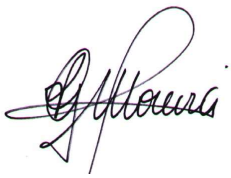
Tese (Doutorado) -- Instituto de Biociências da Universidade de São Paulo. Departamento de Fisiologia.

1. Ecotoxicologia. 2. Reprodução. 3. Peixe. 4. Metal. 5. Espermatozoide. I. Moreira Whitton, Profa. Dra. Renata Guimarães , orient. II. Título.

Comissão Julgadora:

Profa. Dra. Rachel Ann Hauser-Davis

Prof. Dr. Luis David Solis Murgas



Prof. Dr. Camilo Dias Seabra Pereira

Profa. Dra. Renata Guimarães Moreira Whitton - Orientadora

Agradecimentos

Agradeço, primeiramente, a Deus, por ser a minha fonte espiritual, me guiar pelos melhores caminhos e me possibilitar o encontro de pessoas tão incríveis em minha vida.

Aos meus pais, Suzete e Borges, por sempre cuidarem de mim, oferecerem amor, educação de qualidade e tudo o que estava ao alcance deles.

Ao meu querido irmão, Rodrigo, pelo seu apoio em minha vida.

Ao Max, gratidão e amor, por todo o companheirismo e incentivo durante esses anos.

Aos meus amigos, que contribuíram de forma direta ou indireta para a realização desse objetivo de vida.

À Universidade de São Paulo (USP), mais precisamente ao Instituto de Biociências (IB) e ao Programa de Pós-Graduação em Ciências (Fisiologia Geral), por conceder toda estrutura física e profissional para o desenvolvimento de todas as etapas do meu doutorado.

À Profa. Renata, essa pessoa tão fenomenal, que além de ser uma excelente orientadora e professora, tornou-se uma grande amiga.

A todos os "amiguinhos" e "coleguinhas" do LAMEROA pelo suporte durante esses anos, por me auxiliar a tornar os dias mais leves e mais produtivos. Em especial, ao Vagner por sempre estar disposto a resolver qualquer problema logístico. Além do mais, destaco minhas amigas e parceiras Marcela, Aline, Giovana e Cecília ("a melhor IC") por enfrentar e conduzir comigo todas as dificuldades experimentais, por passar noites acordadas e depois celebrar todas as conquistas obtidas.

Aos demais parceiros, que me ensinaram e/ou facilitaram a condução de algumas metodologias: Profa. Cláudia Bueno, Prof. Federico Brown, Prof.

Eduardo Sanches, Prof. Fernando Barbosa Júnior, Prof. Tiago Gabriel, Profa. Fabiana Lo Nostro (grande amiga) e Prof. Charles Tyler.

À Companhia Energética do Estado de São Paulo (CESP), em especial ao Danilo Caneppele, por ceder todos os exemplares de peixes para a pesquisa.

À FAPESP, por todo auxílio financeiro no doutorado (2014/16320-7 e 2016/08770-8) e para a realização dos estágios de pesquisa no exterior (2018/10790-2 e 2019/23816-2).

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Processo 140197/2016-8).

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Resumo

As ações antrópicas, como a introdução de metais na água, podem causar efeitos genotóxicos nos animais, e afetar processos fisiológicos, como a reprodução. A toxicidade desses metais, dentre eles o alumínio (Al), pode ser potencializada por mudanças de temperatura, influenciando na saúde dos peixes e na qualidade dos gametas, ameaçando o sucesso reprodutivo. O objetivo geral deste estudo foi avaliar os efeitos da exposição aguda ao Al, ao pH ácido e as ações sinérgicas da temperatura da água sobre a toxicidade sanguínea, os parâmetros seminais, as porcentagens de fertilização dos gametas, eclosão das larvas, e o desenvolvimento embrionário de *Astyanax altiparanae*. Machos foram expostos por 24 h e 96 h em diferentes grupos experimentais resultantes da combinação de cada uma das temperaturas (20, 25 e 30 ºC), com pH neutro (7,0) ou pH ácido (5,5) e com ou sem a presença de 0,5 mg L^{-1} de Al. Após a exposição, foram analisados: bioconcentração do Al (testículos e sêmen), teste cometa (eritrócitos e espermatozoides), teste de anormalidade nucleares (micronúcleo e eritrócitos), pH e osmolalidade seminais, concentração, morfologia, cinética e ultraestrutura espermáticas, perfil de ácidos graxos seminais, porcentagem de fertilização e de eclosão, assim como o desenvolvimento embrionário. A elevação da temperatura aumentou a bioconcentração de Al nos testículos após 96 h, enquanto após 24 h já desencadeou a bioconcentração de Al no sêmen. A temperatura e o pH potencializaram os efeitos genotóxicos do Al. Além disso, a exposição ao Al e à temperatura da água de 30ºC reduziram a osmolalidade seminal após 24 h e 96 h de exposição, assim como foi reduzida a concentração espermática (24 h). A água ácida induziu mudanças na cinética espermática, mas o acréscimo de Al acentuou essa redução, principalmente na motilidade e na velocidade curvilinear dos espermatozoides. O Al também afetou a ultraestrutura espermática (96 h), a fertilidade, a porcentagem de alguns ácidos graxos polinsaturados (PUFA)

seminais, como C20:4n6 e C22:5n3, além da morfologia das larvas. O meio de ativação/incubação em pH ácido e/ou com Al prejudicou a fertilização, a eclosão e o desenvolvimento embrionário. Por fim, foi observado que a porcentagem de fertilização, o perfil de ácidos graxos e o desenvolvimento embrionário são temperatura-dependente. Os dados permitiram concluir que o Al, de forma temperatura-dependente, teve efeitos genotóxicos sanguíneos e espermáticos, além de reduzir a qualidade seminal interferindo na fertilização, na eclosão e no desenvolvimento embrionário de *A. altiparanae*. Já a acidez, influenciou a qualidade seminal e os parâmetros reprodutivos, mas em menor proporção.

Palavras-chave: Água doce; Fisiologia espermática; Metal; Neotropical; Peixes; Poluição; Qualidade da água; Reprodução.

Abstract

Anthropic actions, such as the introduction of metals into the water, can cause genotoxic effects in animals, and affect physiological processes, such as reproduction. The toxicity of these metals, among them aluminum (Al), can be potentiated by changes in temperature, influencing fish health and the quality of gametes, threatening the reproductive success. The general objective of this study was to evaluate the effects of acute exposure to Al, to acid pH and synergistic actions of water temperature on blood toxicity, seminal parameters, gamete fertilization and larval hatching, and the embryonic development of *Astyanax altiparanae*. Males were exposed for 24 h and 96 h in different experimental groups, resulting from the combination of each temperature (20, 25 and 30 ºC), with neutral pH (7.0) or acidic pH (5.5) and with or without the presence of 0.5 mg L^{-1} of Al. After exposure, Al bioconcentration (testicles and semen), comet test (erythrocytes and sperm), nuclear abnormality test (micronucleus and erythrocytes), pH and seminal osmolality, sperm concentration, morphology, kinetics, and sperm ultrastructure, fatty acid seminal profile, fertilization, and hatching percentages, as well as the embryonic development were analyzed. The temperature rise increased the bioconcentration of Al in the testes after 96 h, while after 24 h it already triggered the bioconcentration of Al in the semen. Temperature and pH potentiated Al's genotoxic effects. Besides, the exposure to Al and water temperature at 30ºC reduced seminal osmolality after 24 h and 96 h of exposure, as well as the sperm concentration (24 h) was reduced. Acid water induced changes in sperm kinetics, but the addition of Al accentuated this reduction, mainly in motility and curvilinear velocity of sperm. Al also affected the sperm ultrastructure (96 h), fertility, the percentage of some seminal polyunsaturated fatty acids (PUFA), such as C20:4n6 and C22:5n3, besides the morphology of the larvae. The activation/incubation medium at acid pH and/or

with Al impaired fertilization, hatching, and embryonic development. Finally, it was observed that the percentage of fertilization, the fatty acid profile, and the embryonic development are temperature-dependent. Therefore, the data allowed to conclude that Al, in a temperature-dependent way, triggered genotoxic effects in blood and sperm, in addition to reducing seminal quality, interfering in the fertilization, hatching, and embryonic development in *A. altiparanae*. Acidity, instead, influenced the seminal quality and the reproductive parameters, but to a lesser extent.

Keywords: Freshwater; Sperm physiology; Metal; Neotropical; Fish; Pollution; Water quality; Reproduction.

Introdução Geral

A poluição, derivada a partir de ações antrópicas, tais como os lançamentos de efluentes domésticos, industriais e agrícolas, rejeitos de mineração, resíduos de estações de tratamento de esgoto entre outras, é considerada uma das principais ameaças ao ambiente aquático (Reid et al., 2013). Estas descargas contêm uma mistura de poluentes, como compostos farmacêuticos, plastificantes, pesticidas, solventes e metais, influenciando em diversos processos morfofisiológicos dos organismos, por exemplo, no comportamento animal, na morfologia tecidual, na atividade de enzimas antioxidantes, no DNA e no perfil de hormônios sexuais (Kida et al., 2016; Abdalla et al., 2019; Lombó et al., 2019; Weber et al., 2019; Martin et al., 2019; Jia et al., 2020).

Dentre esses poluentes, destaca-se o metal alumínio (Al), que é um elemento natural e um dos metais mais comuns na crosta terrestre. O Al é encontrado na maioria dos solos e rochas, e pode estar presente na água por meio de processos naturais, como o desgaste de rochas, mas também por meio de atividades antrópicas, por exemplo a mineração, processos industriais e águas residuais tratadas com um composto de Al (Wilson, 2011). Diferentes órgãos regulatórios ambientais, como o Conselho Nacional do Meio Ambiente (CONAMA) no Brasil e o *United States Environmental Protection Agency* (US EPA) nos Estados Unidos, preconizam um valor máximo de 0,1 mg L^{-1} (CONAMA) e de 0,2 mg L^{-1} de Al dissolvido na água (US EPA). Entretanto, observa-se nos diferentes rios que tais concentrações estão acima das recomendadas, como as reportadas em rios do estado de São Paulo (ex. Rios Grande e Tamanduateí: 0,35 mg L^{-1} Al; Rio Ribeirão Perová: 0,38 a 1,88 mg L^{-1} Al e Rio Guaió: 0,32 a 1,67 mg L^{-1}; CETESB, 2018).

A solubilidade do Al, assim como a biodisponibilidade, é influenciada pelos parâmetros físicos e químicos da água, tais como pH, temperatura, dureza

e carbono orgânico dissolvido (Wilson, 2011; Gensemer et al., 2018). Com isso, o Al sofre o fenômeno de especiação, no qual as espécies monoméricas inorgânicas de Al são as mais tóxicas. As espécies químicas diferem entre os diferentes pHs de água, sendo que em condições de pH ácido predominam as espécies catiônicas de Al [Al^{3+}, $AlOH^{2+}$, e $Al(OH)_2^{+}$], em valores intermediários de pH prevalece $Al(OH)_3$ e sob condições alcalinas destaca-se $Al(OH)_4^{-}$. Além do mais, o Al aquoso pode formar complexos inorgânicos, afetando a biodisponibilidade e a toxicidade, com fluoreto (F^{-}), sulfato (SO_4^{2-}), fosfato (PO_4^{3-}) e ácido silícico [$Si(OH)_4$] (Wilson, 2011). Com relação à temperatura, a associação de baixos valores com baixo pH maximiza a solubilidade do Al em água (Wilson, 2011). Além desse efeito, a temperatura influencia no metabolismo dos peixes, uma vez que altas temperaturas aceleram o metabolismo promovendo uma maior frequência respiratória e, por sua vez, causando um aumento na absorção de poluentes, como o Al, por estruturas respiratórias, como brânquias, levando à morte de animais aquáticos (Poleó e Muniz, 1993; Wilson, 2011).

O Al não é considerado como metal essencial para os organismos aquáticos, como os peixes, uma vez que não exercem qualquer papel fisiológico no organismo e seu mecanismo de entrada na célula ainda não é completamente compreendido (Nayak, 2002; Exley, 2009; Fernández-Dávila et al., 2012; Exley e Mold, 2015). Entretanto, estudos demonstram a capacidade do mesmo de se bioconcentrar em diferentes tecidos (Allin e Wilson, 2000; Correia, 2012; Fernández-Dávila et al., 2012; Voigt et al., 2015) e de afetar a homeostase de algumas espécies de peixes, uma vez que desencadeia desregulação iônica (Camargo et al., 2009), inibe funções respiratórias (acúmulo do Al na superfície branquial; Poléo e Muniz, 1993; Allin e Wilson, 2000; Wilson, 2011), conduzindo, possivelmente, à morte (Poléo e Muniz, 1993; Wilson, 2011). Além do mais, o Al pode influenciar na atividade de enzimas antioxidantes (Ramírez-Duarte et al., 2017; Abdalla et al., 2019), no comportamento animal (Senger et al., 2011; Grassie et al., 2013), nos níveis de diferentes hormônios (Correia et al.,

2010; Vieira et al., 2013; Kida et al., 2016; Höglund et al., 2020) e causar efeitos genotóxicos e citotóxicos (Galindo et al., 2010; García-Medina et al., 2011, 2013).

Dentre esses efeitos deletérios dos xenobióticos, pode-se destacar aqueles que afetam a reprodução de teleósteos, uma vez que esse processo é modulado por fatores ambientais, tais como a temperatura, pH e fotoperíodo, dentre os principais (Nagahama et al., 1995; Nagahama e Yamashita, 2008; Rizzo e Bazzoli, 2020; Servili et al., 2020), e a presença de poluentes tem influência no eixo hipotálamo-hipófise-gônadas (HPG), que controla a reprodução. Essa modulação influencia a síntese e a liberação de neurohormônios hipotalâmicos, no sistema nervoso central, como o hormônio liberador de gonadotropinas (GnRH; estimulador do eixo), hormônio inibidor de gonadotropinas (GnIH; inibidor do eixo), dopamina (inibidor do eixo) e kisspeptina (Levavi-Sivan et al., 2010; Maugars et al., 2020; Muñoz-Cueto et al., 2020; Somoza et al., 2020; Trudeau e Somoza, 2020). O GnRH atua na síntese e na secreção de gonadotropinas, que são o hormônio folículo estimulante (FSH) e o hormônio luteinizante (LH). Esses hormônios participam do processo de esteroidogênese gonadal por meio da regulação da síntese e da liberação de esteroides sexuais relacionados que modulam a vitelogênese (estrógenos), ovulação (progestágenos) e espermatogênese (andrógenos) (Lubzens et al., 2010). Nos machos, as gonadotropinas (FSH e LH) estimulam a secreção dos andrógenos, testosterona e 11-cetotestosterona, que atuam no desenvolvimento testicular e nas características sexuais secundárias. Além dessas funções, os andrógenos agem no processo de formação das células gaméticas masculinas (espermatozoides) (Schulz et al., 2010). Sob condições controladas, a espermatogênese resulta em espermatozoides de boa qualidade, ou seja, viáveis à fertilização. Todavia, quando ocorrem variações nos parâmetros físicos e químicos da água e a presença de poluentes no ambiente aquático, podem ocorrer interferências ao longo do eixo reprodutivo e, consequentemente, a redução da qualidade dos gametas (Brooks et

al., 1997; Alavi e Cosson, 2005; Valdebenito et al., 2015; Kowalski e Cejko, 2019; Kholodnyy et al., 2020).

A qualidade gamética consiste na capacidade do gameta masculino em fertilizar o gameta feminino, permitir o desenvolvimento embrionário normal, possibilitando o sucesso reprodutivo da espécie (Bobe e Labbé, 2010; Herráez et al., 2017; Kowalski e Cejko, 2019). Os gametas de teleósteos constituem uma excelente ferramenta para estudos ecotoxicológicos, já que são células sensíveis a diferentes fatores presentes no ambiente aquático, e possuem a fisiologia espermática relacionada diretamente com o meio. Dentre os biomarcadores de qualidade seminal, pode-se destacar os quantitativos (ex. volume e concentração espermática) e os qualitativos (ex. cinética espermática, morfologia, integridade do DNA, pH seminal, osmolalidade, composição do plasma seminal – íons, lipídios, proteínas – entre outros) que possuem relação direta tanto na capacidade de fertilização dos ovócitos quanto no sucesso reprodutivo da espécie (Bobe e Labbé, 2010; Kowalski e Cejko, 2019).

No tocante à temperatura, mudanças termais podem afetar a sínteses de hormônios reprodutivos, assim como, a qualidade gamética, a fertilização e o desenvolvimento embrionário (Servili et al., 2020). Essas alterações térmicas durante a espermatogênese, bem como no ciclo reprodutivo do animal, podem resultar na redução de diferentes parâmetros seminais, por exemplo, no volume, na cinética e na integridade do DNA espermáticos (revisado por Alix et al., 2020; Servili et al., 2020). Somado a isso, mudanças sazonais de temperatura podem modificar o perfil de ácidos graxos das membranas dos espermatozoides e, consequentemente, influenciar na fluidez da membrana (Engel et al., 2019). Ademais, a temperatura da água tem papel fundamental no desenvolvimento embrionário, quando os eventos morfofisiológicos ocorrem de maneira temperatura-dependente, sendo acelerados ou retardados (Bobe e Labbé, 2010; Santos et al., 2016; Kholodnyy et al., 2020).

Já com relação ao pH, são escassos os estudos que avaliam os potenciais efeitos da acidez ou da alcalinidade da água sob a qualidade seminal, os estudos são focados basicamente no papel desta variável no momento da ativação espermática (Sanches et al., 2015; Öğretmen et al., 2016; Castro et al., 2020), demonstrando que o pH pode interferir em diferentes variáveis espermáticas, como a motilidade (Sanches et al., 2015; Öğretmen et al., 2016), além de influenciar na fertilização e na eclosão (Keinänen et al., 2003, 2004; Santos et al., 2020). As mudanças no pH seminal podem afetar negativamente a qualidade gamética e o sucesso reprodutivo de teleósteos (Servili et al., 2020).

Os estudos dos efeitos do Al que demonstram os efeitos deletérios na quantidade e na qualidade dos espermatozoides são focados principalmente em mamíferos (revisado por Yokel, 2020). Estes efeitos podem ser observados ao longo do eixo HPG, bem como em diferentes parâmetros seminais, por exemplo na redução dos níveis de FSH e de LH (Sajjad et al., 2020), de testosterona (Mouro et al., 2018; Sajjad et al., 2020), aumento em danos mitocondriais espermáticos (Cao et al., 2020), redução na concentração espermática (Miska-Schramm et al., 2017; Güvenç et al., 2020; Sajjad et al., 2020), na motilidade espermática (Miska-Schramm et al., 2017; Mouro et al., 2018; Yuan et al., 2019; Güvenç et al., 2020; Sajjad et al., 2020) e no percentual de espermatozoides morfologicamente normais (Miska-Schramm et al., 2017; Yuan et al., 2019) e com membranas íntegras (Mouro et al., 2018). Já em teleósteos, esse metal também interfere na reprodução, tanto em fêmeas quanto em machos, uma vez que pode alterar a produção de hormônios do eixo HPG (Correia et al., 2010; Correia, 2012; Narcizo, 2014; Kida et al., 2016; Assis et al., 2021; Narcizo et al., 2021) e consequentemente a formação dos gametas. Kida et al. (2016) ao estudarem machos de *Astyanax altiparanae*, expostos ao Al na fase ativa do ciclo reprodutivo, observaram uma elevação na concentração de 11-cetotestosterona e de testosterona após 96 horas de exposição, o que sugere alterações da fisiologia reprodutiva dos machos.

A capacidade de alguns metais em interferir negativamente na qualidade seminal de teleósteos, como na motilidade, na integridade de membrana, na morfologia, no DNA, na atividade mitocondrial espermáticas, assim como nas taxas de fertilização, nas de eclosão e no desenvolvimento embrionário, vem sendo observada *in vitro* com mercúrio ([Hg] Dietrich et al., 2010; Hayati et al., 2019), cobre ([Cu]; Bombardelli et al., 2016; Zebral et al., 2019 – exposição dos machos ao metal; Gárriz e Miranda, 2020) e cádmio ([Cd]; Dietrich et al., 2010; Gárriz e Miranda, 2020). No entanto, os estudos que avaliam a qualidade seminal, após a exposição de machos de teleósteos a diferentes metais ainda são escassos.

Os teleósteos são modelos utilizados frequentemente em estudos ecotoxicológicos em virtude de razões econômicas e ecológicas, além de serem de fácil cultivo e representarem os vertebrados de forma geral (Padilla e Glaberman, 2020). Somado a isso, existem diferentes diretrizes internacionais (ex. *Organization for Economic Co-operation and Development – OECD* e *United States Environmental Protection Agency – US EPA*) aceitas amplamente para testes toxicológicos de compostos químicos com diferentes espécies de peixes, que podem ser adaptadas para outras, contemplando diversos estágios de vida, tempos de exposição, fase reprodutiva entre outros aspectos (Padilla e Glaberman, 2020). Dentre as espécies de peixes que vem sendo utilizadas frequentemente em ensaios ecotoxicológicos e/ou de reprodução encontra-se o lambari-do-rabo-amarelo, *Astyanax altiparanae*[1] (Gomes et al., 2013; Vieira et al., 2013; Chehade et al., 2015, 2020; Bettim et al., 2016; Kida et al., 2016; Abdalla et al., 2019; Brambila-Souza et al., 2019; Pinheiro et al., 2019, 2020, 2021; Godoi et al., 2020; Assis et al., 2021; Muñoz-Peñuela et al., 2021), uma vez que é uma espécie nativa e representativa do sudeste do Brasil, habita rios com a presença de Al, representa um bom bioindicador para toxicidade de diferentes

[1] Está sendo proposta uma alteração do nome da espécie para *Astyanax lacustris* (Lucena e Soares, 2016). Como as questões relacionadas à sistemática do gênero *Astyanax* são muito complexas, e esta revisão de nome da espécie ainda está em fase de consenso da comunidade científica, nesse trabalho preconizou-se pela manutenção da classificação anterior até que seja estabelecida por completo a nova nomenclatura.

compostos, como os metais, em curtos ou longos períodos de exposição e é de fácil manuseio em condições laboratoriais.

Logo, apesar da importância da qualidade seminal, existe pouca informação sobre o efeito dos poluentes nesta variável e suas consequências na fertilização, na eclosão e no desenvolvimento embrionário de teleósteos. Além do mais, apesar dos parâmetros físicos e químicos da água interferirem na biodisponibilidade e na toxicidade de diferentes poluentes e, consequentemente, na fisiologia dos organismos aquáticos (Wlasow et al., 2010; Paiva Magalhães et al., 2015), estudos que associem tais variáveis sobre os parâmetros reprodutivos são praticamente inexistentes.

Os dados da literatura demonstram a capacidade genotóxica do Al em animais jovens, mas os efeitos genotóxicos deste metal durante a fase reprodutiva de teleósteos ainda não foram avaliados, assim como os efeitos desta exposição combinada com variações na temperatura da água (co-exposição). Estas alterações podem prejudicar populações em ambientes contaminados, sendo importante a avaliação do potencial mutagênico e genotóxico do Al aos organismos, identificando possíveis alterações cromossômicas e a influência da contaminação sobre os componentes dos fusos ou da região centromérica. Somado a isso, verificou-se a escassez de dados disponíveis que possam complementar o entendimento dos efeitos dos metais na biota aquática, inclusive do Al, em associação com fatores ambientais, como temperatura, durante o período reprodutivo, na qualidade seminal de teleósteos (*in vivo*), no perfil de ácidos graxos seminal, e as consequências na fertilização, na eclosão e no desenvolvimento embrionário de teleósteos.

Portanto, o presente estudo teve como objetivo geral avaliar os efeitos da exposição aguda ao Al, ao pH ácido, assim como as ações sinérgicas da temperatura da água sobre os efeitos genotóxicos (eritrócitos e sêmen), os parâmetros seminais, as porcentagens de fertilização e eclosão, e o desenvolvimento embrionário de *A. altiparanae*.

O presente estudo foi dividido em três capítulos, com os seguintes objetivos:

- Capítulo 1: Avaliar a bioconcentração de Al nos testículos e no sêmen de *A. altiparanae* e o potencial desse metal, combinado com mudanças de temperatura e pH ácido, em causar citotoxicidade e genotoxicidade nos eritrócitos e espermatozoides *de A. altiparanae*.

- Capítulo 2: Avaliar os efeitos da exposição subaguda de machos de *A. altiparanae* ao Al em concentrações ambientais, além das ações individuais e/ou sinérgicas da temperatura da água e do pH ácido sobre a qualidade seminal desta espécie.

- Capítulo 3: Avaliar os efeitos de machos de *A. altiparanae* expostos ao Al, em diferentes temperaturas e pH ácido, sobre o perfil de ácidos graxos do sêmen, a fertilização, a eclosão e o desenvolvimento embrionário, correlacionando essas variáveis com os parâmetros de qualidade espermática. Além dos efeitos nos gametas, após a exposição do macho, objetiva-se avaliar o efeito aditivo da água utilizada na ativação dos gametas.

As seguintes hipóteses foram testadas:

- Capítulo 1: o Al se bioconcentra nos testículos e no sêmen de *A. altiparanae* de maneira temperatura-dependente, tem efeito genotóxico no sangue e nos espermatozoides desta espécie e o aumento da temperatura potencializa este efeito;

- Capítulos 2 e 3: o sinergismo entre o Al e o aumento da temperatura modifica as características seminais, reduz o potencial fecundante e afeta o

desenvolvimento embrionário de *A. altiparanae.* A presença de Al na água de ativação dos gametas e incubação dos embriões afeta negativamente a fertilização, a eclosão e o desenvolvimento embrionário.

Capítulo 1

A temperatura da água e o pH ácido influenciam os efeitos citotóxicos e genotóxicos do alumínio no teleósteo de água doce *Astyanax altiparanae* (Teleostei: Characidae)

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

Publicado na revista Chemosphere 220, 266-274, 2019 [2].
DOI: 10.1016/j.chemosphere.2018.12.143

[2] Pinheiro, J.P.S., Assis, C.B., Muñoz-Peñuela, M., Barbosa Junior, F., Correia, T.G., Moreira, R.G., 2019. Water temperature and acid pH influence the cytotoxic and genotoxic effects of aluminum in the freshwater teleost *Astyanax altiparanae* (Teleostei: characidae). Chemosphere 220, 266-274. https://doi.org/10.1016/j.chemosphere.2018.12.143

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

João Paulo Silva Pinheiro[1], Cecília Bertacini de Assis[1], Marcela Muñoz-Peñuela[1], Fernando Barbosa Júnior[2], Tiago Gabriel Correia[3], Renata Guimarães Moreira[1]

1. Universidade de São Paulo, Institute of Biosciences, Department of Physiology, Laboratory of Metabolism and Reproduction of Aquatic Organisms - LAMEROA; Matão Street, 14 lane, number 101 - room 220, Cidade Universitária - São Paulo – SP- Brazil.
2. Universidade de São Paulo, Faculty of Pharmaceutical Sciences of Ribeirão Preto (FCFRP); Café Avenue, Ribeirão Preto – SP – Brazil.
3. Universidade Federal do Amapá, Biological Sciences Course; Juscelino Kubitschek Highway, Km 02 - Jardim Marco Zero - Macapá – AP – Brazil.

João Paulo Silva Pinheiro: joaopaulospinheiro@yahoo.com.br
Cecília Bertacini de Assis: cbertacini4@gmail.com
Marcela Muñoz-Peñuela: marce8407@gmail.com
Fernando Barbosa Júnior: fbarbosa@fcfrp.usp.br
Tiago Gabriel Correia: correia.tg@gmail.com
Renata Guimarães Moreira: renata.fish@gmail.com

Corresponding author: João Paulo Silva Pinheiro: joaopaulospinheiro@yahoo.com.br
+551130917531

Resumo

A toxicidade de metais, entre eles o alumínio (Al), pode ser potencializada pela temperatura e pH ácido, uma preocupação diante do atual cenário de aquecimento global. O objetivo deste estudo foi avaliar a bioconcentração de Al nos testículos e sêmen de *Astyanax altiparanae* e o potencial desse metal, em diferentes temperaturas ambientais e pH ácido, em causar citotoxicidade e genotoxicidade em eritrócitos e espermatozoides. Machos de *A. altiparanae* foram divididos em nove grupos experimentais: em cada uma das três diferentes temperaturas da água (20, 25 e 30 ºC), os peixes foram expostos a pH neutro, pH ácido e água ácida contendo Al (0,5 mg L^{-1}). Os peixes foram submetidos à exposição semi-estática subaguda e amostrados às 24 h e 96 h. Após cada período de exposição foi realizado o ensaio cometa (sangue e sêmen) e o teste do micronúcleo (sangue). A bioconcentração de Al foi avaliada nos testículos e no sêmen. O tempo de exposição e a temperatura influenciaram o padrão de bioconcentração de Al nos testículos. A concentração de Al no sêmen foi maior nos peixes expostos a 20 e 25 ºC (24 h). O escore de fragmentação do DNA para o sêmen e sangue foi maior nos peixes expostos ao Al em 20 ºC (24 h) e 30 ºC (96 h). A frequência de anormalidades nucleares em eritrócitos foi maior nos animais expostos ao Al em 30 ºC (96 h). Concluiu-se que o Al bioconcentra-se nos testículos e sêmen de *A. altiparanae* em diferentes temperaturas e é potencialmente citotóxico e genotóxico para eritrócitos e espermatozoides nesta espécie.

Palavras-chave: pH; Espermatozoides; Sêmen; Metal; Temperatura

Abstract

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

Keywords: pH; Spermatozoa; Semen; Metal; Temperature

Highlights

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

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

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

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

1. Introduction

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

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

Kida *et al.* (2016) analyzed *A. altiparanae* males exposed to Al during the active phase of the reproductive cycle and observed an increase in plasma levels of testosterone and 11-ketotestosterone after exposure for 96 hours, suggesting alterations in the reproductive physiology of males impairing sperm production and spermiation. Genotoxic effects of Al were also observed in the erythrocytes

of juveniles of the tropical species *Prochilodus lineatus* (Galindo *et al.*, 2010), oxidative stress in *C. carpio* (García-Medina *et al.*, 2013), ionic imbalance and inhibition of Na^+/K^+–ATPase enzymatic activity in *P. lineatus* (Camargo *et al.*, 2009), and increased DNA damage (time-dependent) and a higher level of oxidized DNA in *C. carpio* (García-Medina *et al.*, 2011).

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

Teleosts inhabit different trophic levels in the food chain, and for this reason are widely used in ecotoxicological trials and studies to evaluate the genotoxic potential of various substances, including metals. They are also sensitive to environmental changes and capable of bioconcentrating contaminants, even at low concentrations in water bodies (Jesus *et al.*, 2014, 2016; Chua *et al.*, 2018). Furthermore, fish bioassay data correlate well with genotoxicity in human cells exposed to environmental mutagenic agents (Marcon *et al.*, 2010), and some of the results obtained using teleosts can be extrapolated to humans. *Astyanax altiparanae* has also been used in bioassay studies (Gomes *et al.*, 2013; Vieira *et al.*, 2013; Chehade *et al.*, 2014; Bettim *et al.*, 2016; Kida *et al.*, 2016; Abdalla *et al.*, 2019), mainly due to its high plasticity to different environments, small size so that experiments can be conducted in tanks, and ease of handling under laboratory conditions.

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

2. Material and Methods

2.1. Experimental conditions

Sexually mature *A. altiparanae* (n=360) males were donated by the Paraibuna Hydrobiology and Aquaculture Station (*Companhia Energética de São Paulo*, CESP, Paraibuna, São Paulo, Brazil). Prior to the experiment, the fish were kept for seven days in the ectotherm facility at the Universidade de São Paulo, Institute of Biosciences, Department of Physiology (IB/USP). They were then transferred to 18 glass tanks (132 L water/tank, 10 fish/tank) with water replenishment (90% every 24 h) and fed daily *ad libitum* with extruded feed (32% crude protein). They were deprived of food for 24 hours before beginning the experiments and until the end of subacute exposure, in order to obviate feces as a further experimental variable.

Separate studies were conducted at two exposure times: 1) 180 fish were exposed to the experimental conditions for 24 hours; and 2) 180 fish were exposed

to the experimental conditions for 96 hours. These periods were chosen based on previous studies (Correia *et al.*, 2010; Kida *et al.*, 2016; Abdalla et al., 2019) to verify responses over different time intervals, since some physiological responses occur rapidly due to the plasticity of the species studied.

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

The Al solution was prepared at a concentration of 0.5 $mg.L^{-1}$ (5.67 g $Al_2(SO_4)_3.18H_2O$ - Sigma Aldrich - diluted in 700 mL of deionized water, adjusting the pH to 2.5 with 65% HNO_3 - Suprapur, Merck). A quantity of 8.148 ml of this solution was added to the 132-L tanks in order to obtain an initial concentration of 0.5 mg.L-1 of Al and a pH of 5.5 (required for Al bioavailability), since Al is relatively insoluble at pH 6 to 8 due to hydrolysis and the formation of $Al(OH)_3$ (Driscoll and Schecher, 1990; Gensemer and Playle, 1999). This concentration of Al was chosen as representative of contamination levels in some basins in the State of São Paulo, according to reports published by *Companhia Ambiental do Estado de São Paulo* (CETESB, 2016), and is below the LC_{50} value (1 $mg.L^{-1}$) previously obtained (unpublished data).

Water temperatures were determined according to the known adequate survival temperatures (Siqueira-Silva *et al.*, 2015). The 24- or 96-hour period started from temperature stabilization for each experimental group, and temperature transitions were controlled at a rate of 1 $°C.h^{-1}$ (Trueman *et al.*, 2000).

For each experimental group, the water temperature was kept constant using the ectotherm facility cooling system, and thermostat-controlled water heaters monitored periodically.

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

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

2.2. Water collection

Water samples (150 mL) from each tank were collected every 24 hours and placed in amber flasks previously washed with nitric acid. The sample was always taken with a 20 ml syringe at the same point inside the tank. It was aliquoted into two flasks (one for total Al - unfiltered, one for dissolved Al - filtered with a Valuprep PVDF 0.45 µM, Hexis filter syringe) and acidified with 65% nitric acid (Suprapur, Merck) at pH<2.5. The vials were then kept refrigerated at 4 °C and Al quantified by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). To determine Al in the water samples, they were diluted 10x in 2% HNO_3 and directly injected into the ICP-MS unit.

2.3. Collection of semen

The fish were always collected in the morning. Before collection, spermiation was induced with pituitary extract of common carp, *C. carpio*, at a dose of 5 $mg.kg^{-1}$ body mass. Induction was carried out at different times during exposure, taking the treatment temperature into account. Spermiation is faster at higher temperatures: for fish kept at 20 ºC – hypophysation (induced breeding) was initiated 13 h before collection; for fish kept at 25 ºC, 11 h before collection;

and for fish kept at 30 ºC, 9 h before collection. Fish from different experimental groups were anesthetized with eugenol-based solution (clove oil) (1 ml eugenol: 10 ml ethanol: 10000 ml tank water). After collecting the semen and blood, the anesthetized fish were euthanized by transection of the spinal cord at the operculum (Schreck and Moyle, 1990).

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

2.4. Quantification of aluminum in semen and testes

Al was quantified in an aliquot of 5 µL semen, diluted in 100 µL saline solution (0.9% NaCl). Samples were frozen at -20 °C and Al quantified by ICP-MS following the protocol in Batista *et al.* (2009). The removed testes were frozen for Al quantification by ICP-MS, following the protocol in Aguiar *et al.* (2012).

2.5. Comet assay on spermatozoa

A quantity of semen (10 µL) was removed, diluted in 1000µL fetal bovine serum (Gibco; Thermo Fisher Scientific) and the solution from each fish/treatment stored at 4 °C for three days before performing the comet assay (as described for blood samples).

2.6. Blood collection and blood tests

Blood was collected by caudal vasculature puncture using a heparin coated syringe (5000 UI, Hepamax®). The blood (10 µL) was diluted in 1000 µL of cryopreservation buffered solution (250 mM Saccharose, 40 mM Trisodic Citrate, 5% DMSO, pH 7.6) (Evrard *et al.*, 2010; Jackson *et al.*, 2013). The samples were

frozen for one month and thawed in a polystyrene box at 4 ºC. Then, 10 µL of the cell suspension was added to 120 µL of low melting agarose (0.8%) at 37 °C. To run the comet assay, the slides were precoated with agarose (1% w/v in PBS buffer). Two slides were prepared for each fish/treatment and incubated in the refrigerator in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% TRITON X-100, 0.01M Sarcosine) at pH 10 for one hour. The slides were then transferred to a horizontal electrophoresis cell and placed in denaturation buffer (300 mM NaOH, 100 mM EDTA, pH 13) for 30 minutes in the dark. The samples were then electrophoresed (25V and 300mA) for 20 minutes, and the slides placed in neutralization buffer (0.4M Tris - 15 minutes), fixed with 100% ethanol (10 minutes) and stored in the refrigerator for further analysis. For fluorescence microscopy analysis (200x), 50 µL of GelRed (Biotium) were deposited on each slide and 100 nucleoids counted, according to fragment migration, and classified as: class 0 (no damage); class 1 (low damage); class 2 (average damage) and class 3 (high damage), based on Kobayashi *et al.* (1995). The scores of each experimental group were calculated by multiplying the number of nucleoids visualized in each class by the value of the class (0, 1, 2 or 3). Finally, the scores of all the individuals subjected to a given treatment were summed and divided by the number of individuals to obtain the mean score. The DNA data produced by the comet assay were ranked based on scores from zero (minimum degree of fragmentation) to 300 (maximum degree of fragmentation).

To run the micronuclei and nuclear abnormalities test, blood smears (5µL) were prepared on clean glass slides (duplicate) and left to dry overnight. The slides were then fixed in methanol PA (100%) for 10 minutes, washed with distilled water, stained with 10% Giemsa (25 minutes), washed with distilled water, dried under ambient conditions (24 hours), coated with Permount coverslips (Fisher-SP15-500) for analysis with an optical microscope (1000x) and the micronuclei and nuclear abnormalities (binucleate, segmented, kidney, lobed and blebbed) counted and identified according to Carrasco *et al.* (1990). A

thousand cells (totaling 12000/group) were counted on each slide and the results expressed as percentage frequency (%).

2.7. Statistical analysis

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

3. Results

3.1. Water chemistry and animal survival

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

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

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

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

Treatment	pH	Temperature	Dissolved Oxygen	Total Al	Dissolved Al
24 h		ºC	$mg.L^{-1}$	$mg.L^{-1}$	$mg.L^{-1}$
1	7.40 ± 0.05	19.37 ± 0.06	8.63 ± 0.06	0.08 ± 0	-
2	5.65 ± 0.08	18.68 ± 0.17	8.63 ± 0.08	0.08 ± 0	-
3	5.49 ± 0.08	18.37 ± 0.13	8.71 ± 0.13	0.45 ± 0.05	0.36 ± 0.09
4	7.50 ± 0.10	24.52 ± 0.19	6.82 ± 0.19	0.08 ± 0	-
5	5.73 ± 0.04	24.62 ± 0.23	7.04 ± 0.10	0.06 ± 0	-
6	5.50 ± 0.07	24.97 ± 0.09	7.26 ± 0.11	0.42 ± 0.04	0.38 ± 0.08
7	7.72 ± 0.06	30.88 ± 0.25	5.87 ± 0.11	0.08 ± 0	-
8	5.65 ± 0.16	29.92 ± 0.10	5.77 ± 0.07	0.07 ± 0	-
9	5.38 ± 0.09	31.27 ± 0.07	5.71 ± 0.05	0.47 ± 0.01	0.44 ± 0.02
Treatment	pH	Temperature	Dissolved Oxygen	Total Al	Dissolved Al
96 h		ºC	$mg.L^{-1}$	$mg.L^{-1}$	$mg.L^{-1}$
1	7.62 ± 0.02	20.69 ± 0.07	7.72 ± 0.08	0.08 ± 0	-
2	5.38 ± 0.02	19.95 ± 0.17	8.06 ± 0.10	0.07 ± 0	-
3	5.37 ± 0.02	20.44 ± 0.15	8.05 ± 0.11	0.54 ± 0.01	0.48 ± 0.02
4	7.49 ± 0.06	24.56 ± 0.11	7.53 ± 0.06	0.08 ± 0	-
5	5.42 ± 0.07	24.48 ± 0.11	7.28 ± 0.12	0.09 ± 0	-
6	5.47 ± 0.05	24.90 ± 0.13	7.25 ± 0.10	0.53 ± 0.01	0.50 ± 0.02
7	7.66 ± 0.03	31.24 ± 0.07	6.15 ± 0.04	0.07 ± 0	-
8	5.31 ± 0.07	30.44 ± 0.20	6.19 ± 0.06	0.07 ± 0	-
9	5.39 ± 0.05	31.27 ± 0.20	6.29 ± 0.07	0.53 ± 0.01	0.49 ± 0.02

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

3.2. Bioconcentration of Al in testes and semen

In the 24 h bioassay (Fig. 1A), there was no difference in Al concentration in the testes for any of the treatments. In the fish exposed for 96 h, the effect of water pH on Al levels in the testes was temperature-dependent (P=0.004). No significant difference was observed in the bioconcentration of Al in the testes at different temperatures in the fish exposed to neutral pH. In the fish exposed to acid pH (with or without Al) there was an increase in Al concentration in the testes as the temperature increased (P<0.001). On analyzing the experimental groups at different temperatures, there was no difference between groups at 20 ºC (P=0.30). At 25 ºC, the fish exposed to acid pH with Al exhibited a higher bioconcentration of Al in the testes compared to the other groups (neutral pH - P=0.004; acid pH- P=0.01). At 30 ºC, the fish kept at neutral pH exhibited a lower concentration of Al in the testes than those in the other experimental groups, and the fish exposed to acid pH with Al exhibited the highest concentration of Al (13.7 ± 1.98 µg.g^{-1}; P<0.001) (Fig. 1B).

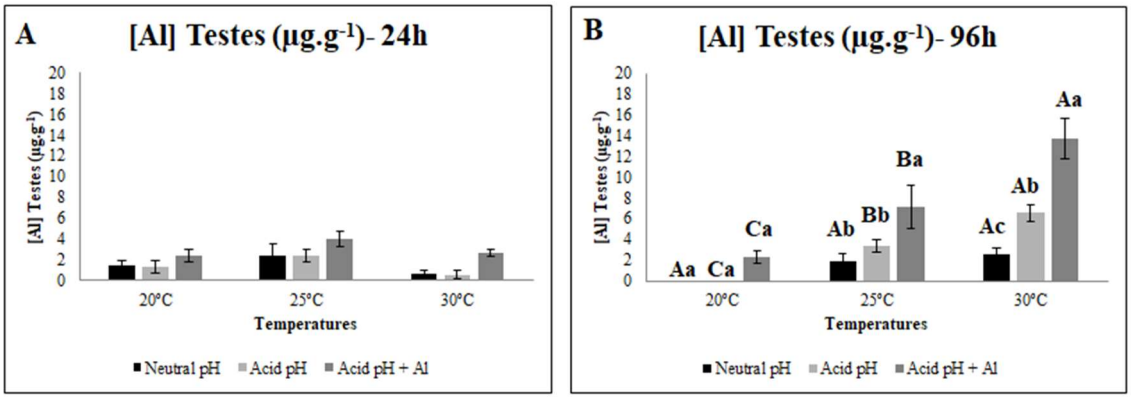

Fig. 1. Concentration of aluminum in the testes (µg.g^{-1}) for each experimental group at exposure times of 24 h (A) and 96 h (B) (n=6). T1) water at 20 ºC, no Al, neutral pH; T2) water at 20 °C, no Al, acid pH; T3) water at 20 ºC, with Al, acid pH; T4) water at 25 °C, no Al, neutral pH (control group);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

In the semen, Al was observed in the ejaculate of all experimental groups at both 24 (Fig. 2A) and 96 h (Fig. 2B). In the 24 h assay at acid pH, the concentration of Al was higher at 25 ºC than at 30 ºC (P=0.02). In acid pH + Al, Al concentration was higher at 20 and 25 ºC compared to the fish exposed at 30 ºC (P=0.04, P=0.02). For different treatments at the same temperature, a significant difference was observed only at 20 ºC; fish exposed to acid pH + Al exhibited a higher concentration of Al in the semen than those exposed to neutral and acid pH (Fig. 2A). In the fish exposed for 96 h at 30 °C, the concentration of Al was higher in the semen of fish exposed to acid pH + Al than in those in the other experimental groups (P=0.006) (Fig. 2B).

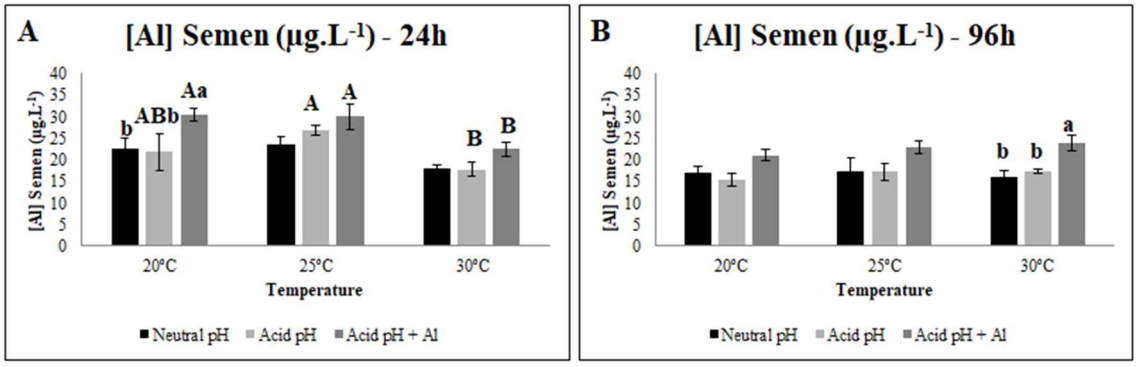

Fig. 2. Concentration of aluminum in the semen (µg.L^{-1}) for each experimental group at exposure times of 24 h (A) and of 96 h (B) (n=6). T1) water at 20 ºC, no Al, neutral pH; T2) water at 20 °C, no Al, acid pH; T3) water at 20 ºC, with Al, acid pH; T4) water at 25 °C no Al, neutral pH (control group);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

3.3. Comet assay: blood and semen

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

In the 24-h experimental groups, the DNA fragmentation score in the semen of fish exposed to neutral pH was higher at 25 ºC and 30 ºC than at 20 ºC

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

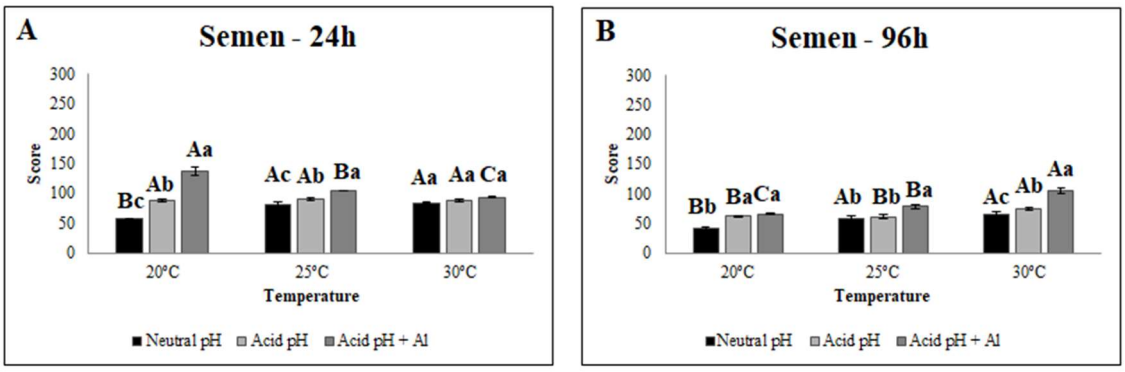

Fig. 3. DNA fragmentation score (0-300) for spermatozoa of *Astyanax altiparanae,* obtained by the comet test. The fish were exposed in experimental groups for 24 h (A) and 96 h (B) (n=6). T1) water at 20 ºC, no Al, neutral pH; T2) water at 20 °C, no Al, acid pH; T3) water at 20 ºC, with Al, acid pH; T4) water at 25 °C no Al, neutral pH (control group); T5) water at 25 °C, no Al, acid pH; T6) water at 25 °C, with Al, acid pH; T7) water at 30 °C, no Al, neutral pH; T8) water at 30 °C, no Al, acid pH; T9) water at 30 °C, with Al, acid pH. Different uppercase letters indicate significant differences (P<0.05) for the same treatment at different temperatures; different lowercase letters indicate significant differences (P <0.05) at the same temperature for different treatments.

In the erythrocytes (24 h, Fig. 4A), DNA fragmentation in fish exposed to neutral and acid pH (no Al) increased with rising temperature. With the addition

of Al, fragmentation was higher at 20 and 30 ºC than at 25 ºC (P=0.02). An analysis of erythrocyte fragmentation scores at each temperature showed that, at 20 ºC, fish exposed to acid pH + Al exhibited a higher degree of fragmentation than fish in the other experimental groups. At 25 ºC, fish exposed to acid pH (with and without Al) exhibited higher fragmentation compared to fish exposed to neutral pH. At 30 ºC, the fragmentation score was higher in fish exposed to neutral pH compared to the other groups (P=0.02). At 96 h (Fig. 4B), fish exposed to neutral and acid pH (no Al) exhibited a significant increase in the fragmentation score with increasing temperature. When Al was added, the fragmentation score was higher at 20 ºC than at 25 and 30 ºC (P=0.02). In overall terms, DNA fragmentation at 20 and 25 ºC was higher in fish exposed to Al compared to the other groups.

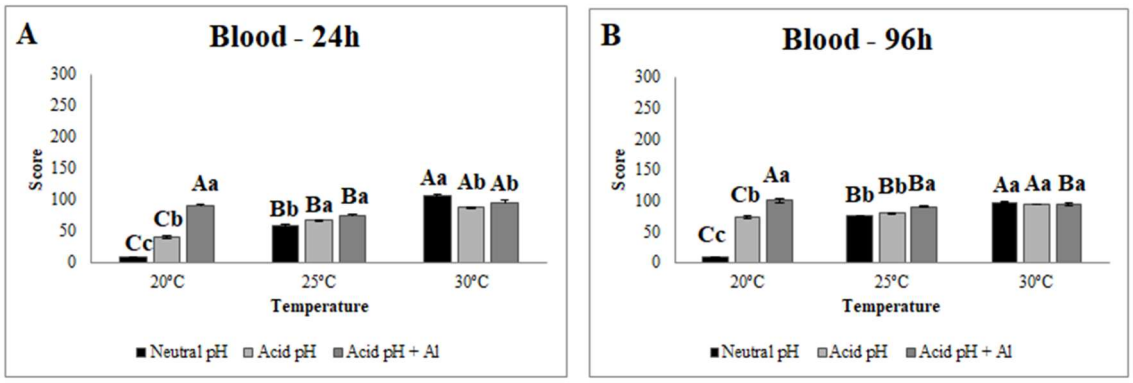

Fig. 4. DNA fragmentation score (0-300) for erythrocytes of *Astyanax altiparanae,* obtained by the comet test. The animals were exposed in experimental groups for 24 h (A) and 96 h (B) (n=6). T1) water at 20 ºC, no Al, neutral pH; T2) water at 20 °C, no Al, acid pH; T3) water at 20 ºC, with Al, acid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

3.4. Micronucleus blood test

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

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

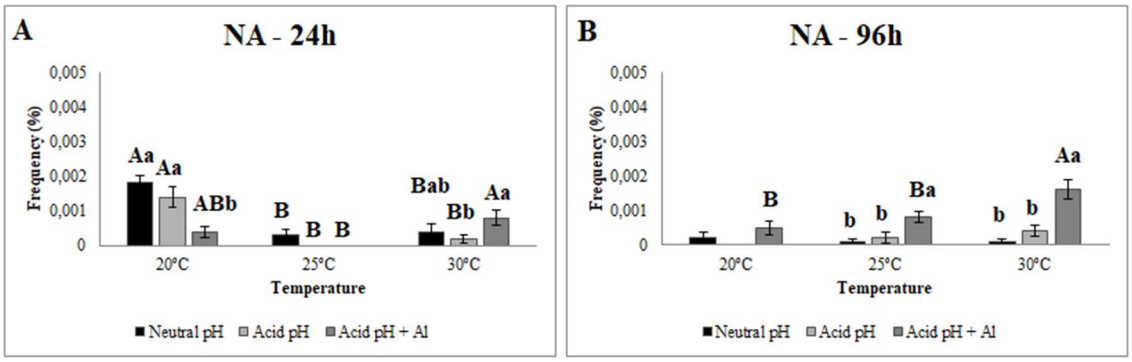

Fig. 5. Frequency of nuclear abnormalities (NA) in erythrocytes of *Astyanax altiparanae*. The experimental groups of fish were exposed for 24 h (A) and 96 h (B) (n=6). T1) water at 20 ºC, no Al, neutral pH; T2) water at 20 °C, no Al, acid pH; T3) water at 20 ºC, with Al, acid pH; T4) water at 25 °C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

4. Discussion

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

4.1. Water physicochemical properties and fish survival

The physicochemical properties of the water (e.g. temperature, pH and dissolved oxygen) are of fundamental importance in ecotoxicological bioassays, since they can influence biomarkers and fish survival. Moreover, the properties of the water directly affect the solubility of metals and their bioavailability to aquatic organisms, since changes in these properties promote the speciation of the metals, making them more or less toxic (Namiesnik and Rabajczyk, 2010). Al is amphoteric, and therefore changes in pH are followed by Al speciation. In addition to pH, other parameters that influence the toxicity and bioavailability of Al are temperature, binder concentration and the ionic strength of the solution (Matus and Kubova, 2005).

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

The survival of the fish in this study was influenced by high temperature (30 ºC) combined with the presence of Al in the water (96 h). It is possible that the lower survival rate at 30 ºC with the addition of Al was due to the Al potentiation effect in combination with higher temperature and acid pH. Under

these conditions, polymerization of Al in the gills increases leading to functional hypoxia, a fact corroborated by the behavior of the fish in this group (seeking oxygen on the surface of the water when oxygen was available in the water). These findings are corroborated by Poleó and Muniz (1993), who reported that Al toxicity in *Salmo salar* depends not only on pH but also on temperature, emphasizing the importance of these variables when evaluating the effects of Al on fish.

4.2. Bioconcentration of Al in the testes and semen

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

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

These results show the plasticity of fish under Al stress, responding by reproducing as fast as possible.

In teleosts, the effects of Al on male reproductive organs/cells have not been investigated to date. In the nineties, it was been shown that Al affects mammals by inhibiting testosterone synthesis (*in vitro)* in Leydig cells (Laskey and Phelps, 1991). Exposure of mammals to Al has also been shown to reduce testosterone levels, trigger histopathological changes in the testes, reduce sperm concentration, motility and viability, and increase sperm abnormalities in Wistar rats (Cheraghi *et al.*, 2017). Wistar rats fed for 60 days on a diet containing 1.5 $mg.kg^{-1}$ of Al (mimicking human exposure to this metal) exhibited Al in germ cells, and even at low concentration Al was found to impair spermatogenesis and sperm quality (Martinez *et al.*, 2017). The same authors reported an increase in oxidative stress and inflammation, highlighting the toxic action pathways of Al in the male reproductive system (Martinez *et al.*, 2017). In human semen, Klein *et al.* (2014) found high concentrations of Al in French patients and discussed the implications of these levels for spermatogenesis and sperm concentration.

In regard to the effects of temperature, the data show that the pattern of Al bioconcentration in the testes and semen differs according to exposure time. Exposure to Al for 24 h was not sufficient to trigger Al bioconcentration in the testes, even at different temperatures, but after 96 h exposure at acid pH, higher temperature boosted the bioconcentration of Al in the testes. In the semen, the effects were observable after only 24 h, with higher levels of Al at 20 and 25 ºC compared to 30 ºC. However, after 96 h exposure the pattern changed and the fish exposed to Al at 30 ºC exhibited higher levels of Al in their semen. We suggest that the bioconcentration of Al in the semen occurred faster than in testes due to its fluid consistency and the spermatozoa renewal rate during spermatogenesis. However, no studies have been carried out so far comparing the concentration of metals in the testes and semen.

4.3. Comet assay

One of the tools used to verify the effects of a compound on tissue is the comet assay, which is a standard method for determining *in vivo/in vitro* genotoxicity, and is sensitive, robust, versatile and powerful enough to evaluate damage caused by clastogenic agents by measuring DNA strand breaks in animal and plant cells (Lapuente *et al.*, 2015). The comet assay has therefore been used in several ecotoxicological studies to measure reversible DNA damage caused by caffeine (Santos-Silva *et al.*, 2018), pesticides and hydrocarbons (Bianchi *et al.*, 2017) and trace metals (D'Costa *et al.*, 2017). The degree of fragmentation detected herein by the comet assay suggests that Al binds to the DNA-histone complex causing conformational chromatin changes, as found in the human nervous system (Lukiw *et al.*, 1987), and may cause cell DNA fragmentation. Using the comet assay, Al genotoxicity has been found in the erythrocytes of *Prochilodus lineatus* (Galindo *et al.*, 2010) and *Cyprinus carpio* (Gómez-Oliván *et al.*, 2017), and in the liver of *Rhamdia quelen* (Klingelfus *et al.*, 2015).

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

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

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

4.4. Micronucleus test

In addition to the comet assay, the micronucleus test and the presence of nuclear abnormalities are widely used as *in vivo/in vitro* biomarkers in molecular epidemiology and the investigation of cytogenetic damage. A micronucleus consists of acentric fragments expelled from the main nucleus in the late stages of anaphase (Kirsch-Volders *et al.*, 2003). These fragments can be formed by two mechanisms: clastogenesis (chromosomal breaks) and aneugenesis (dysfunction of the mitotic apparatus). Nuclear erythrocyte abnormalities may be indicative of errors during cell division (Carrasco *et al.*, 1990; Udroiu, 2006). It is important to

combine the results of the comet assay and micronucleus test with mutagenic tests, since they are sensitive, powerful, simple, versatile and cost-effective (Araldi *et al.*, 2015). In this study, a low frequency of nuclear abnormalities was observed, possibly due to the short exposure time. Nuclear abnormalities in fish erythrocytes are more frequent when the fish are exposed for long periods to genotoxic agents (Osman *et al.*, 2010; Vicari *et al.*, 2012). This fact can be observed in the natural environment, where a high number of nuclear abnormalities are observed as a result of the impacts of human activities, providing a bioindicator for water quality and organism survival potential (Gutiérrez *et al.*, 2015; Viana *et al.*, 2017; Hussain *et al.*, 2018). According to Alink *et al.* (2007), prolonged exposure to low concentrations of genotoxic agents aggravates these effects on fish cells, and in other aquatic organisms. In this study, the micronucleus test, a biomarker that evaluates irreversible erythrocyte DNA damage, was not useful, suggesting that Al causes reversible DNA damage (evaluated by the comet assay) to the erythrocytes and spermatozoa of *A. altiparanae*. The data suggest that semen quality parameters, such as semen physicochemical characteristics, morphology and sperm kinetics, should also be investigated to predict impacts on the reproduction of these organisms.

5. Conclusion

Under the experimental conditions described herein, it can be stated that Al, even at environmental concentrations, bioconcentrates in the testes after 96 hours of exposure and this accumulation increases at higher temperature. In the semen, accumulation occurs sooner, just after 24 h exposure. In conjunction with water temperature and acid pH, this accumulation triggers cytotoxic and genotoxic effects, causing reversible DNA damage to the erythrocytes and spermatozoa of *A. altiparanae*.

Acknowledgments

This study was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; Processes 2016/08770-8 and 2014/16320-7), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Process 140197/2016-8) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) - Finance Code 001. The authors would also like to thank the fish farm at *Companhia Energética de São Paulo* (CESP, Paraibuna, SP) for donating the fish, the staff at LAMEROA (IB-USP) for their help, the Evolution and Development of Invertebrates Lab (Prof. Dr. Federico Brown) at the University of São Paulo (IB-USP) for microscopy support, the staff at Laboratório de Ecofisiologia Animal (Prof. Dr. Claudia Martinez; UEL – Londrina/PR) for the training provided in genotoxic testing and IB/USP for providing logistics and facilities.

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Capítulo 2

O alumínio, em concentração ambiental, associado ao pH ácido e à alta temperatura da água, causa prejuízo na qualidade espermática do teleósteo de água doce *Astyanax altiparanae* (Teleostei: Characidae)

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)

Publicado na Revista Environmental Pollution 262, 114252, 2020 [3].

DOI: 10.1016/j.envpol.2020.114252

[3] Pinheiro, J.P.S., Assis, C.B., Sanches, E.A., Moreira, R.G., 2020. 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). Environ. Pollut. 262, 114252. https://doi.org/10.1016/j.envpol.2020.114252

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)

João Paulo Silva Pinheiro[1], Cecilia Bertacini de Assis[1], Eduardo Antônio Sanches[2], Renata Guimarães Moreira[1]

1. Universidade de São Paulo, Institute of Biosciences, Department of Physiology, Laboratory of Metabolism and Reproduction of Aquatic Organisms - LAMEROA; Matão Street, 14 lane, number 101 - room 220, Cidade Universitária - São Paulo – SP- Brazil.
2. Universidade Estadual Paulista, Fishery Engineering Course, Nelson Brihi Badur Avenue, 430, Registro – São Paulo - SP – Brazil.

João Paulo Silva Pinheiro: joaopaulospinheiro@yahoo.com.br
Cecília Bertacini de Assis: cbertacini4@gmail.com
Eduardo Antônio Sanches: sanches@registro.unesp.br
Renata Guimarães Moreira: renatagm@ib.usp.br

Corresponding author: João Paulo Silva Pinheiro: joaopaulospinheiro@yahoo.com.br
+551130917531

Resumo

Dada a toxicidade de metais, incluindo alumínio (Al), e os efeitos da temperatura da água sobre os ectotérmicos, investigamos o efeito individual ou de associação dessas variáveis (Al + pH ácido + mudanças de temperatura) na qualidade seminal de *Astyanax altiparanae*. Machos adultos foram divididos em nove grupos experimentais com base na combinação de cada uma das três temperaturas da água (20, 25 e 30 ºC) com valores de pH neutro e ácido (7,0 e 5,5, respectivamente) com ou sem 0,5 mg L^{-1} de Al. Os peixes foram submetidos à exposição subaguda e semiestática e às 24 e 96 horas foram avaliados quanto aos parâmetros seminais: (1) pH; (2) osmolalidade; (3) concentração espermática; (4) morfologia espermática; (5) cinética espermática; e (6) ultraestrutura espermática. Em 30 ºC, o Al reduziu a osmolalidade (24 e 96 h) e a concentração espermática (24 h). Ao analisar a cinética espermática (30 segundos pós-ativação), o Al causou uma redução na motilidade total em todas as temperaturas (24h), e quando este tempo de exposição foi maior (96 h), tanto o pH ácido quanto a adição de Al na água causaram redução da motilidade. Ao analisar a velocidade curvilinear (VCL) 30 segundos após a ativação do esperma (24 e 96 h), o pH ácido causou uma redução na motilidade dos espermatozoides em 20 e 30 ºC, mas em 25 ºC o Al desencadeou essa redução. Finalmente, o Al na água causou mudanças ultraestruturais na cabeça do espermatozoide, peça intermediária e flagelo, independentemente da temperatura da água. Além disso, verificou-se que a combinação de Al em 30 ºC causou uma redução na área da cabeça do esperma, enquanto em 20 ºC, o Al desencadeou uma redução na área da peça intermediária. Portanto, a acidez influenciou alguns parâmetros do espermatozoide de *A. altiparanae*, mas o Al na água acentuou esses efeitos na qualidade seminal, especialmente na osmolalidade e na concentração, na cinética e na ultraestrutura espermáticas. Essa toxicidade também foi influenciada por mudanças na temperatura da água.

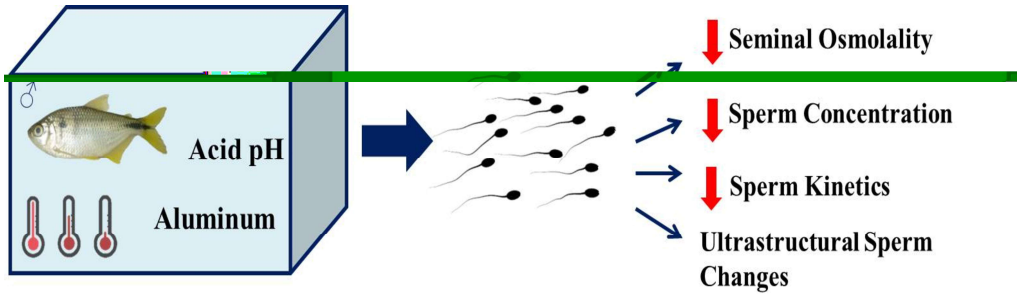
Palavras-chave: Peixe; Metal; Reprodução; Espermatozoides; Exposição subaguda

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 ºC) 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 ºC, 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 ºC, but at 25 ºC 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 ºC caused a reduction in sperm head area while at 20 ºC, 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

Graphical Abstract



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 (Poléo 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; Chehade 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 30ºC) 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, Lt = 8.40 ± 0.05 cm; Wt = 7.45 ± 0.16 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 ºC) *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 ºC, no Al, neutral pH; (2) water at 20 °C, no Al, acidic pH; (3) water at 20 ºC, 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 ºC 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 $Al_2(SO_4)_3.18H_2O$ (Sigma Aldrich) and 65% HNO_3 (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 ºC; 11 h before collection for animals kept at 25 ºC; 9 h before collection for animals kept at 30 ºC). 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 ºC). 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; 25ºC; 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 μm s^{-1}, VAP = 6 μm s^{-1} and VSL = 1 μm 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 Al in the testes of the control groups for each temperature and the presence of Al 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 Al 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 ± 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 Al 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 ºC 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 ºC *versus* 25 ºC; P = 0.14 – 25 ºC *versus* 30 ºC; P = 0.05 – 20 ºC *versus* 30 ºC). However, at acidic pH and acidic pH + Al, seminal pH decreased in animals kept at 30 ºC (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 ºC.

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.

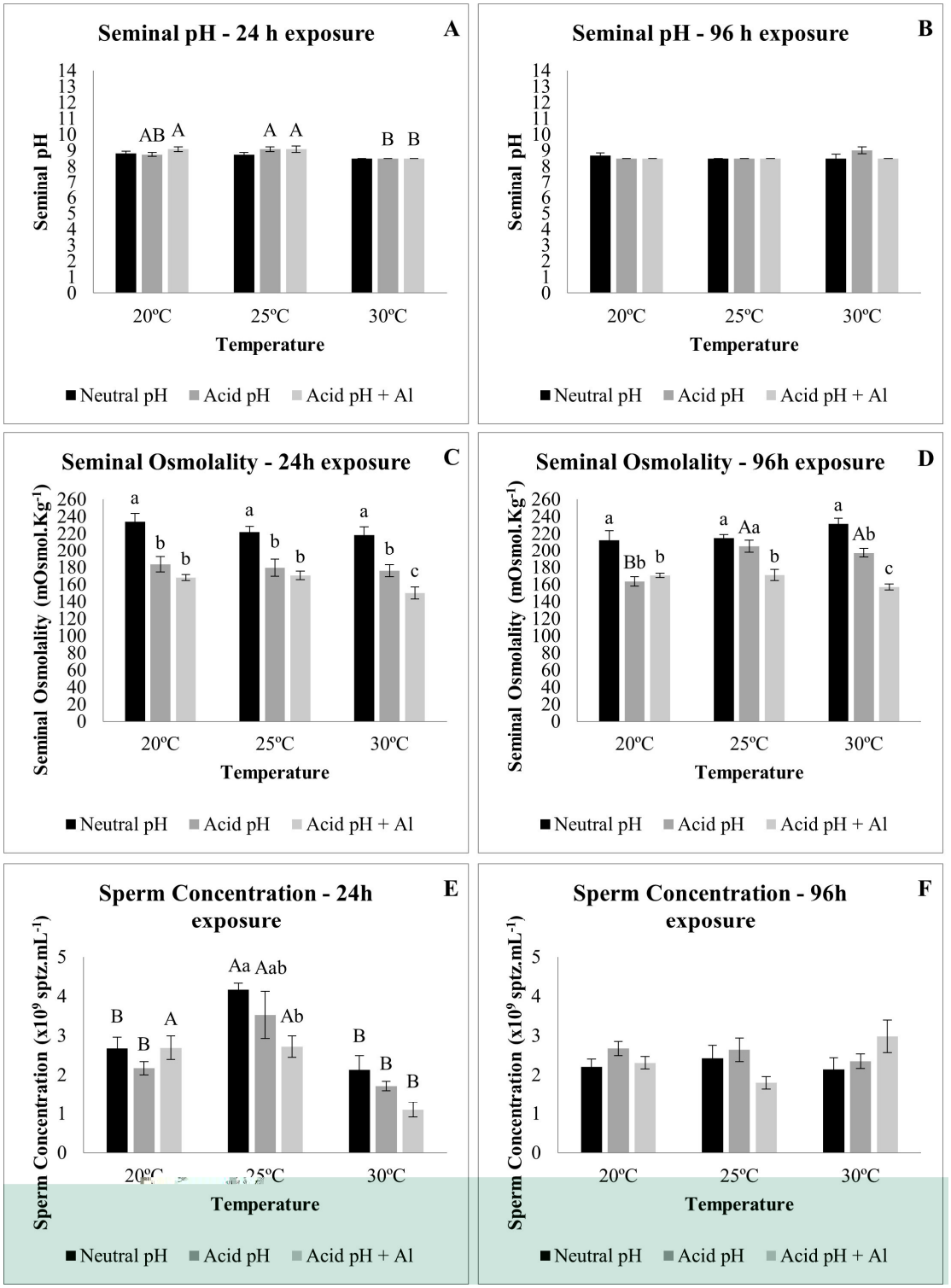


Fig. 1. Physicochemical characteristics of Astyanax altiparanae semen after exposure to different temperatures, pH values, and presence or absence of aluminum (mean ± standard error of the mean). A. Seminal pH (exposure for 24 h); B. Seminal pH (exposure for 96 h); C. Seminal Osmolality (exposure for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

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 ± 18.26 mOsmol kg^{-1} in cyprinids, from 232 ± 13 to 332 ± 5.1 mOsmol.kg^{-1} in salmonids, and from 38 ± 3 to 93.6 ± 7.3 mOsmol.kg^{-1} in acipenserids (Alavi and Cosson, 2006). The seminal osmolality of *A. altiparanae* was 224.83 ± 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 ºC 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 ºC, 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 ºC, 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 ºC, this decrease was only observed in the group exposed to acid pH and Al ($P < 0.001$). Already at 30 ºC, 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 ºC ($P < 0.001$) and 30 ºC ($P < 0.001$) compared to 20 ºC. Thus, it can be emphasized that the association of higher temperatures (25 and 30 ºC), 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

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.

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 ± standard error of the mean.

Sperm Morphology - % Normal			
Treatment	**24 h Exposure**		
	20ºC	**25ºC**	**30ºC**
Neutral pH	99.92 ± 0.08	99.83 ± 0.17	99.92 ± 0.08
Acid pH	99.80 ± 0.11	100 ± 0	99.83 ± 0.11
Acid pH + Al	99.83 ± 0.11	99.58 ± 0.20	99.50 ± 0.30

Treatment

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 Al at 30 ºC compared to 25 ºC ($P = 0.006$). Also, by analysing the different groups within the same temperature, it was observed that in acid pH with Al at 20 ºC and 30 ºC 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 Al. When comparing the experimental treatments within the same temperature, at 20 ºC, sperm motility was reduced when the animals were exposed to acidic pH ($30.53\% \pm 4.59\%$; $P < 0.001$) and acidic pH with Al ($19.16\% \pm 3.26\%$, $P < 0.001$) compared to neutral pH ($56.20\% \pm 4.42\%$). Already at both 25 and 30 ºC, sperm motility was reduced by more than 20% only when Al 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 Al presented lower sperm motility at 30 ºC than at 20 and 25 ºC. After comparing the different treatments at the same temperature, males at 30 ºC and acidic pH with Al 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 ºC ($P < 0.001$) and 30 ºC ($P < 0.001$) compared to 20 ºC. At acidic pH, sperm motility was also higher at 25 ºC and reduced at 30 ºC ($P < 0.001$) and 20 ºC ($P < 0.001$). At acidic pH with Al, the percentage of mobile sperm was higher at 25 and 30 ºC ($P < 0.001$ in both cases) than at 20 ºC. After comparing the different groups within the same temperature, the same pattern was observed at 20, 25, and 30 ºC: 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).

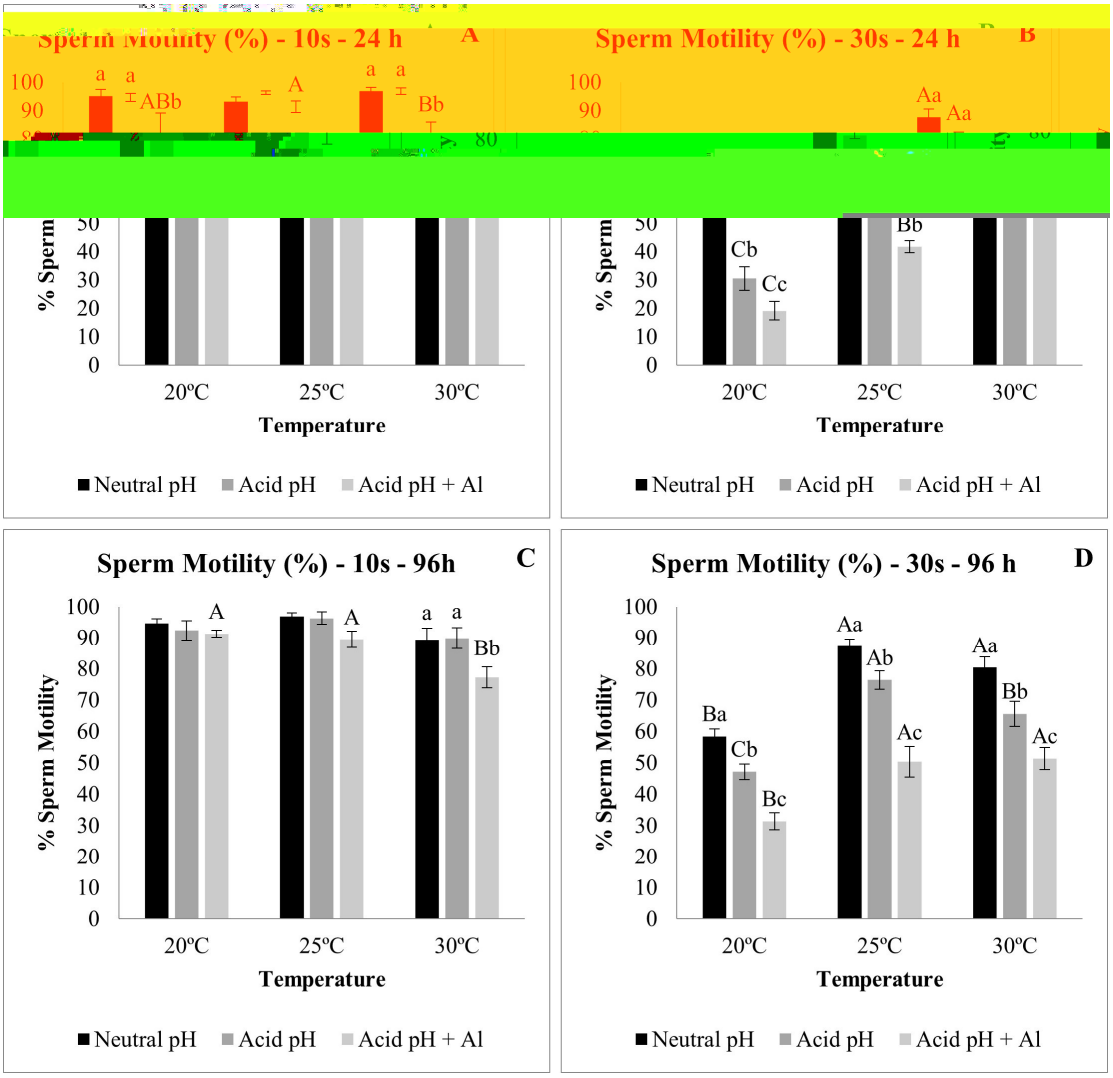


Fig. 2. Sperm motility (%) of male *A. altiparanae* after exposure at different temperatures, pHs and the presence or absence of Al. A. Sperm motility after 10 sec post-activation (animal exposure for 24 h); B. Sperm motility after 30 sec post-activation (animal exposure for 24 h); C. Sperm motility 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).

Adriaenssens et al. (2012) studied long-term exposure (five weeks) of mosquitofish males (*Gambusia holbrooki*) at different temperatures (cold acclimation: 18 ºC and warm acclimation: 30 ºC) 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 ºC < 25 ºC < 30 ºC). Already at acidic pH at 20 ºC and acid pH + Al at 20 and 25

ºC, 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.

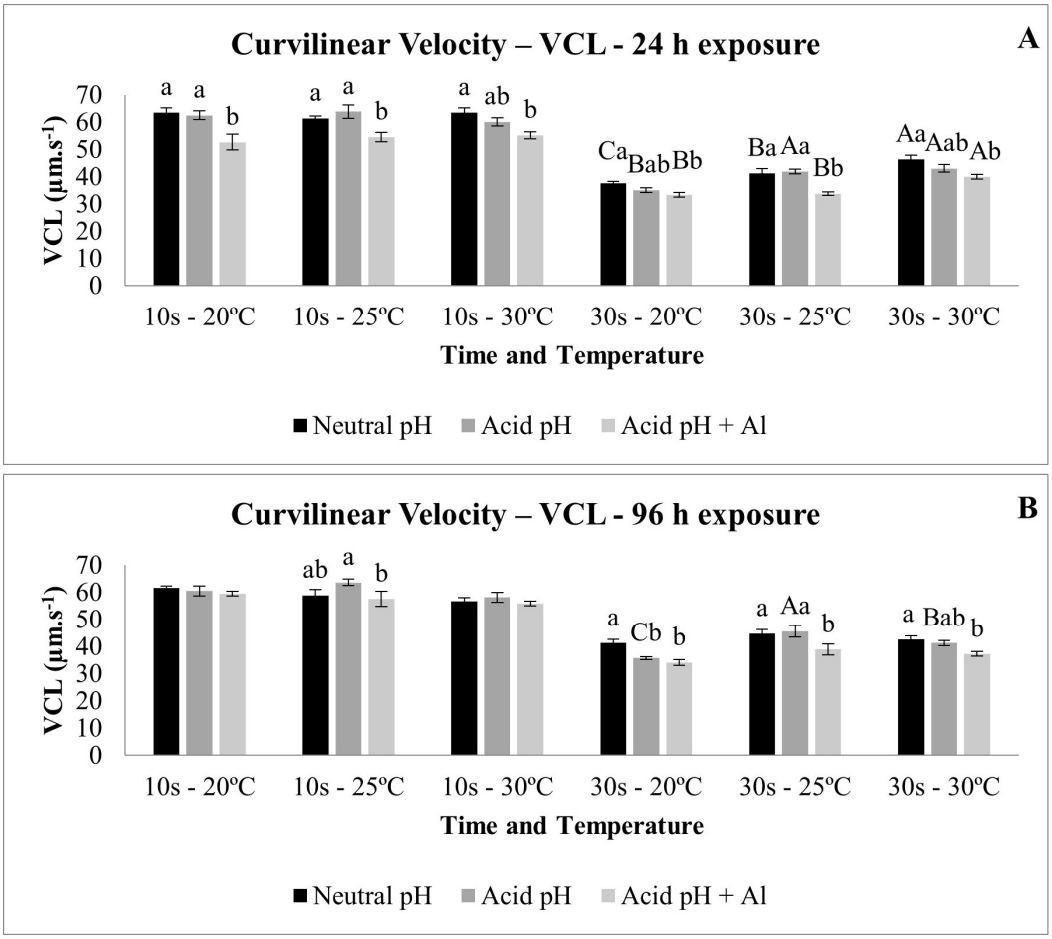


Fig. 3. Curvilinear velocity (VCL) of *A. altiparanae* sperm after 24 h (A) and 96 h (B) exposure at different temperatures, pHs, and the presence or absence of Al. Uppercase letters indicate differences within the same treatment at different temperatures; Lowercase letters indicate differences within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

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 ºC and 30 ºC) 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 ºC, 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 ºC ($P = 0.002$; $P = 0.016$).

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 ºC at 10 sec post-activation. At 30 sec pot-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 ºC and acidity triggered a decrease in VAP, but at 25 ºC, only the presence of Al caused this reduction (similar to 24 h).

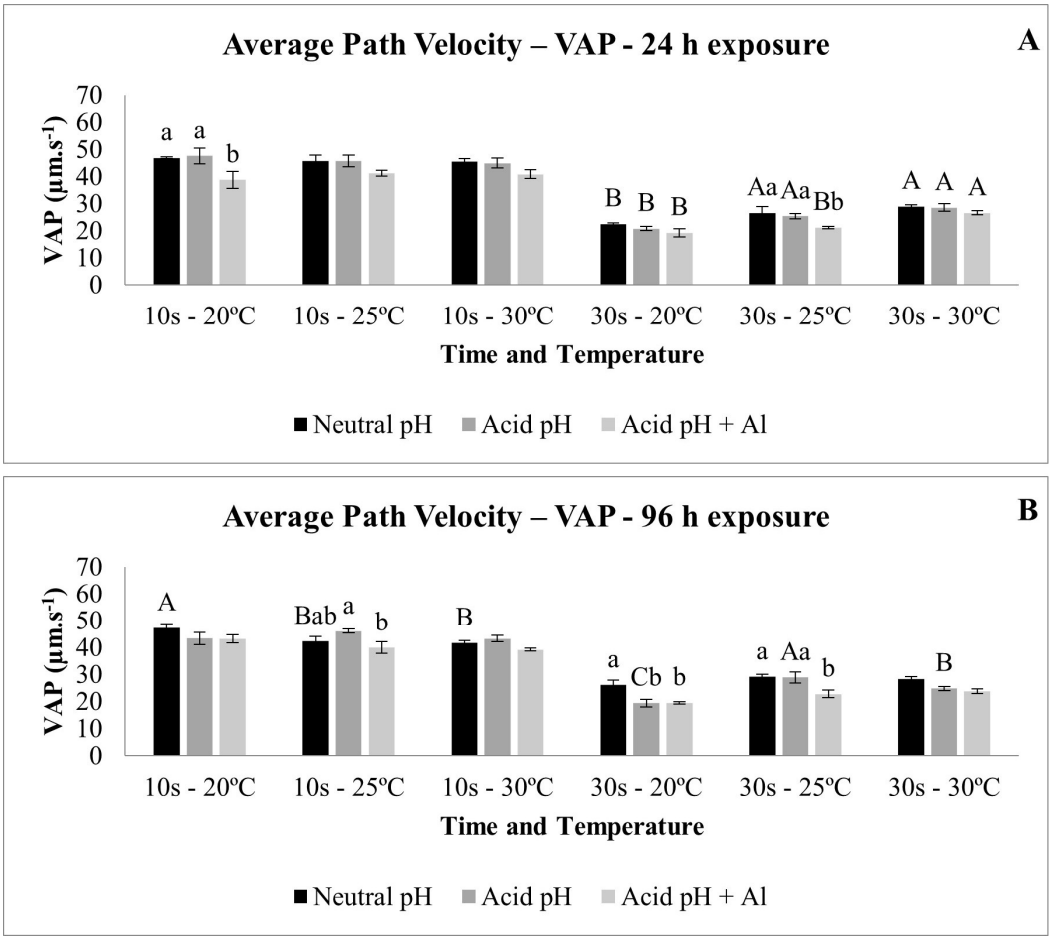


Fig. 4. Average path velocity (VAP) of *A. altiparanae* sperm after 24 h (A) and 96 h (B) exposure at different temperatures, pHs, and the presence or absence of Al. Uppercase letters indicate differences within the same treatment at different temperatures; lowercase letters indicate differences within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

Regarding VSL, after 24 h of exposure (Fig. 5A) was observed that the presence of Al at 20 ºC 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 ºC compared to 25 and 30 ºC was seen. However, at acidic pH + Al, the lowest values were observed at 20 and 25 ºC.

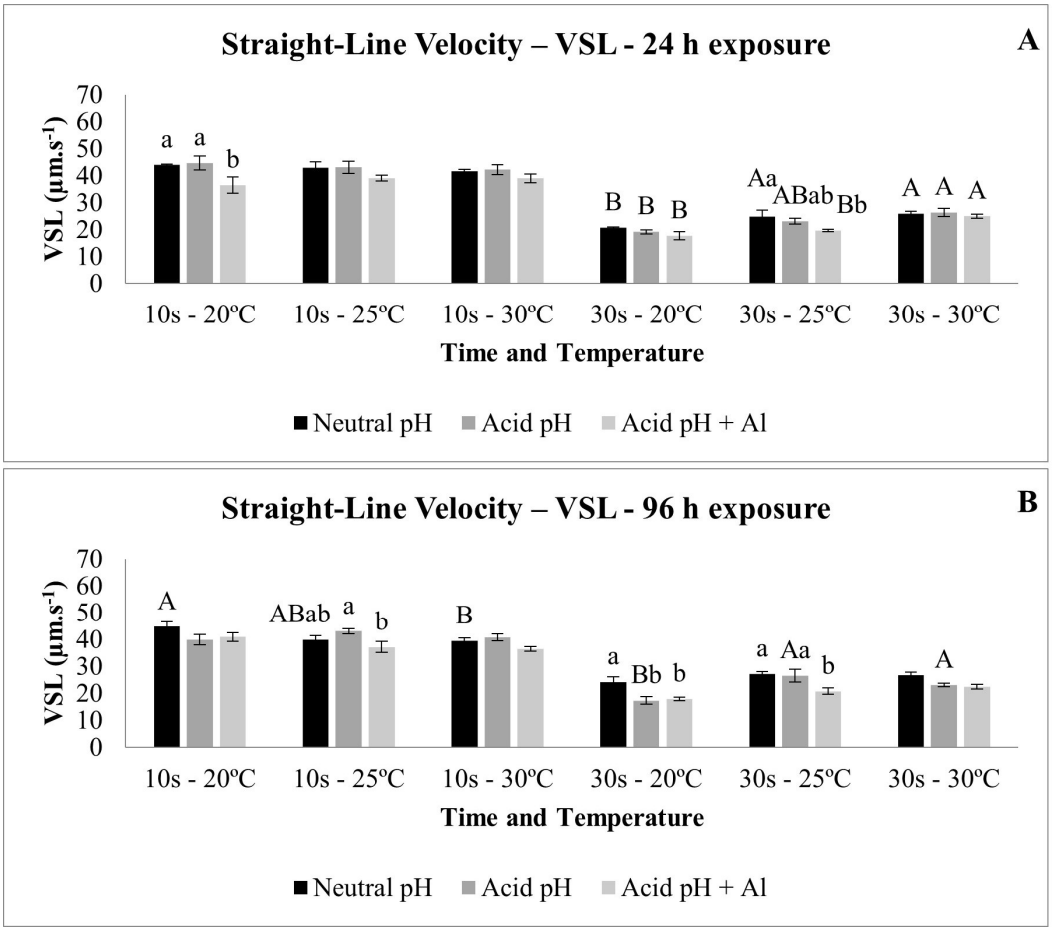


Fig. 5. Straight line velocities (VSL) of *A. altiparanae* sperm after 24 h (A) and 96 h (B) exposure at different temperatures, pHs, and the presence or absence of Al. Uppercase letters indicate differences within the same treatment at different temperatures; lowercase letters indicate differences within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

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 ºC. Also, when comparing the different groups at the same temperature, it was verified that acidity was responsible for the reduction in VSL at 20 ºC. However, at 25 ºC, 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-Góralska 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 ºC. 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).

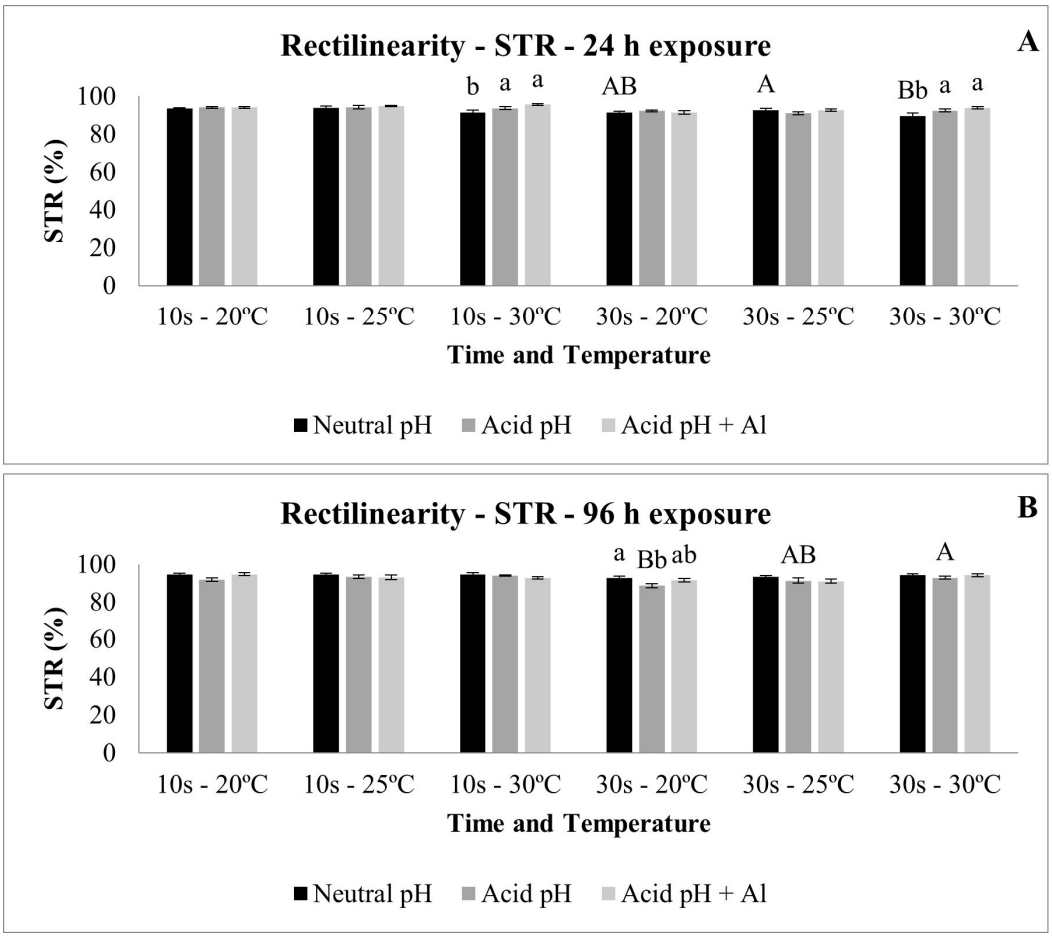


Fig. 6. Rectilinearity (STR) of *A. altiparanae* sperm after 24 h (A) and 96 h (B) exposure at different temperatures, pHs, and the presence or absence of Al. Uppercase letters indicate differences within the same treatment at different temperatures; lowercase letters indicate differences within the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

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 ± 0.02 μm, surrounded by a plasma membrane, totalling a nuclear area of 0.49 ± 0.01 μm^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 ± 0.04 μm and an area of 0.28 ± 0.01 μm^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 ºC. 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).

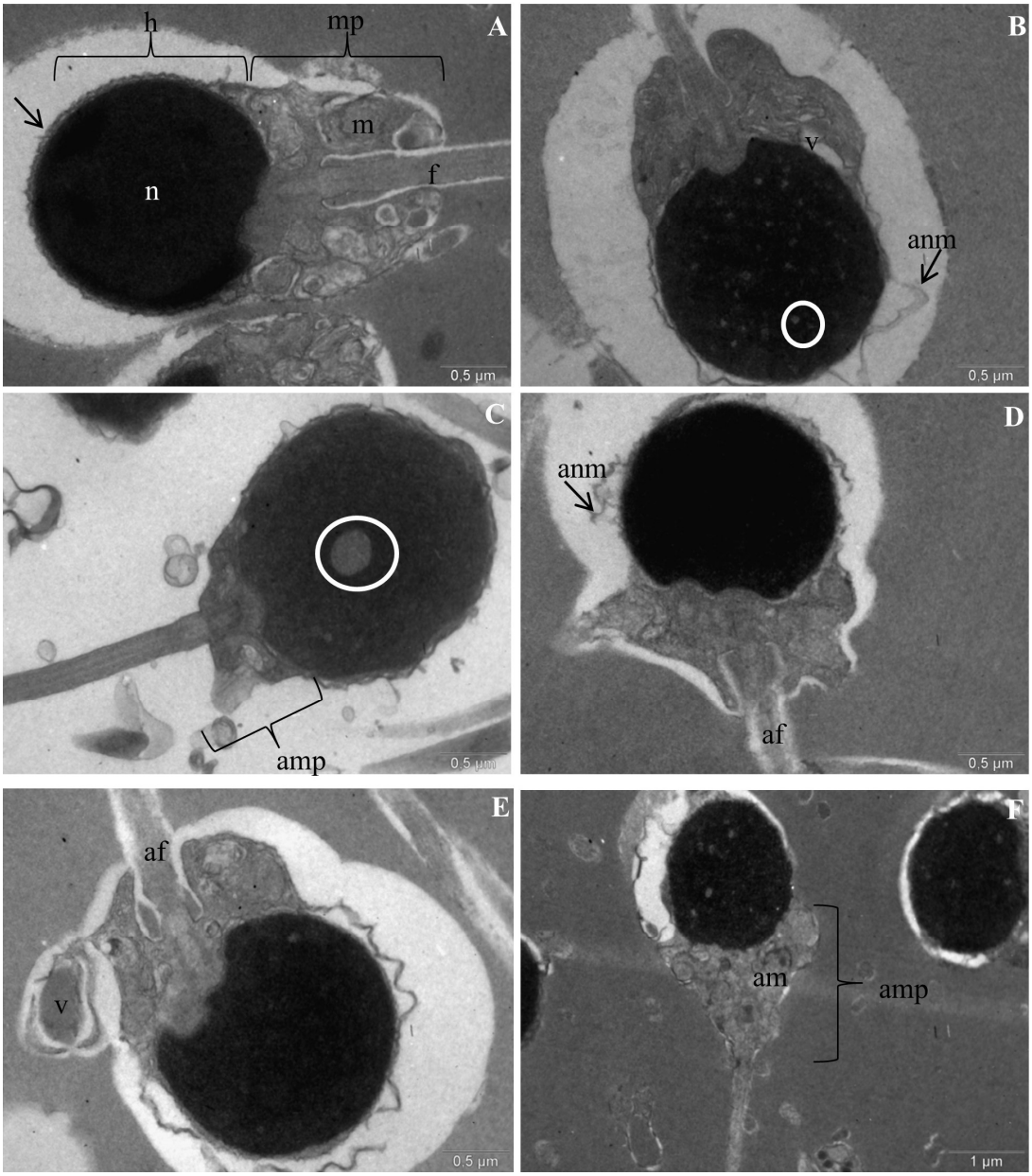


Fig. 7. Sperm ultrastructure of *A. altiparanae* after exposure to different temperatures and the presence or absence of Al. A. Normal spermatozoa (12.000 x; arrow: nuclear membrane; n: nucleus; h: head; mp: midpiece; m: mitochondria; f: flagellum; treatment: 25ºC, and neutral pH). B–F. Abnormal spermatozoa (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 20ºC, acid pH, and Al; E-F: treatment 30ºC, acid pH, and Al).

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 ºC 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 ºC (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 ºC.

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 ± standard error of the mean.

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

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 micropyle 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 Al 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 Al 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.

Acknowledgments

This study was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; Grants 2016/08770-8 and 2014/16320-7), the Conselho

Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Grant 140197/2016-8) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) - Finance Code 001. The authors would also like to thank the fish farm at *Companhia Energética de São Paulo* (CESP, Paraibuna, SP) for donating the fish, the staff at LAMEROA (IB-USP) for their help, the Centro de Aquisição de Imagens e Microscopia do Instituto de Biociências (Waldir Caldeira) at the University of São Paulo (IB-USP) for microscopy support, and IB/USP for providing logistics and facilities.

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Capítulo 3

A exposição paterna ao alumínio, acidez e temperatura afeta o perfil de ácidos graxos seminal, o desenvolvimento embrionário e larval de *Astyanax altiparanae*

Paternal exposure to aluminum, acidity, and temperature affect fatty acid seminal profile, embryonic and larval development of *Astyanax altiparanae*

Publicado na Revista Chemosphere 264, 128935, 2021 [4].
DOI: 10.1016/j.chemosphere.2020.128935

[4] Pinheiro, J.P.S., Lima, J., Assis, C.B., Branco, G.S., Gomes, A.D., Moreira, R.G., 2021. Paternal exposure to aluminum, acidity, and temperature affect fatty acid seminal profile, embryonic and larval development of *Astyanax altiparanae*. Chemosphere, 128935. https://doi.org/10.1016/j.chemosphere.2020.128935

Paternal exposure to aluminum, acidity, and temperature affect fatty acid seminal profile, embryonic and larval development of *Astyanax altiparanae*

João Paulo Silva Pinheiro [a#*], Jennifer Lima[a#], Cecília Bertacini de Assis [a], Giovana Souza Branco [a], Aline Dal'Olio Gomes [a], Renata Guimarães Moreira [a]

[a] Universidade de São Paulo, Instituto de Biociências, Departamento de Fisiologia, Laboratory of Metabolism and Reproduction of Aquatic Organisms - LAMEROA, Matão Street, 14 lane, number 101, Room 220, Cidade Universitária, São Paulo, SP, Brazil.

João Paulo Silva Pinheiro: joaopaulospinheiro@yahoo.com.br
Jennifer Lima: mjdacamara@gmail.com
Cecília Bertacini de Assis: cbertacini4@gmail.com
Giovana Souza Branco: giovana_branco@hotmail.com
Aline Dal'Olio Gomes: eniladal@gmail.com
Renata Guimarães Moreira: renata.fish@gmail.com

[#]Two authors have contributed equally to the paper (dual first authorship)

*Corresponding author: João Paulo Silva Pinheiro: joaopaulospinheiro@yahoo.com.br
+551130917531

Resumo

Nós investigamos os efeitos da acidez da água, temperatura e alumínio (Al) no perfil seminal de ácidos graxos (AG), parâmetros reprodutivos (fertilização e eclosão) e desenvolvimento embrionário de *Astyanax altiparanae*. Expomos machos a diferentes tratamentos experimentais, correspondendo à combinação de temperatura da água (20 ºC; 25 ºC), pH (neutro - 7,0; ácido - 5,5) e ausência ou presença de Al (0,5 mg L^{-1}). Após 96 h, analisamos o perfil de AG do sêmen e realizamos a fertilização artificial em meio ativador em pH neutro ou meio ativador nas mesmas condições experimentais dos machos (pH neutro, pH ácido e Al) para avaliar as taxas de fertilização e de eclosão e monitorar o desenvolvimento embrionário. A porcentagem de ácidos graxos polinsaturados diminuiu no sêmen de peixes nos animais mantidos em pH neutro, enquanto os ácidos graxos monoinsaturados aumentaram em todos os grupos mantidos em 20 °C em comparação com 25 ºC. A exposição ao Al diminuiu a porcentagem de C20:4n6 e aumentou a porcentagem de C22:5n3 em 20 ºC. Machos expostos ao pH ácido e Al apresentaram menores taxas de fertilização e eclosão, bem como aumento da mortalidade de embriões e larvas. Além disso, a exposição ao Al causou uma porcentagem maior de larvas anormais. A fertilização em meio ativador com Al prejudicou os embriões e larvas, uma vez que as taxas de fertilização e de eclosão diminuíram. Finalmente, a temperatura influenciou no tempo de fertilização, na taxa de eclosão e morfologia dos embriões e larvas. Machos expostos ao Al apresentaram menor capacidade fertilizante, o que afetou negativamente o desenvolvimento embrionário. Além disso, o meio ativador com Al reduziu o número de oócitos fertilizados, embriões eclodidos e larvas normais. Todos os eventos dependeram da temperatura.

Palavras-chave: Mudanças climáticas; Água doce; Poluição; Reprodução; Qualidade da água.

Abstract

We investigated the effects of water acidity, temperature, and aluminum (Al) on the fatty acid (FA) seminal profile, reproductive parameters (fertilization and hatching) and embryonic development of *Astyanax altiparanae*. We treated males with different experimental treatments, corresponding to the combination of water temperature (20 ºC; 25 ºC), pH (neutral - 7.0; acidic - 5.5), and the absence or presence of Al (0.5 mg L^{-1}). After 96 hours, we analyzed the FA profile of semen and performed artificial fertilization in activating medium with neutral pH or activating medium in the same experimental conditions of the males (neutral pH, acidic pH, and Al) to evaluate fertilization and hatching rates and to monitor embryonic development. Polyunsaturated FA percentage decreased in semen of fish from the neutral group, while monounsaturated FA increased in all groups maintained at 20 °C compared to 25 ºC. Aluminum exposure decreased the percentage of C20:4n6 and increased the percentage of C22:5n3 at 20 ºC. Males exposed to acidic pH and Al showed lower fertilization and hatching rates, as well as increased mortality of embryos and larvae. Moreover, Al favoured a higher percentage of abnormal larvae. Fertilization in Al activating medium harmed the embryos and larvae since fertilization and hatching rates decreased. Finally, temperature influenced fertilization time, hatching rate, and the morphology of embryos and larvae. Males exposed to Al had lower fertilizing capacity, which negatively affected the embryonic development of the species. Furthermore, Al activating medium reduced the number of fertilized oocytes, hatched embryos, and normal larvae. All events were temperature dependent.

Keywords: Climate change; Freshwater; Pollution; Reproduction; Water quality.

Highlights

Al exposure impairs *Astyanax altiparanae* male fertility and larvae morphology;

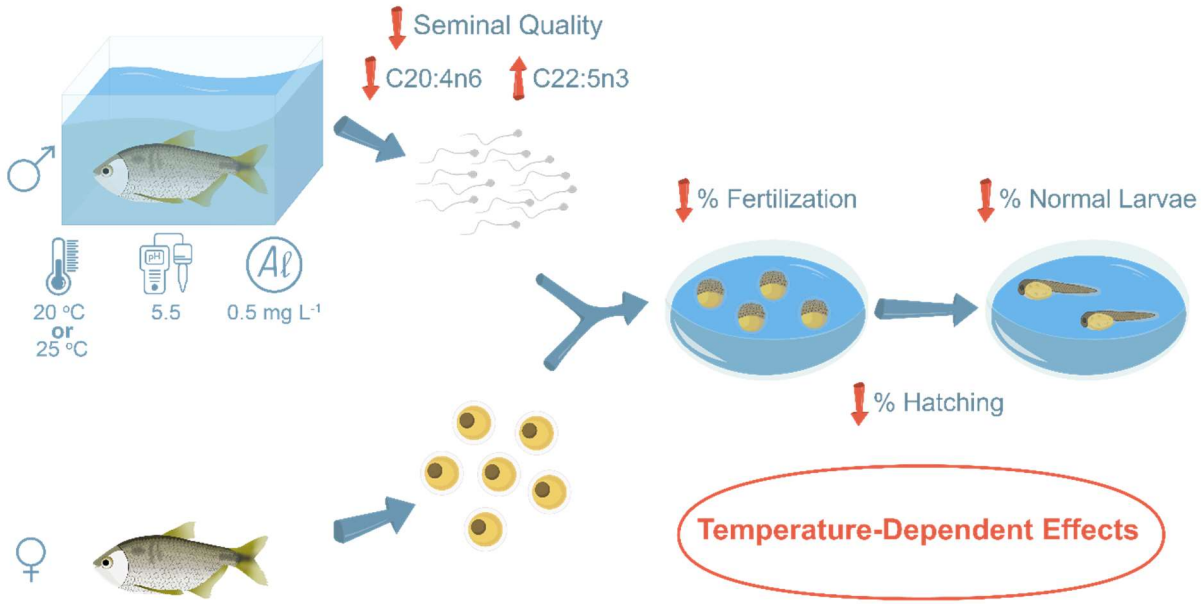
Al exposure affects the percentage of C20:4n6 and C22:5n3 in semen;

Acidic pH activating medium impairs fertilization and hatching;

Al activating medium impairs fertilization, hatching, and embryonic development;

Fertility, fatty acid profile and embryonic development are temperature dependent.

Graphical Abstract



1. Introduction

Aluminum (Al) is one of the most abundant metallic elements in the earth's crust. This element occurs in the combined form in rocks and soils, and in the water through natural processes such as rock weathering. Anthropic activities release Al into the water. Examples of these activities are mining, water treatment (flocculation and filtration), and wastewater discharge into rivers (Wilson, 2011; Fernández-Dávila et al., 2012). However, Al solubility correlates with water pH, as it is almost insoluble between pH 6 and 8 (Wilson, 2011). Temperature also interferes with Al toxicity, influencing solubility, cell membrane permeability, and the respiratory metabolism of fish (Wilson, 2011). In many rivers, Al concentration is above that recommended by environmental protection agencies such as the United States Environmental Protection Agency (US EPA - maximum concentration of 0.2 mg L^{-1}) and the Brazilian National Environmental Council (CONAMA - maximum concentration of 0.1 mg L^{-1}). For example, the Mogi Guaçu River in São Paulo State, Brazil, has an aluminum concentration that ranges between 0.1 and 1.0 mg L^{-1} (CETESB, 2018).

Aluminum can bioconcentrate in different organisms, such as phytoplankton, macroinvertebrates, amphibians (Oberholster et al., 2012), fish (Correia, 2012; Pinheiro et al., 2019), and humans (Mold et al., 2018). Notwithstanding, it does not present any biological function (Nayak, 2002; Fernández-Dávila et al., 2012). This element causes adverse effects in fish, such as changes in the antioxidant defense system (Ramírez-Duarte et al., 2017; Abdalla et al., 2019), ionic imbalance, inhibition of the activity of Na^+/K^+–ATPase (Camargo et al., 2009), deregulation of hormones of the hypothalamic-pituitary-gonadal axis (Correia et al., 2010; Correia, 2012; Kida et al., 2016), and impairment of gamete quality (Pinheiro et al., 2019, 2020). The latter also affects mammals (Cheraghi et al., 2017; Martinez et al., 2017), including men (Klein et al., 2014).

In the neotropical teleost *Astyanax altiparanae*, Al can bioconcentrate in the testes and semen as a function of exposure time, acidic pH, and water temperature (Pinheiro et al., 2019). Furthermore, changes in the physical and chemical parameters of water from the presence of Al caused genotoxic damage to the sperm of this species, reducing seminal osmolality, sperm concentration, and kinetics, affecting the sperm ultrastructure (Pinheiro et al., 2019, 2020).

Due to their high sensitivity to stressors and environmental changes, lipids can also be considered important biomarkers to assess the effect of adverse conditions since several organisms alter their lipid classes and proportions to maintain the fluidity of biological membranes (Rodríguez-Vargas et al., 2007). Fatty acids (FAs) are very important in the gametic composition of different vertebrates (Furuita et al., 2007; Ling et al., 2006; Vassallo-Agius et al., 2001; Lund et al., 2008; Samaee et al., 2009). They correlate with the fluidity and structure of sperm membranes, influencing motility and fertilization capacity (Wassall and Stillwell, 2009; Gholami et al., 2011; Mansour et al., 2011; Baeza et al., 2015). Exposure to metals can alter the FA profile of vertebrate gonads (Castellanos et al., 2010; Kida et al., 2012), affecting gamete quality (Kida et al., 2012). However, there are no reports of the effects of Al on the FA profile of fish testes and sperm.

Sperm quality refers to its ability to fertilize the oocyte and, consequently, develop into a normal embryo, thus determining the reproductive success of the species (Bobe and Labbé, 2010; Herráez et al., 2017; Kowalski and Cejko, 2019). Factors such as diet, age, and environmental conditions can affect this quality (Kowalski and Cejko, 2019). The contribution of sperm to the offspring goes beyond the delivery of the haploid genome to compose the genomic information of the zygote. This is because different events occur during spermatogenesis (e.g., processing and packaging of chromatin, reorganization of contacts between DNA and the nuclear matrix, epigenetic remodelling, and interruption of transcription).

These events have a specific contribution to embryonic development (Herráez et al., 2017).

Despite the importance of sperm quality, most studies focus on evaluating the effect of pollutants on embryo development and oocyte quality. In turn, there is little information about the effect of pollutants on seminal quality and its consequences on fertilization, hatching, and embryonic development of teleosts. Therefore, the present study aimed to evaluate the effects of *A. altiparanae* males exposed to Al, at different temperatures and acidic pH on the FA profile of sperm, fertilization, hatching, and embryonic development, correlating these variables with the sperm quality parameters. Furthermore, beyond the effects of these variables in the gametes, after male exposure, we evaluated the additive effect of the water (activating medium) used in the activation of gametes.

2. Material and Methods

2.1. Male exposure

We chose *A. altiparanae* as a neotropical model species in this study. This species inhabits the southeast of Brazil, represents a good bioindicator for metal toxicity in short or long exposure periods (Pinheiro et al., 2019, 2020); and has already been used in ecotoxicological and reproduction studies due to its easy handling in laboratory conditions (Gomes et al., 2013; Vieira et al., 2013; Chehade et al., 2015, 2020; Bettim et al., 2016; Kida et al., 2016; Abdalla et al., 2019; Brambila-Souza et al., 2019; Pinheiro et al., 2019, 2020; Godoi et al., 2020; Muñoz-Peñuela et al., 2021).

A. altiparanae mature males (18.73 ± 1.14 g; 10.59 ± 0.21 cm) were donated by the Votorantim Energia Group (Paraibuna, SP) and kept for seven days at the Ectothermic Facility in the Department of Physiology of *Universidade de São Paulo* (IB/USP) prior to the experimental period. The animals were divided into glass aquariums (10 animals/aquarium; 132 L of water/aquarium),

with water renewal every 24 h (90%) and fed *ad libitum* daily on extruded feed (32% crude protein) until 24 h before the experiments.

After this period, the animals were exposed for 96 hours (acute exposure) to different experimental treatments (each group: 10 animals/aquarium in duplicates; totalizing 20 animals/group), which consisted of the combination of water temperature (20 ºC; 25 ºC), pH (neutral - 7.0; acidic - 5.5), and the absence or presence of Al, resulting in six experimental treatments: 1) water at 20 ºC, neutral pH, absence of Al; 2) water at 20 ºC, acidic pH, absence of Al; 3) water at 20 ºC, acidic pH, presence of Al; 4) water at 25 ºC, neutral pH, absence of Al (control); 5) water at 25 ºC, acidic pH, absence of Al; 6) water at 25 ºC, acidic pH, presence of Al. The acidic pH was considered to achieve Al solubility and bioavailability in acidic pH (Wilson, 2011). This study considered the Al concentration of 0.5 mg L^{-1}, corresponding to literature values for different rivers in the state of São Paulo (CETESB, 2018).

The Al solution added to the experimental groups (three and six) was prepared with $Al_2(SO_4)_3.18H_2O$ (Sigma Aldrich), deionized water, and nitric acid (65% HNO_3; Suprapur, Merck). Aquarium water was renewed every 24h (semi-static system 90%) in order to maintain the Al concentration and water quality. The metal concentration in the water was analyzed by inductively coupled plasma mass spectrometry (ICP-MS); and was within the range proposed in the study. To reach the temperature of each experimental treatment, the water was heated or cooled at a rate of 1 ºC h^{-1} (Trueman et al., 2000) with the aid of a thermostat. The water temperature (19.8 ± 0.20 ºC; 24.8 ± 0.20 ºC), dissolved oxygen (oximeter YSI 55; 7.97 ± 0.05 mg L^{-1} at 20 ºC; 7.49 ± 0.06 mg L^{-1} at 25 ºC), and pH (Gehaka pH meter; neutral groups: 7.20 ± 0.20; acid and Al groups: 5.40 ± 0.10) were analyzed daily.

2.2. Gamete collection

Before the end of the acute exposure (96 h), the males were induced to spermiation with pituitary extract from common carp (Danúbio Aquaculture; 5 mg Kg^{-1} body mass). For that, the macerate was diluted in physiological solution and injected at the base of the pectoral fin with 1 mL syringes. Because the release of gametes is faster as the water temperature increases, different injection periods were adopted for each treatment according to the temperature (13 h before the end of the experiment - temperature of 20 ºC - 260 accumulated thermal units [ATU]; 11 h before the end of the experiment - temperature of 25 ºC - 275 ATU). During the experimental period, females (from the same location as the males: Votorantim Energia Group - Paraibuna; without exposure to experimental treatments) were kept in 180 L black boxes (24.9 ± 0.30 ºC; pH 7.3 ± 0.10; OD 7.83 ± 0.12 mg L^{-1}) and were induced to spawning with two doses of common carp pituitary extract (1st dose: 0.5 mg kg^{-1}/body mass, after 10 h [250 ATU]; 2nd dose: 5 mg kg^{-1}/body mass, at the same time as the male induction). All females were maintained in the same conditions to avoid any maternal influence in the results (they were not treated with Al or different pH/temperature). These protocols to induce sperm release and spawning have previously been tested in *A. altiparanae* from previous literature reports (Machado-Evangelista et al., 2019; Pinheiro et al., 2019, 2020).

For semen collection, the males were sedated with a eugenol-based solution (clove oil; 100 mg L^{-1}), subsequently wrapped with a damp cloth, and the urogenital papilla was dried to avoid contamination with blood, feces, urine, or water. The semen was collected through abdominal massage (craniocaudal) using an automatic pipette and graduated polyethylene tubes. After collection, the samples were analyzed under optical microscopy to discard contaminated samples and to select those with motility greater than 80% after sperm activation, and only semen samples with this characteristic were frozen in ultrafreezer (-80 ºC) for fatty acid analysis.

For oocytes collection, the females (n = 20) had their urogenital papilla dried and extruded through abdominal massage in the craniocaudal direction. The oocytes were placed in porcelain flasks, using a pool of gametes obtained from all females.

2.3 Fatty acids analysis

Samples with sufficient volume and sperm motility greater than 80% after sperm activation were used for the analysis of fatty acids. Total semen lipids were extracted using chloroform, methanol, and water (2: 1: 0.5) according to Folch et al. (1957), adapted for aquatic organisms by Parrish (1999). The samples were methylated with acetyl chloride (5% HCL in methanol), converted to fatty acid methyl esters (FAMEs), and identified in a gas chromatograph (GC; model 436, Walnut Creek, CA, USA) equipped with flame ionization detector (FID) and autoinjector (CP-8400).

Fatty acids (FA) were analyzed using a CP Wax 52 CB capillary column (0.25 μm film thickness, 0.25 mm internal diameter, and 30 m length). For that, hydrogen was used as a carrier gas at a linear speed of 22 cm s^{-1}. The temperature was established as follows: 170 °C for 1 minute, followed by a ramp of 2.5 °C $minute^{-1}$ to 240 °C, and a final waiting time of 5 minutes, totaling 31 minutes of running. The detector temperature was maintained at 250 °C, and the FID temperature was 260 °C. Fatty acid methyl esters (FAMEs) were identified by comparison with the retention times of commercial standards (Supelco, 37 components; Sigma-Aldrich). Data are presented in percentage (%), based on peak area analysis.

2.4. Fertilization

The pool of oocytes from all females (n = 40) were aliquoted in 150 Petri dishes (1 g oocytes/dish) and 20 μL of semen from a treated male exposed was added to each dish (3 dishes/male). After mixing the gametes (in dry conditions),

2 mL of water (activating medium; neutral pH) was added to activate the gametes (2 minutes). Finally, 40 mL of the same water was added to each Petri dish and kept until the analysis of fertilization and hatching rates. In total, 5 replicates were obtained in triplicate of each experimental treatment. After fertilization, 25 embryos in the blastula stage were separated from each dish (triplicate; 75 embryos/treatment/male totalizing 375 embryos/treatment) for analysis and recording of the stages of *A. altiparanae* embryonic development in a stereoscope (adapted from Santos et al., 2016). Each treatment in fertilization means the combination of the male experimental exposure group (neutral pH, acidic pH and aluminum) with the activating medium (neutral pH and experimental), in a total of 15 dishes/neutral pH (neutral pH + neutral activating medium), 30 dishes/acidic condition (15 – acidic pH + neutral activating medium; 15 – acidic pH + acidic activating medium) and 30 dishes/Al condition (15 – Al + neutral activating medium; 15 – Al + Al activating medium) in each temperature.

Spermatozoa of freshwater and marine fishes are immotile in the seminal plasma, and their motility is started after their contact with the water into the environment (osmolality difference). Thus, to evaluate the effect of water quality on gamete activation and embryonic development, the same procedures described above were performed, but replacing the neutral pH activating medium with an activating medium with the same characteristics of the water used in each experimental treatment of male exposure. Hence, fertilization took place in neutral pH activating medium, acidic pH activating medium, and Al activating medium. With this step, we analyzed whether the activating medium and incubation water (simulating that the sperm will be released in the same environment of male exposure) has additive effect or not on the success of fertilization, hatching, and embryonic development. Additionally, it is possible to dissociate which effect can be assigned due to male exposure and which one was due to the characteristic of the activating medium. Fig. 1 summarizes the experimental design of the study.

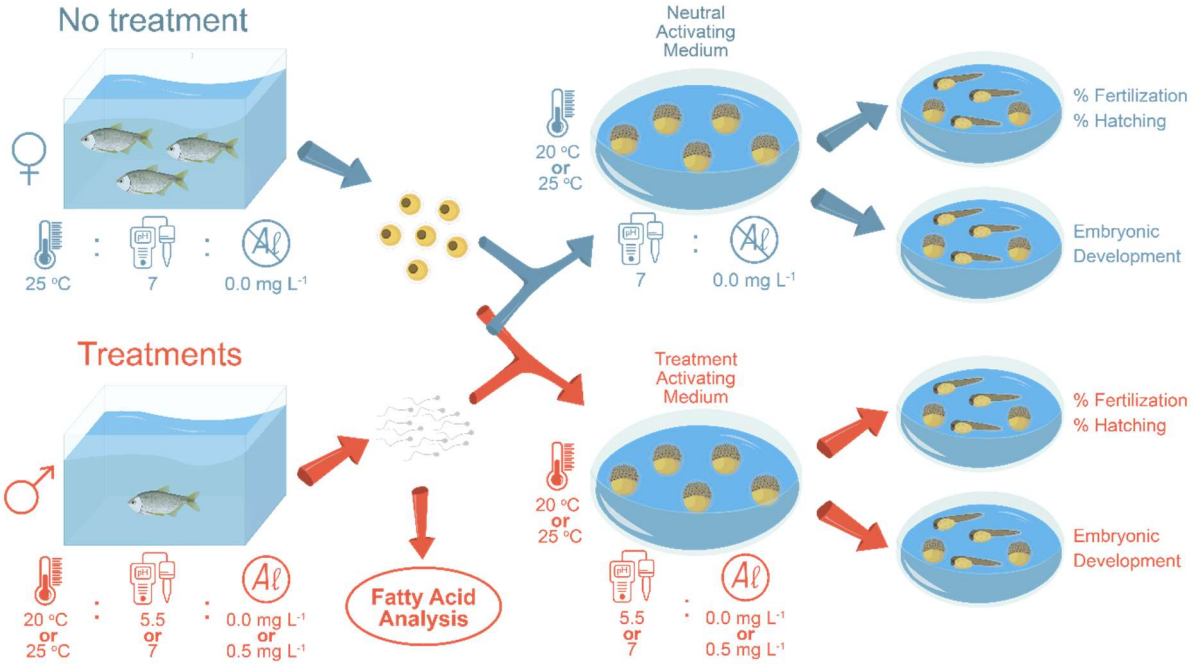


Fig. 1. Experimental design. *Astyanax altiparanae* males exposed to experimental treatments for 96 hours - combination of three factors (temperature [20 ºC or 25 ºC], pH [neutral or acidic], and Al [absence or presence]) – 10 animals/treatment in duplicate totalizing 20 animals/treatment. To evaluate the fatty acid profile of semen, fertilization and hatching rates and to monitor embryonic development, gamete fertilization took place in neutral activating medium and treatment water (neutral, acidic, and Al activating medium). In fertilization, each treatment means the combination of the male experimental exposure group (neutral pH, acidic pH and aluminum) with the activating medium (neutral pH and experimental), in a total of 15 dishes/neutral pH (neutral pH + neutral activating medium), 30 dishes/acidic condition (15 in acidic pH + neutral activating medium; 15 in acidic pH + acidic activating medium) and 30 dishes/Al condition (15 in Al + neutral activating medium; 15 in Al + Al activating medium) in each temperature (20 ºC or 25 ºC). Females were not exposed to experimental treatments. Illustration by Jardim (2020).

The embryos were placed in acrylic trays in biochemical oxygen demand (BOD) incubators to maintain temperature, photoperiod (LD 12:12), humidity, and oxygen in each experimental treatment (20 ºC and 25 ºC) until hatching.

2.5. Reproductive parameters

The fertilization rates were calculated at the blastula stage (128 cells), and the hatching rates when the larvae were free and swimming in the dishes. The fertilization rate was obtained by the ratio between the quantity of all fertilized oocytes in the dish (time and phase described by Santos et al., 2016) divided by the total number of oocytes, multiplying the result by 100. The hatching rate consisted of dividing the number of hatched embryos by the number of fertilized oocytes, multiplying the result by 100 (Bardon-Albaret and Saillant, 2017).

2.6. Analysis of embryos and larvae

The embryos were classified as normal embryos (no rupture of the chorion and the normal structures within the egg) and abnormal embryos (no rupture of the chorion and structures with malformations within the egg). After chorion disruption and hatching, the larvae were classified as normal (without anomalies in the body structure), abnormal (hatched but with body malformations), and dead larvae (lethal endpoints - embryo coagulation, absence of somites, no tail detachment, or absence of heartbeat). All embryos (25 from each replica) were considered in the calculation of the percentage of mortality, embryos, and larvae (normal and abnormal). Embryonic malformations included sublethal and developmental endpoints: completion of gastrulation, somite formation, development of eyes, spontaneous movements, heartbeat and blood circulation, pigmentation and oedema; and teratogenic endpoints: malformation of the head, tail, and heart, modified corda structure, scoliosis, yolk sac deformity, delayed growth, and tail length. These lethal, sublethal/developmental, and teratogenic

endpoints were established according to an adaptation from Nagel (2002). Thus, it was possible to verify the effect of semen quality on the F1 generation and the "additive" effect of the activating medium. The analysis of the aforementioned endpoints occurred in all experimental groups from the moment when all viable embryos in the control group (neutral pH) were in the larval stage (20 ºC: 26 h 05 min after fertilization; 25 ºC: 16 h 32 min after fertilization).

2.7. Statistical analyses

Data were expressed as Mean ± Standard Error of the Mean (SEM), submitted to the Kolmogorov-Smirnov normality test and Spearman homoscedasticity test. Seminal FA percentages in the experimental groups were compared using two-way ANOVA analysis of variance, considering "temperature" and "treatment" as factors, followed by the Holm-Sidak post-hoc test. The data from the reproductive parameters and embryo development were analyzed by the Generalized Linear Model (GLM) and the Bonferroni post hoc test with the factors "Temperature" (20 and 25 ºC) and "Treatment" (neutral pH + neutral pH activating medium; acidic pH + neutral pH activating medium; Al + neutral pH activating medium) and their interactions. Moreover, a subsequent GLM analysis was carried out focusing on the same factors, but replacing the activating medium for each treatment water: "Temperature" (20 and 25 ºC) and "Treatment" (neutral pH + neutral pH activating medium; acidic pH + acidic pH activating medium; Al + Al activating medium) and their interactions. The Pearson correlation test was used to assess the relationship between the fatty acid profile of semen and seminal quality variables at 10 and 30 s after sperm activation (sperm motility, [MOT,%]; curvilinear velocity [VCL, $\mu m.s^{-1}$]; average path velocity of sperm [VAP, $\mu m.s^{-1}$]; straight line velocity of sperm [VSL, $\mu m.s^{-1}$]); and fertilization rate (%). Seminal quality data were obtained from Pinheiro et al. (2020). Statistical analyses were performed using the IBM SPSS Statistic 22 software and Sigma Stat 3.5 for Windows, considering a 95% significance ($P < 0.05$).

3. Results

3.1 Fatty acids

Table 1 shows the seminal FA profile after 96 h. In general, 14 FAs were identified with a minimum percentage of 0.5%, varying from C16:0 to C22:6n3. They were grouped into saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA).

In the semen of animals exposed to 20 °C, the percentages of C17:0 and C22:5n3 were higher in groups undergoing acid and Al treatment ($P < 0.001$ in both cases) than in the control group (neutral treatment). Similarly, at 25 °C, the percentage of C17:0 was also higher in the semen of animals from the acid and Al groups than in those from the neutral group, with a difference also between acid and Al treatment ($P < 0.001$).

Regarding the effect of temperature alone, C16:0 and C18:0 decreased ($P = 0.007$, $P = 0.015$, respectively) while C17:0 increased in the acid treatment at 20 °C compared to the same treatment at 25 °C ($P < 0.001$), resulting in a lower percentage of SFA at the lowest temperature ($P = 0.003$). On the other hand, the percentage of MUFA in the semen of fish from all treatments increased at 20 °C compared to 25 °C ($P < 0.001$), with C18:1 being the most abundant MUFA and, consequently, the responsible for this difference ($P < 0.001$).

Among the PUFA, C20:4n6 (arachidonic acid, ARA) and C22:6n3 (docosahexaenoic acid, DHA) were the most abundant. However, there was no significant difference between the groups, except for a decrease in the percentage of ARA in the semen of fish from the Al group at 20 °C compared to 25 °C ($P = 0.038$). This same effect of temperature was observed in C22:5n6 in the semen of fish from neutral and Al groups ($P < 0.001$). These changes led to a decrease in the percentage of total PUFA in the neutral group at 20 °C ($P = 0.002$) and n6 PUFA in the neutral and Al groups at 20 °C compared to 25 °C ($P < 0.001$ in both cases). The percentage of PUFA C22:5n3 in semen was also altered by Al and

acidic pH exposure, with higher values in these groups than in the control group, at 20 ºC ($P < 0.001$).

Table 1. Total fatty acid profile (%) of *Astyanax altiparanae* semen after exposure to different temperatures, pH values and presence or absence of aluminum (mean ± standard error of mean).

	20 °C			**25 °C**		
Fatty Acid (%)	**Neutral pH**	**Acidic pH**	**Aluminum**	**Neutral pH**	**Acidic pH**	**Aluminum**
	n = 4	**n = 9**	**n = 9**	**n = 4**	**n = 8**	**n = 8**
C16:0	14.5 ± 2.14	13.3 ± 0.61*	13.6 ± 0.24	15.5 ± 0.55	16.1 ± 0.64**	15.5 ± 0.72
C17:0	0^{B}	2.1 ± 0.34^{A}*	1.9 ± 0.32^{A}	0^{C}	1.2 ± 0.13^{B}**	2.4 ± 0.30^{A}
C18:0	10.5 ± 1.66	9.2 ± 0.31*	9.4 ± 0.29	9.7 ± 0.42	11.4 ± 0.45**	11.8 ± 0.58
∑ SFA	**25.1 ± 3.79**	**24.5 ± 1.08***	**24.5 ± 0.64**	**25.2 ± 0.88**	**28.3 ± 1.03****	**29.7 ± 1.40**
C16:1n7	2.3 ± 0.46	1.8 ± 0.10	1.8 ± 0.14	1.8 ± 0.15	2.0 ± 0.14	1.9 ± 0.21
C18:1	35.5 ± 1.97*	33.0 ± 1.21*	31.2 ± 1.19*	27.3 ± 1.85**	24.4 ± 1.05**	23.1 ± 0.85**
C24:1	3.0 ± 0.65	2.5 ± 0.14	2.8 ± 0.11	2.9 ± 0.18	2.9 ± 0.16	2.4 ± 0.18
∑ MUFA	**40.2 ± 1.75***	**37.3 ± 1.14***	**35.6 ± 1.16***	**32.0 ± 1.97****	**29.4 ± 1.13****	**27.3 ± 0.80****
C18:2n6	3.5 ± 1.05	2.7 ± 0.28	2.7 ± 0.22	2.3 ± 0.28	3.1 ± 0.48	3.3 ± 0.61
C20:2n6	0	2.6 ± 0.32	2.1 ± 0.26	2.1 ± 0.09	2.4 ± 0.19	2.0 ± 0.25
C20:3n6	2.5 ± 0.41	2.1 ± 0.18	1.8 ± 0.18	2.1 ± 0.20	2.3 ± 0.21	1.8 ± 0.10
C20:4n6	13.0 ± 1.62	13.2 ± 0.62	12.9 ± 0.67*	14.2 ± 0.19	14.2 ± 0.75	15.3 ± 0.86**
C22:4n6	1.8 ± 0.19	2.0 ± 0.16	1.5 ± 0.17	2.0 ± 0.28	2.0 ± 0.18	1.9 ± 0.17
C22:5n6	3.8 ± 0.73*	3.7 ± 0.26	3.5 ± 0.32*	5.3 ± 0.31**	4.5 ± 0.28	5.3 ± 0.39**
C22:5n3	0^{B}*	1.7 ± 0.15^{A}*	1.4 ± 0.20^{A}	1.1 ± 0.02**	1.4 ± 0.08**	1.1 ± 0.18
C22:6n3	11.1 ± 0.99	11.4 ± 0.63	14.6 ± 1.26	14.3 ± 1.48	13.7 ± 1.29	12.9 ± 0.89
∑ t PUFA	**34.7 ± 3.06***	**38.2 ± 1.44**	**40.0 ± 1.49**	**42.8 ± 1.58****	**42.3 ± 1.55**	**43.0 ± 1.63**
∑ n6 PUFA	**23.6 ± 2.33***	**25.2 ± 0.97**	**24.4 ± 0.86***	**28.0 ± 0.51****	**27.6 ± 0.90**	**29.8 ± 0.92****
∑ n3 PUFA	**11.1 ± 0.99**	**13.0 ± 0.65**	**15.6 ± 1.42**	**14.8 ± 1.75**	**14.8 ± 1.48**	**13.2 ± 1.02**
n3/n6	**0.5 ± 0.04**	**0.5 ± 0.02**	**0.6 ± 0.08**	**0.5 ± 0.07**	**0.5 ± 0.06**	**0.4 ± 0.03**

*Different symbols indicate statistical differences between temperatures within the same treatment. ^{ab}Different letters indicate statistical differences among treatments within the same temperature. Two-way ANOVA $P < 0.05$. Nd – not detected. *SFA* saturated fatty acids; *MUFA* monounsaturated fatty acids; t *PUFA* total polyunsaturated fatty acids.

3.2. Embryo development

At the temperature of 20 ºC, fertilization occurred 5 h 20 min after activation of gametes (blastula stage, 128 cells), and hatching after 31 h 25 min.

At 25 ºC, fertilization occurred 1 h 48 min (blastula stage) after activation of gametes, and hatching after 18 h 20 min. In addition to this temperature effect during fertilization and hatching times, embryos fertilized with gametes from males exposed to Al, both at 20 ºC and 25 ºC, did not develop beyond the gastrula stage. The morphological analysis also showed that this effect was dependent on the activating medium. In the group of males exposed to Al and fertilized with Al activating medium, the embryos stopped developing in the gastrula stage, with 17 somites (no tail detachment). In turn, those fertilized with neutral pH activating medium stopped developing in the 30 somites stage, which is among the final stages before larvae hatching.

Fig. 2 shows the morphology of embryos and larvae (normal – Fig. 2 A; abnormal – Fig. 2 B - F), regardless of experimental treatment. In the larvae from Al-exposed males (both fertilized with neutral pH activating medium and with Al activating medium), the most common deformities were: oedema heart (oe; Fig. 2 B and E), yolk sac deformity (yd; Fig. 2 B, D, and F), modified structure of the corda (mc; Fig. 2 B, C, D, and F), absence of pigmentation (ap; Fig. 2 E), and short tail (st; Fig. 2 E). Acidic pH (male exposure or activating medium) did not lead to morphological alterations of embryos and larvae.

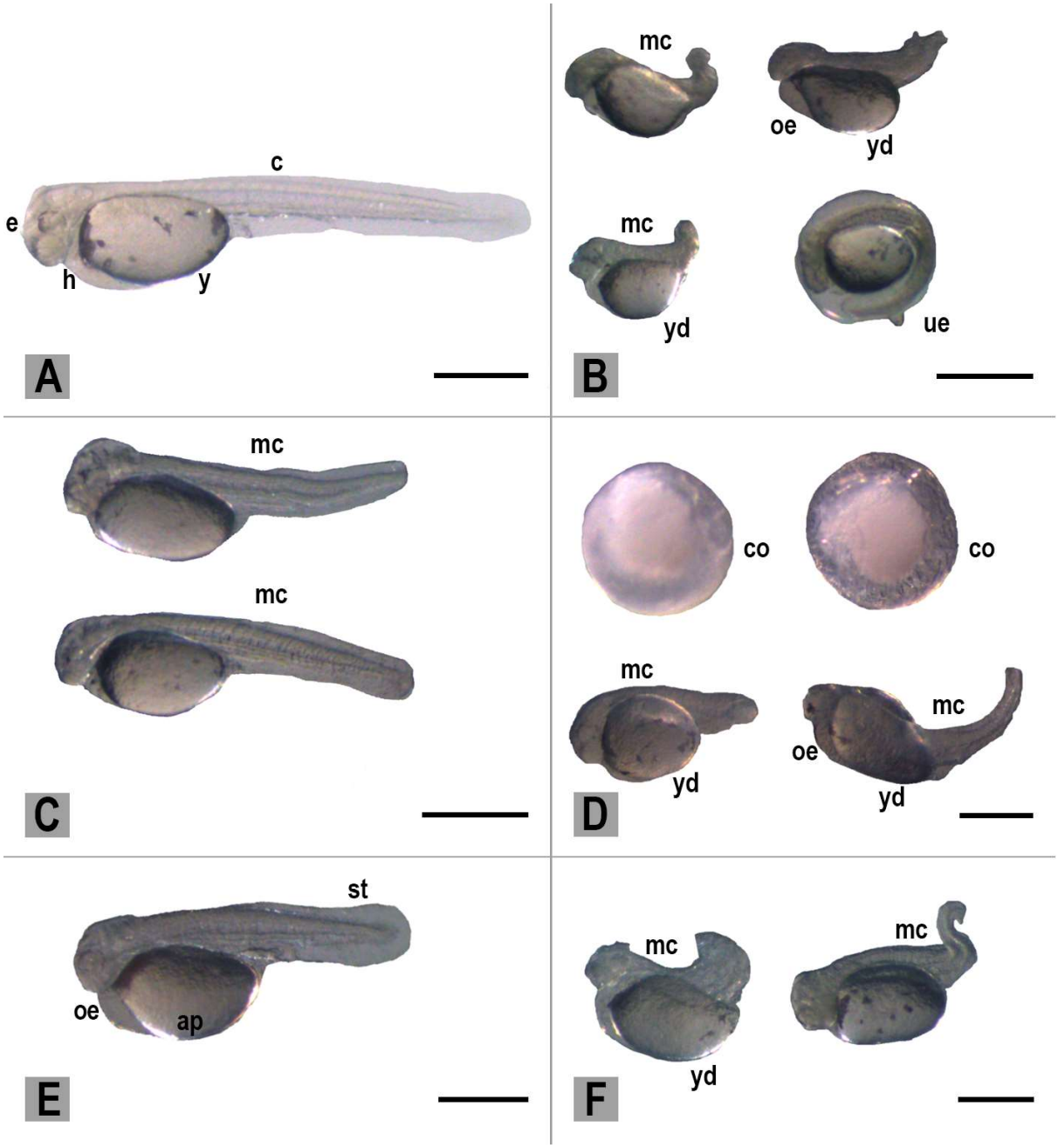


Fig. 2. Representative photographs of *Astyanax altiparanae* embryos and larvae, regardless of experimental treatment. (A) indicates normal larvae (e – eye; h – heart; y – yolk sac; c – corda). (B to F) indicate changes in the morphology of embryos and larvae, including modified structure of the corda (mc), yolk sac deformity (yd), oedema heart (oe), embryo coagulation (co), unhatched embryo (delay; ue), absence of pigmentation (ap), and short tail (st). Scale bar: 0.88 mm.

3.2.1. Effects of male exposure (fertilization at neutral pH activating medium)

A. altiparanae males exposed to acidic pH and Al released sperm that provided lower fertilization (20 ºC and 25 ºC, $P < 0.001$ in all cases) and hatching (20 ºC; $P < 0.001$; $P < 0.001$, respectively) in comparison to those exposed to neutral pH (Fig. 3). When considering the embryos (at the hatching time described; Fig. 4 A), males exposed to Al and acidic pH provided the lowest percentages of abnormal embryos at 20 ºC ($P < 0.001$; $P < 0.011$, respectively). However, at 25 ºC, males exposed to Al provided the highest percentage of abnormal embryos, but only compared to the acidic pH treatment ($P = 0.037$). Regarding the larvae, at both temperatures, 20 ºC and 25 ºC, Al contributed to the highest percentage of abnormal larvae ($P < 0.001$, in both cases) (Fig. 4 B). Finally, at 20 ºC, the mortality of embryos/larvae was higher after male exposure to acidic pH and Al compared to neutral pH ($P < 0.001$, in both cases), and the Al treatment group had the lowest mortality at 25 ºC ($P = 0.001$) (Figure 4 C).

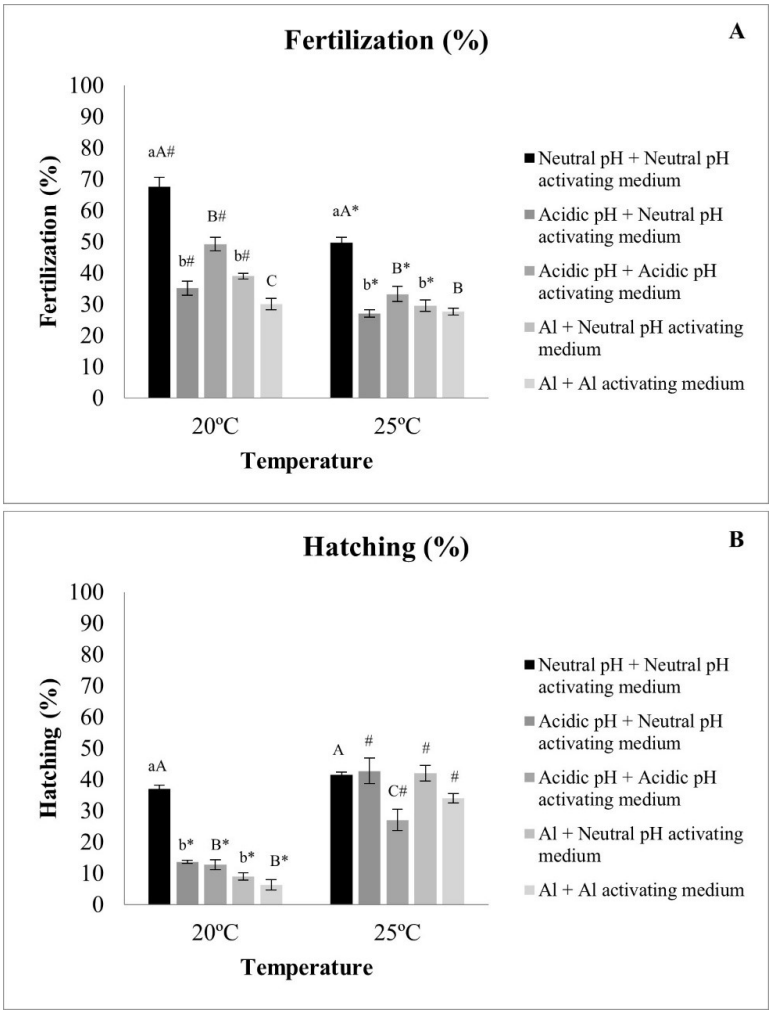


Fig. 3. Reproductive Parameters. A. Fertilization (%); B. Hatching (%).[a,b] Different lowercase letters indicate a difference in the same temperature, in neutral pH activating medium, in different treatments; [A,B] Different uppercase letters indicate a difference at the same temperature, comparing treatments that maintained the same characteristic of the activating medium (neutral pH + neutral pH activating medium; acidic pH + acidic pH activating medium; Al + Al activating medium); [#,*] Different symbols indicate a difference in the same treatment, in the same activating medium, at different temperatures. $P < 0.05$.

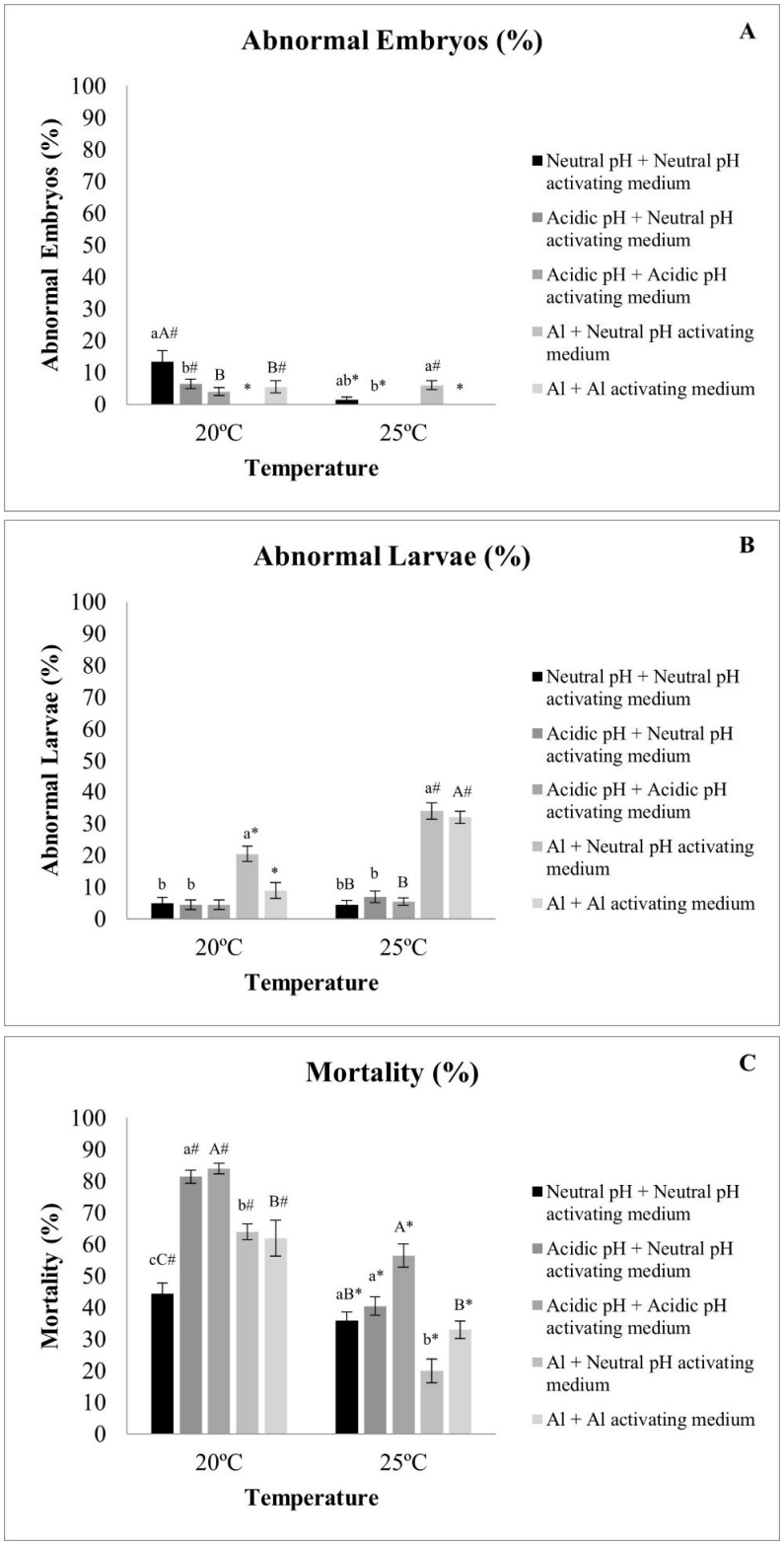


Fig. 4. Abnormal and mortality of embryos and larvae. A. Abnormal Embryos (%); B. Abnormal Larvae (%); C. Mortality (%).[a,b] Different lowercase letters indicate a difference in the same temperature, in neutral pH activating medium, in different treatments; [A,B] Different uppercase letters indicate a difference at the same temperature, comparing treatments that maintained the

same characteristic of the activating medium (neutral pH + neutral pH activating medium; acidic pH + acidic pH activating medium; Al + Al activating medium); [#,*] Different symbols indicate a difference in the same treatment, in the same activating medium, at different temperatures. $P < 0.05$.

3.2.2. Additive effect of male exposure and the activating medium

The combination of male exposure to Al and acidic pH with the same characteristics in the activating medium negatively affected fertilization (Fig. 3 A) and hatching (Fig. 3 B). At 20 ºC, males exposed to Al combined with Al activating medium showed the lowest percentage of fertilization, followed by males maintained in acidic pH and fertilized in acidic activating medium, in comparison to males maintained and fertilized in neutral water ($P < 0.001$, in both cases). At 25 ºC, males in the Al and acidic pH groups showed the same reduction of those maintained in neutral pH with gamete fertilization in neutral pH activating medium ($P < 0.001$, in both cases), but with no difference between them ($P = 0.186$). At 20 ºC, hatching rate was lower after male exposure to acidic pH and Al with the same conditions in the activating medium in comparison to neutral pH ($P < 0.001$, in both cases). At 25 ºC, this decrease in hatching rate was greater in acidic pH ($P < 0.001$), followed by Al ($P = 0.020$), in comparison to neutral pH.

For embryos evaluated during the hatching period (Fig. 4 A), at 20 ºC, there was a lower percentage of abnormal embryos in acidic pH ($P = 0.001$) and Al treatment ($P = 0.004$). In turn, at 25 ºC, the activating medium did not influence the percentage of abnormal embryos. Regarding the larval stage (Fig. 4 B), at 20 ºC, the activating medium did not alter the percentage of abnormal larvae ($P = 1.00$ – neutral and acidic; $P = 0.36$ – neutral and Al; $P = 0.24$ – acidic and Al). In turn, at 25 ºC, there was a higher percentage of abnormal larvae in males exposed to Al with Al activating medium in comparison to neutral pH and acidic pH ($P < 0.001$, in both cases). At 20 ºC, acidic pH ($P < 0.001$) and Al ($P = 0.003$) led to a

higher mortality of *A. altiparanae* embryo/larvae, which was even higher in acidic pH. At 25 ºC, mortality was higher in the acidic pH treatment with acidic pH activating medium in comparison to the other treatments ($P < 0.001$, in both cases) (Fig. 4 C).

3.2.3. Effects of temperature

The fertilization rate (Fig. 3 A) was higher in all experimental treatments at 20 ºC compared to 25 ºC, regardless of the activating medium ($P < 0.001$ in all cases, except for acidic pH with neutral pH activating medium, $P = 0.002$), except for the Al group with Al activating medium, which showed no difference between temperatures ($P = 0.414$). The hatching rate (Fig. 3 B) was lower in all experimental treatments at 20 ºC compared to 25 ºC ($P < 0.001$, in all cases), except for the neutral pH groups ($P = 0.102$). At 20 ºC, the percentage of abnormal embryos (Fig. 4 A) was higher when males were in neutral pH ($P < 0.001$), acidic pH (neutral activating medium; $P = 0.007$), and Al (Al activating medium, $P = 0.024$) in comparison to the respective groups at 25 ºC. However, males exposed to Al (neutral pH activating medium) had a higher percentage of abnormal embryos at 25 ºC compared to 20 ºC ($P = 0.012$). Furthermore, the lowest temperature of the study (20 ºC) provided a lower percentage of abnormal larvae in the Al treatment, both in neutral and Al activating medium ($P < 0.001$, in both cases; Fig. 4 B). The temperature of 20 ºC led to a higher percentage of mortality of embryos and larvae (Fig. 4 C) in all experimental groups ($P < 0.001$ in all cases, except for neutral pH [$P = 0.045$]) in comparison to the temperature of 25 ºC.

3.3 Sperm variables x FA profile

Table 2 shows the correlations between semen FA profile and sperm quality variables, fertilization rate, and normal hatched embryos. MUFA percentage correlated positively with VAP and VSL (10 s). On the other hand, the percentages of total PUFA and n6 PUFA correlated negatively with these

same sperm quality variables. Additionally, n3 PUFA correlated positively with MOT, while n6 PUFA and ARA correlated negatively with VCL (10 s). Fertilization rate correlated positively with MUFA and negatively with total PUFA, n6 PUFA, and C22:5n3.

Table 2. Correlations between semen fatty acid profile and sperm quality variables, fertilization rate, and normal hatched embryos.

FA (%)	**MOT – 10s**	**VCL – 10s**	**VAP – 10s**	**VSL – 10s**	**MOT -30s**	**VCL – 30s**	**VAP – 30s**	**VSL – 30s**	**FR**	**NHE**
	n = 28	n = 28	n = 28	n = 28	n = 28	n = 28	n = 28	n = 28	n = 42	n = 42
16:0	0.02	0.06	-0.00	-0.02	0.372	0.284	0.193	0.157	-0.07	0.430*
18:0	-0.172	-0.07	-0.04	-0.02	0.177	0.244	0.184	0.176	-0.134	0.284
SFA	-0.187	-0.06	-0.07	-0.07	0.126	0.140	0.06	0.04	-0.180	0.374*
18:1	-0.05	0.147	0.357	0.391*	-0.291	-0.231	-0.130	-0.09	0.490**	-0.456*
MUFA	-0.03	0.165	0.389*	0.424*	-0.239	-0.188	-0.07	-0.04	0.495**	-0.445*
20:4n6	-0.05	-0.412*	0.347	-0.328	0.02	-0.07	-0.04	-0.05	-0.220	0.301
22:4n6	0.115	-0.02	-0.193	-0.220	0.118	0.217	0.103	0.09	-0.06	0.106
22:5n6	0.214	-0.03	-0.279	-0.333	0.179	0.185	0.115	0.08	-0.104	0.408*
22:5n3	0.05	0.103	-0.120	-0.229	-0.03	-0.181	-0.266	-0.300	-0.680**	-0.353*
22:6n3	0.362	0.140	-0.142	-0.174	0.253	0.303	0.246	0.240	-0.09	0.07
T PUFA	-0.161	-0.139	-0.386*	-0.413*	0.196	0.153	0.09	0.07	-0.342*	0.265
n6 PUFA	-0.08	-0.373*	-0.468*	-0.469*	0.112	0.02	-0.00	-0.02	-0.342*	0.417*
n3 PUFA	0.347*	0.166	-0.125	-0.167	0.194	0.218	0.153	0.147	-0.158	-0.02

FA (%) – fatty acid; MOT - Sperm motility (%); VCL – curvilinear velocity ($\mu m.s^{-1}$);VAP – average path velocity of sperm ($\mu m.s^{-1}$); VSL – straight line velocity of sperm ($\mu m.s^{-1}$); FR – fertilization rate (%); NHE – normal hatched embryos. Asterisks indicate significant correlations between sperm quality parameters and fatty acids. *$P < 0.05$. **$P < 0.001$.

4. Discussion

The present study showed that *A. altiparanae* males exposed to acidic pH and Al had lower fertilization, hatching rates, and higher mortality of embryos/larvae. Aluminum also contributed to a higher percentage of abnormal larvae (mainly with oedema heart, yolk sac deformity, and modified structure of

the corda). Moreover, the activating medium/incubation water of the embryos/larvae directly contributed to the percentage of fertilized oocytes, hatching, and normal larvae. Besides, temperature influenced fertilization time, hatching time, and the morphology of *A. altiparanae* embryos and larvae. Regarding fatty acids, Al exposure changed the percentage of seminal C20:4n6 and C22:5n3; FAs, such as MUFA, total PUFA, n3 and n6 PUFA, correlated with sperm kinetics and fertilization rate.

Aquatic organisms show a decrease in PUFA after exposure to metals, mainly due to lipid peroxidation or alteration of membrane permeability. This has been reported for blue mussels (*Mytilus edulis*) exposed to cadmium and copper (Fokina et al., 2013). However, this is not a classic scenario because stressful conditions, such as changing pH and the presence of pollutants, are normally followed by an increase in PUFA. This increase is considered a defense mechanism (Oda, 2016; Silva et al., 2017), as seen in the sperm and testicles of *Cervus elaphus* (Castellanos et al., 2010). According to Oda (2016), n3 PUFA exhibited protective and antioxidant effects in many organs, including testicles and epididymis in rats, with histopathological and biochemical changes after Al administration. In *A. altiparanae*, however, no effects were observed in total PUFA in response to exposure to Al. These FAs tend to increase in the semen of fish exposed to Al at 20 °C due to a trend of increase in C22:6n3 and C22:5n3. This may be a response to protect the semen from the probable antioxidant effects caused by Al, as previously showed by Abdalla et al. (2019) in the liver and gills of mature males of this same species.

Some teleosts reproduce throughout the year and can modify the FA composition of gametic membranes, thus resisting seasonal variations in temperature (Engel et al., 2019). In *A. altiparanae*, a clear adjustment in FA composition was the decrease in the percentage of PUFA and an increase in MUFA in all groups maintained at 20 °C. Although the analysis of the present study considered total fatty acids, we cannot discard the possibility of a response

to a homeoviscous adaptation, common in cell membranes. This indicates that MUFA, especially C18:1, play an important function in the fluidity of membranes under temperature changes in tropical fish (Brooks et al., 2002).

Although fish exposed to Al and neutral pH also exhibited a decrease in C22:5n6 percentage at 20 °C, the temperature potentialized the effect of Al in ARA percentage, since only fish exposed to Al at 20 ºC presented a lower ARA percentage in semen. Arachidonic acid (ARA) increases the fluidity of cell membranes, in addition to acting as a substrate for the synthesis of eicosanoids that are involved in the regulation of inflammation and immune responses (Bagga et al., 2003; Calder, 2009). Thus, the reduction of ARA in the Al group can decrease membrane permeability if this alteration is also present in membrane phospholipids. This is a possible mechanism to reduce metal entry into the cell, and/or a result of ARA metabolization to activate the immune system via eicosanoids production.

The role of FA in regulating semen quality was evidenced in the correlations between the main FA and the sperm quality variables. These correlations clearly demonstrated the importance of n3 PUFA as a protective response to the sperm, and the metabolism of ARA for the formation of prostaglandins that are involved in the production of steroids and immune response (Wade and Van der Kraak, 1993), increasing sperm velocities. This relationship has also been observed in rainbow trout (*Oncorhynchus mykiss*) (Lahnsteiner et al., 2009) and European eel (*Anguilla anguilla* L.), highlighting the negative correlation between ARA in the testes and VCL (Baeza et al., 2015), which is the most important velocity parameter for a good fertilization (Viveiros et al., 2010). Additionally, ARA, DHA, and C18:1 were the main FAs in the semen of *A. altiparanae* and several other species. These FAs are important for the sperm membrane (Bobe and Labbé, 2010), being closely related to sperm quality patterns.

Another valuable biomarker to evaluate the action of pollutants on reproduction is the occurrence of malformations during embryonic and larval development. The exposure to cadmium reduced the sperm kinetics of *Odontesthes bonariensis* and *Prochilodus magdalenae*, this reduction negatively interfered with larval survival (Sierra-Marquez et al., 2019; Gárriz and Miranda, 2020). In the present study, male exposure to Al decreased fertilization by 40% at both temperatures, and hatching by 76% (20 ºC), also leading to a higher number of abnormal larvae (4-fold at 20 ºC; 6-fold at 25 ºC) and higher mortality (at 20 ºC). This occurred due to the influence of sperm on fertilization. Previous studies using the same Al concentration of the present study showed that the element bioconcentrated in the testes and semen (Pinheiro et al., 2019) and reduced seminal quality (Pinheiro et al., 2020). These gametic changes may also have resulted from changes in the hormonal levels of the hypothalamic-pituitary-gonadal axis, such as the increase in plasma testosterone levels reported by Kida et al. (2016) for males of the same species (25 ºC).

Physical and chemical parameters of water affect the bioavailability and toxicity of pollutants, which may interfere with the physiology of aquatic organisms (Wlasow et al., 2010; Paiva Magalhães et al., 2015). Bioassays demonstrate that the acidity of some rivers, in addition to favouring Al bioavailability, increases the plasma levels of testosterone and 11-ketotestosterone in *A. altiparanae* males (pH 5.5; 25 ºC; Kida et al., 2016). This endocrine disruption may have influenced the reduction of sperm quality (Pinheiro et al., 2020), the lower percentage of fertilization and hatching, and the higher percentage of embryo/larvae mortality in the present study.

Acidic pH activating medium reduced both fertilization and hatching. Some studies highlight that embryos and larvae have a high sensitivity to acidic pH (i.e., pH < 6.0; McCormick et al., 1989 [*Pimephales promelas*]; Keinänen et al., 2003 [*Coregonus lavetus lavaretus*]; Keinänen et al., 2004 [*Rutilus rutilus*]; Santos et al., 2020 [*Astyanax lacustris*; *Piaractus mesopotamicus*]). This may be

due to high surface/volume ratio, absence of specialized mechanisms for acid-base regulation (Kikkawa et al., 2003; Ishimatsu et al., 2008), and energy inhibition or slow yolk absorption (Baker and Schofield, 1982; Leino et al., 1988). As ionoregulation is a process that requires energy consumption, the lethal effects of acidic pH may be due to the combination of these bioenergetically linked ionic and osmoregulatory interferences (McCormick et al., 1989).

In addition to water pH, temperature variation in the activating medium/incubation water can interfere with reproductive parameters and embryonic development. The highest temperature of the present study accelerated the fertilization and hatching events which was expected since temperature affects the metabolism, activity, and structure of the developing embryo (Bobe and Labbé, 2010). Santos et al. (2016) observed the same events in *A. altiparanae*. However, although these processes occurred more quickly in the treatments at 25 ºC, the fertilization rate was higher at 20 ºC in most experimental treatments (except in the Al treatment with Al activating medium). Sperm motility period is usually higher at lower temperatures since higher temperatures trigger higher cell motility and velocities, which reduces the motility period; consequently, the sperm will have less time to find the micropyle and fertilize the oocyte (Alavi and Cosson, 2005; Kholodnyy et al., 2020). However, hatching rate was higher at the highest temperature, except for the neutral pH treatment. These observations, together with the higher mortality at 20 ºC, reinforce that the optimal temperature for *A. altiparanae* embryonic development is between 25 ºC and 26 ºC, which corresponds to the tropical climate (Santos et al., 2016, 2020).

The oocyte permeability can favour the entry of metals and, consequently, alter the structure and permeability of the chorion (Jezierska et al., 2009; Cerdà et al., 2013). In *Cyprinus carpio* eggs, copper, cadmium, and lead reduced swelling by about 37% (with an increase in egg diameter) (Jezierska and Słominska, 1997; Sikorska and Ługowska, 2005). The degree of inhibition of egg swelling is dependent on the concentration and hydration energy of the metallic

ion. In this sense, the high hydration energy of aluminum strongly inhibit colloidal processes (Eddy and Talbot, 1983). Thus, the malformation of embryos/larvae in *A. altiparanae* may be due both, the quality of the sperm and the effect of the activating medium on oocyte swelling.

In addition to these negative effects, agglutination of embryos and the presence of precipitates in the chorion were noticeable at 20 and 25 ºC (Al + Al activating medium). Some authors also observed these events in the perivitelline fluid of whitefish (*Coregonus lavaretus lavaretus*) exposed to acidic pH (5.5) and/or Al (at pH 5.5 and 6.5) (Keinänen et al., 2003). This may have occurred due to the accumulation of excretory products from the abnormal chemical reactions at low pH (Dabrowski et al., 1984; Wright and Land, 1998) or due to the coagulation of perivitelline material in adverse conditions (Keinänen et al., 2003). In whitefish, these precipitates correlated with blood circulation disturbances, abnormal spinal curvature, and increased embryo mortality (Keinänen et al., 2003). These disorders were among the main malformations of *A. altiparanae*. Some authors have reported Al toxicity in embryonic/larval development in different vertebrates, including defects in the avian and the zebrafish heart, and neurological damage in zebrafish glial cells (El Mazoudy and Bekhet, 2016; Monaco et al., 2017).

The results point to the need for further studies to evaluate the effects of Al exposure for both parents and progenies, addressing larval development and the mechanisms (e.g., gene regulation, DNA damage, enzymatic activity, etc.) correlated with water toxicity (e.g., changes in pH, temperature, and the presence of Al in the water). Besides, further studies are required to complete the understanding of the metabolic damage caused by the anthropic actions.

5. Conclusion

Under the experimental conditions of the present study, temperature exposure changed the percentage of C20:4n6 and C22:5n3 in *A. altiparanae*

semen. Moreover, Al exerted a more pronounced effect, influencing FA composition regardless of treatment, with a consequent increase in the percentage of MUFA, especially C18:1. Our data showed that males exposed to acidic pH and Al have a lower fertilizing capacity. This negative effect on sperm quality also decreased the percentage of hatching and normal larvae, with a consequent increase in the percentage of embryo/larvae mortality. The presence of Al in the activating medium after previous male exposure to Al favoured a higher percentage of abnormal larvae as a function of temperature. Furthermore, the Al activating medium/incubation water of the embryos/larvae impaired the percentage of fertilized, hatched, and normal larvae. This may be due to changes in the fertilization events, such as chorion permeability and egg swelling. Finally, temperature influenced fertilization time, hatching time, and the morphology of *A. altiparanae* embryos/larvae.

Acknowledgments

This study was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; Grants #2016/08770-8 and #2014/16320-7), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Grant 140197/2016-8) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) - Finance Code 001. The authors would also like to thank the Votorantim Energia Group (Paraibuna-SP) for donating the fish, the staff at LAMEROA (IB-USP; Ethiene F. Oliveira, Marília P. Camargo, Valquíria de O. Pereira, Natália P.V.M. de Faria, Vagner Alberto e Victor H. M. Lorenti) for the assistance in the experiments, IB/USP for providing logistics and facilities, and Victor L. Jardim for the illustrations of this study.

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Discussão Geral e Conclusões

Esse é o primeiro estudo a evidenciar a toxicidade do Al associado a mudanças de temperatura sobre a qualidade seminal, bem como sobre as taxas de fertilização, de eclosão e desenvolvimento embrionário de uma espécie de teleósteo. Além do mais, o estudo buscou utilizar diferentes biomarcadores para explicar a interferência desses fatores ambientais na fisiologia reprodutiva, como a quantificação do metal nos testículos e no sêmen, anormalidades nucleares sanguíneas, fragmentação de DNA dos eritrócitos e dos espermatozoides, aspectos quantitativos (ex. concentração espermática) e qualitativos do sêmen (ex. pH e osmolalidade seminais, perfil de ácidos graxos do sêmen, morfologia, ultraestrutura e cinética espermáticas), além dos parâmetros reprodutivos (fertilização e eclosão) e do desenvolvimento embrionário.

A capacidade do Al de se bioconcentrar em diferentes órgãos já é evidenciada na literatura em mamíferos, como no cérebro (Bondy, 2010; Exley, 2014; Exley e Mold, 2019; Exley e Clarkson, 2020), e em alguns órgãos de peixes, como nas brânquias e no fígado (Vuorinen et al., 2003; Correia, 2012; Narcizo et al., 2021), sendo também encontrado, no presente estudo, nos testículos e no sêmen de teleósteo. Essa bioconcentração ocorreu de maneira dependente do tempo de exposição dos animais e da temperatura, e diferentemente entre os testículos e o sêmen. Entretanto, era esperado que ambos tivessem maiores concentrações de Al após 96h de exposição dos animais e quando fossem expostos na maior temperatura do estudo. Sugerimos que a bioconcentração de Al no sêmen ocorreu mais rápido do que nos testículos em decorrência da consistência fluida e da taxa de renovação dos espermatozoides durante a espermatogênese. No entanto, nenhum estudo foi realizado até agora comparando a concentração de metais nos testículos e no sêmen. Essa bioconcentração de Al pode ter favorecido a interferência desse metal na secreção dos andrógenos

(testosterona e 11-cetotestosterona), como observado por Kida et al. (2016) em *A. altiparanae*, que atuam no processo de formação dos espermatozoides, e, consequentemente, na atividade gamética.

Outra ação do Al que foi observada neste estudo foi a capacidade de gerar genotoxicidade em diferentes tipos celulares, como nos eritrócitos e nos espermatozoides, hipótese inicialmente levantada e confirmada. Esse efeito tóxico também se apresentou dependente do tempo e da temperatura de exposição do animal. Por isso, sugere-se que a fragmentação do DNA foi aumentada pelo processo de desnaturação induzido pela presença do Al, como observado nos espermatozoides após 96 h de exposição dos animais. Além disso, ficou evidente que as temperaturas extremas do experimento modificam a sensibilidade da célula a poluentes (fluidez da membrana) e/ou favorecem a maior toxicidade do Al, uma vez que na menor temperatura (20 ºC) ou na maior (30 ºC) houve aumento da fragmentação do DNA. Também, alguns estudos destacam, de forma isolada, que a elevação de temperatura em relação a ideal para o teleósteo e/ou para os gametas desses indivíduos acarretam estresse termal, induzindo a expressão de proteínas de choque térmico (HSP70, do inglês, *heat shock protein*) e aumentando o grau de fragmentação do DNA (Hassan et al., 2017; Castro et al., 2020), o que pode ter ocorrido no presente estudo. Quanto ao pH ácido, os achados destacam que esse parâmetro químico da água também é capaz de induzir danos no DNA dos eritrócitos e dos espermatozoides de *A. altiparanae*, mas em menor proporção ao se comparar com a presença do Al na água. No tocante às anormalidades nucleares nos eritrócitos, a frequência foi muito baixa, indicando que o tempo de exposição não foi suficiente para induzir tais alterações, uma vez que é reportado na literatura que essas anormalidades são mais presentes em teleósteos expostos a agentes genotóxicos por longos períodos de exposição (Osman et al., 2010; Vicari et al., 2012).

Com isso, todos esses eventos supracitados se relacionam e despertam a interferência do Al nos parâmetros seminais da espécie estudada, que influenciam

na maturação espermática e na capacidade fecundante do óocito. Dentre esses parâmetros, o pH do sêmen não foi perturbado pelo pH ácido da água nem pela presença do Al. Tal efeito pode ter sido resultante do tempo de exposição, que permitiu um reajuste dos valores por meio da capacidade de tamponamento do fluido seminal.

No tocante à osmolalidade seminal, houve uma resposta temperatura-dependente, e tanto o pH ácido quanto a presença de Al reduziram os valores de osmolalidade, sendo esta redução mais acentuada com a presença do metal na maior temperatura do estudo. Essas alterações podem ter ocorrido porque o pH e a temperatura ambientais impõem mudanças na permeabilidade de membrana, na atividade enzimática e no metabolismo energético (Dadras et al., 2016). Além do mais, alguns metais pesados (que não é o caso do Al) podem afetar e bloquear canais de água ou aquaporinas, que são responsáveis pela regulação osmótica e ativação da motilidade celular (Preston et al., 1993; Kuwahara et al., 1997; Dietrich et al., 2010), consequentemente, sendo um dos fatores responsáveis pela redução da cinética espermática observada no estudo.

Mais uma vez, observou-se que as temperaturas extremas do estudo potencializaram o efeito tóxico do Al, desta vez na motilidade espermática (tanto em 24 h quanto em 96 h de exposição). No geral, a maior temperatura do estudo reduziu a duração da motilidade espermática, mas que foi compensada por uma velocidade de natação mais alta em comparação às menores temperaturas (maior duração da motilidade). Tal efeito deve-se a influência da temperatura nos processos metabólicos, reduzindo os estoques energéticos a *posteriori* (Dadras et al., 2016). A redução da motilidade ocasionada pelo Al também já foi observada em mamíferos (Cheraghi et al., 2017; Martinez et al., 2017), sendo relacionada com desequilíbrio redox e com um aumento na produção de espécies reativas de oxigênio, peroxidação lipídica e capacidade antioxidante alterada em órgãos reprodutivos. Além da motilidade, os poluentes podem influenciar nas velocidades espermática, que possuem uma correlação positiva com as taxas de

fertilização em teleósteos (Viveiros et al., 2010; Gallego et al., 2017), principalmente a velocidade curvilinear (VCL), que foi afetada pelo Al em todas as temperaturas do estudo. Sugere-se que o Al pode ter se ligado às proteínas flagelares interferindo na movimentação espermática, além de afetar a atividade de enzimas dos espermatozoides e, consequentemente, o metabolismo (como a inibição da atividade proteica, desnaturação ou mudanças conformacionais nas proteínas). Dessa maneira, a estrutura do flagelo do gameta masculino pode ser alterada, e o processo de deslizamento dos microtúbulos pode ser prejudicado, como ocorre na exposição ao mercúrio (Dietrich et al., 2010). Além do mais, essa redução na cinética é pode ser decorrente das alterações ultraestruturais causadas pelo Al, como observado no presente estudo.

Além desses biomarcadores, o presente estudo avaliou a interferência da acidez, mudanças de temperatura e a presença de Al na água sobre o perfil de ácidos graxos (AG) seminais, configurando o primeiro estudo a utilizar tal ferramenta na área de Ecotoxicologia. Uma vez que a maior temperatura do estudo no grupo Al causou 50% de mortalidade após 96 h de exposição, decidimos conduzir a análise de AG, bem como os testes de fertilização e de eclosão, somente com as outras temperaturas testadas no estudo (20 ºC e 25 ºC). A exposição ao Al alterou a porcentagem de C20:4n6 (ácido araquidônico, ARA) e C22:5n3 (ácido docosapentaenoico, EPA) seminal, com um maior percentual em 25 ºC, que pode ser um mecanismo de proteção do sêmen aos prováveis efeitos oxidantes causados pelo Al. A redução da porcentagem do ARA no grupo Al (20 ºC em relação a 25 ºC) pode também diminuir a permeabilidade da membrana, se essa alteração também estiver presente nos fosfolipídios da membrana. Este é um possível mecanismo para reduzir a entrada de metal na célula, e/ou um resultado da metabolização de ARA para ativar o sistema imunológico via produção de eicosanoides. Da mesma forma que na variáveis já descritas, o efeito da temperatura também foi observado, com um aumento de ácidos graxos monoinsaturados (MUFA) em todos os grupos mantidos a 20 °C, que pode ser

resultado de uma adaptação homeoviscosa, comum nas membranas celulares (Brook et al., 2002). Somado a isso, ácidos graxos, como MUFA, PUFA n3 e n6 PUFA total correlacionam-se com alguma variável da cinética espermática e a taxa de fertilização, o que demostra a importância desse biomarcador para futuros estudos de qualidade seminal frente a exposição a diferentes estressores ambientais.

Dessa maneira, sugere-se que esses efeitos supracitados do Al na qualidade seminal resultaram em menores porcentagens de fertilização e de eclosão, assim como a interferência ao longo do desenvolvimento embrionário da espécie estudada (maior mortalidade de embriões e larvas e surgimento de más-formações, como edema cardíaco, deformidade do saco vitelínico e estrutura modificada da corda). Além disso, ficou evidente o efeito aditivo da água "poluída" na ativação dos gametas e na incubação dos embriões e larvas, conferindo uma maior redução na porcentagem de oócitos fertilizados, de larvas eclodidos e normais.

A figura 1 sintetiza os principais efeitos do Al, pH ácido e da temperatura sobre o eixo hipotálamo-hipófise-gônadas, assim como no sêmen, na fertilização, eclosão, nos embriões e larvas de *A. altiparanae*. Essa análise foi realizada compilando-se os dados de Kida et al. (2016) com os resultados obtidos no presente estudo.

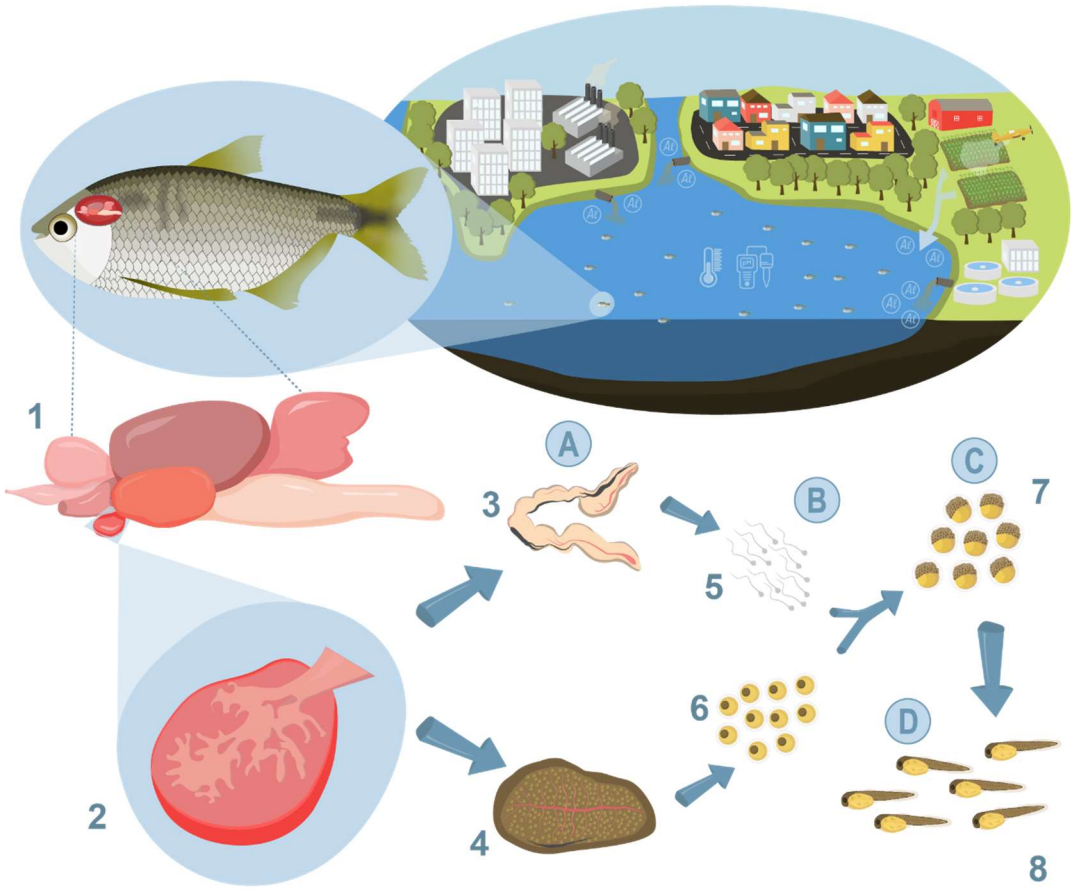

Fig. 1. Alumínio (Al), pH ácido e mudanças de temperatura da água interferem na fisiologia reprodutiva e na qualidade dos gametas de *A. altiparanae*, bem como no desenvolvimento embrionário e larval da espécie. Os números correspondem a: 1. Cérebro; 2. Hipófise; 3. Testículos; 4. Ovários; 5. Espermatozoides; 6. Oócitos; 7. Embriões; 8. Larvas. A exposição ao alumínio (0,5 mg L^{-1}) aumenta os níveis plasmáticos de testosterona e 11-cetotestosterona (A; Kida et al., 2016), e a acidez também aumenta os níveis plasmáticos de andrógenos (A; Kida et al., 2016). Além disso, este metal e/ou a água ácida reduzem a qualidade seminal (B) em função da temperatura; altera ácidos graxos seminais (B; presente estudo); diminui a porcentagem de fertilização e eclosão; interfere negativamente no desenvolvimento embrionário (C; presente estudo); e diminui a porcentagem de larvas normais (D; presente estudo) em função da temperatura. Ilustração de Jardim (2020).

Por fim, as principais conclusões obtidas nos capítulos da tese são:

1. O alumínio, mesmo em concentrações ambientais, bioconcentra-se nos testículos de *A. altiparanae* após 96 h de exposição dos animais e esse acúmulo se intensifica em temperaturas mais altas. Já no sêmen, essa acumulação ocorre mais cedo, logo após 24 h de exposição. A associação de estressores ambientais, principalmente o Al, desencadeia efeitos citotóxicos e genotóxicos, causando danos reversíveis no DNA dos eritrócitos e espermatozoides de *A. altiparanae*;

2. A acidez influencia os parâmetros seminais de *A. altiparanae*, mas a presença de Al na água, em concentrações ambientais, acentua os efeitos na qualidade seminal, especialmente na osmolalidade, na concentração, na cinética e na ultraestrutura espermáticas. Essa toxicidade é influenciada pela temperatura;

3. A temperatura alterou a porcentagem de C20:4n6 e C22:5n3 no sêmen de *A. altiparanae*. Além disso, o Al exerceu efeito mais pronunciado, influenciando a composição de AG independentemente do tratamento, com consequente aumento do percentual de MUFA, principalmente C18:1. Além disso, machos expostos ao pH ácido e ao Al têm menor capacidade fertilizante. Este efeito negativo na qualidade seminal também diminuiu a porcentagem de eclosão e de larvas normais, com consequente aumento na porcentagem de mortalidade de embriões/larvas. Além disso, a presença de Al no meio de ativação espermática e de incubação dos embriões, após exposição prévia dos machos ao Al, prejudicou a porcentagem de fertilização, assim como de larvas eclodidas e normais. Finalmente, a temperatura influenciou o tempo de fertilização, de eclosão e a morfologia dos embriões/larvas de *A. altiparanae*.

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Anexos

ANEXO 1. Matéria publicada na Revista Superinteressante

(https://super.abril.com.br/ciencia/aluminio-na-agua-afeta-semen-e-causa-malformacoes-em-embrioes-de-peixe/)

ANEXO 1. Matéria publicada na Revista Superinteressante

Clique e assine com até 75% de desconto

Ciência

Alumínio em excesso na água afeta sêmen e causa malformações em embriões de peixe

Grupo da USP descobre que alterações na temperatura, acidez e concentração de metais são suficientes para afetar a reprodução de lambaris – mesmo quando os rios não estão perceptivelmente poluídos.

Por **Bruno Vaiano** Atualizado em 16 nov 2020, 08h42 - Publicado em 15 nov 2020, 18h12

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No imaginário popular, rio poluído é sinônimo de esgoto a céu aberto. Mas muitos corpos d'água estão contaminados de maneiras mais sutis – imperceptíveis para quem dá um mergulho, mas mesmo assim bastante danosas para os ecossistemas.

O biólogo João Paulo Pinheiro se dedica a estudar os problemas que o alumínio causa na reprodução de peixes. Ele integra a equipe do Laboratório de Metabolismo e Reprodução de Organismos Aquáticos, na Universidade de São Paulo (USP), que investiga como metais e outros resíduos discretos da atividade humana – como fármacos – afetam a vida debaixo d'água.

"Em quase todos os lugares em que nós trabalhamos existe pesca e lazer. A poluição não é perceptível", diz João, que coleta amostras no interior de São Paulo. "Além disso, o alumínio que nós estudamos está presente

naturalmente no rios – vem da erosão de rochas. O problema é que ele aparece em uma concentração anormal graças à atividade humana."

O grupo da USP trabalha até com a água de partes mais limpas do Rio Tietê – o rio é razoavelmente bem conservado em sua nascente, embora o trecho que cruza a capital paulista seja famoso pelo cheiro (só as capivaras, com sua paciência plácida, aguentam).

O trabalho mais recente de João e seus colegas – publicado no periódico científico *Chemosphere* – trata de um peixe denominado *Astyanax altiparanae*, que você e seu estômago provavelmente conhecem por outro nome: lambari. Mais precisamente, lambari-tambiú.

Em laboratório, a equipe liderada pela professora Renata Moreira avaliou como mudanças na temperatura, na acidez e na quantidade de alumínio diluído na água afetam as propriedades bioquímicas do sêmen produzido pelos machos dessa espécie.

CONTINUA APÓS A PUBLICIDADE

A temperatura da água variou entre 20 °C e 25 °C e o pH foi de 5,5, mais ácido que o neutro, que é 7.

Além de reduzir a qualidade do sêmen, o metal, o calor e pH baixo afetam o desenvolvimento dos peixes bebês tanto no estágio embrionário (ainda dentro dos ovos) quanto no estágio larval (depois que os ovos eclodem).

Os machos que vivem em águas com níveis anormais de alumínio têm dificuldades em fecundar os ovos. Quando a fertilização é bem-sucedida, o número de ovos que efetivamente eclode é menor, e o número de larvas com malformações aumenta.

Esses resultados reforçam conclusões de outros dois artigos sobre alumínio publicados pelo grupo de João em 2019 e no meio de 2020, que analisaram outros parâmetros. Os pesquisadores observam de tudo: do formato da cabeça e do flagelo (o "rabinho") dos espermatozoides a sua motilidade, ou seja: quantos deles nadam com vigor e quantos ficam largados por aí.

Pode parecer óbvio que peixes que vivem em águas poluídas tenham um desempenho pior em todos os estágios de sua reprodução. Mas lembre-se: não estamos falando de um rio sufocado por esgoto. Para um leigo, essas podem soar como alterações discretas no equilíbrio do habitat.

É uma demonstração clara de algo que o naturalista alemão Humboldt – que foi um ambientalista antes de existirem ambientalistas –, já havia percebido no século 18: a natureza é interconectada, o que a torna vulnerável como um tecido. Se você puxa um fio, toda a trama pode se desmanchar.

O ser humano, claro, é parte da trama: os peixes são a porta de entrada do alumínio e de outros metais na cadeia alimentar. Esses elementos pesadinhos da tabela periódica, que passam de animal para animal, também fazem mal a mamíferos como nós. Concentrações anormais de alumínio em seres humanos já foram associadas, por exemplo, à doença de Alzheimer.