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CRESCIMENTO E SOBREVIVÊNCIA DE JUVENIS DE JUNDIÁS (*Rhamdia quelen*) EM DIFERENTES CONCENTRAÇÕES DE ÁCIDO HÚMICO, EM pH ÁCIDO.

TESE DE DOUTORADO

Sílvio Teixeira da Costa

Santa Maria, RS, Brasil
2012

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Sílvio Teixeira da Costa

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Doutor em Zootecnia

Orientador: Prof. Bernardo Baldisserotto

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Universidade Federal de Santa Maria
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Elaborada por
Sílvio Teixeira da Costa

Como requisito parcial para obtenção do grau de
Doutor em Zootecnia

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Santa Maria, 27 de julho de 2012.

Dedico
Às mulheres da minha vida!
Em ordem cronológica, desde o inicio do século XX:
Vó Xeda, Vó Lili, Mãe, Ana Gabriela, Maria Antônia e Maria Clara.

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Aos grandes amigos da UNICRUZ, ao CNPq, FEAPAM – INCT ADAPTA, à Professora Marisa Narciso Fernandes/UFSCar e PPGZ/UFSM.

"Lógica e sermões jamais convencem;
O úmido da noite cala fundo em minha alma...
E eu reexamino filosofias e religiões.
Ambas provam muito bem dentro de quatro paredes, mas nada provam quando temos sobre nós as nuvens, e nos rodeia a paisagem, e nos defronta o caudal.
É que a paisagem, a caudal e as nuvens formigam de vida incalculável."
Will Durant

RESUMO

Tese de Doutorado
Programa de Pós-graduação em Zootecnia
Universidade Federal de Santa Maria, RS, Brasil

CRESCIIMENTO E SOBREVIVÊNCIA DE JUVENIS DE JUNDIÁS (*Rhamdia quelen*) EM DIFERENTES CONCENTRAÇÕES DE ÁCIDO HÚMICO, EM pH ÁCIDO.

Autor: Sílvio Teixeira da Costa
Orientador: Bernardo Baldisserotto
Data e local da defesa: Santa Maria, 27 de julho de 2012.

O ácido húmico é uma substância orgânica identificada nas águas pretas, já utilizada como promotor de crescimento e que confere proteção contra a contaminação ambiental por metais. O objetivo deste estudo foi verificar a sobrevivência e o crescimento de juvenis de jundiá – *Rhamdia quelen* em concentrações distintas de ácido húmico em pH ácido, observando os efeitos morfológicos nas brânquias e nos eritrócitos. No primeiro manuscrito, os juvenis foram expostos a dois pH (5,5 e 6,5) em concentrações (0, 10, 25 e 50 mg L^{-1}) de ácido húmico, por 40 dias. No segundo manuscrito, foram analisados os aspectos histológicos e morfométricos das brânquias dos juvenis expostos a esses tratamentos ao final de 40 dias de experimento. O terceiro manuscrito avaliou a sobrevivência e o efeito na morfometria eritróide de juvenis de jundiá expostos a pH distintos (3,8; 4,0; 4,2 e 7,0) e nas concentrações de ácido húmico já citadas. Após ao experimento, os juvenis de jundiás apresentaram maior peso, comprimento, bioamassa, consumo de ração e taxa de crescimento específico quando expostos ao pH 6,5 sem a presença de ácido húmico. As brânquias dos jundiás expostos ao pH 5,5 em 50 mg L^{-1} de ácido húmico apresentaram maior comprimento dos filamentos, largura das lamelas e da espessura do epitélio do filamento branquial, menor distanciamento entre as lamelas, proliferação de células de cloreto e aumento na área de superfície respiratória GRSA. Também se verificou proliferação das células de cloreto e aumento na área fracional das células de cloreto (CCFA) dos juvenis expostos ao pH 5,5 em 50 mg L^{-1} de ácido húmico. A sobrevivência dos juvenis foi comprometida em pH 3,8 e pH 4,0, diminuindo esta sobrevivência em quanto maior foi a concentração de ácido húmico adicionado ao meio ambiente. Jundiás expostos ao pH 7,0 apresentavam área eritrocitária maior que os peixes expostos aos pH 3,8; 4,0 ou 4,2. Já a presença do ácido húmico causou um efeito contrário, fazendo com que os eritrócitos de peixes expostos às maiores concentrações (50 mg L^{-1}) tivessem maiores diâmetros. Não há alteração nos níveis plasmáticos de Na^+, K^+, porém em pH 3.8 os níveis plasmáticos de Cl^- foram menores que em pH neutro e esse efeito foi potencializado pelo aumento da concentração de ácido húmico. Assim, conclui-se que a presença do ácido húmico sintético na água é prejudicial para o desenvolvimento de juvenis de jundiá; quanto maior a concentração de ácido húmico na água, maior é a barreira água-sangue e a proliferação de células de cloreto, alterando a morfologia branquial de juvenis de jundiá; os parâmetros hematimétricos de juvenis de jundiás também são alterados pela presença do ácido húmico sintético, podendo comprometer a adaptação e sobrevivência desta espécie em pH ácido; o ácido húmico protege juvenis de jundiá contra os efeitos ionorregulatórios negativos da exposição ao pH ácido.

Palavras-chave: Substância húmica. Histologia. Piscicultura.

ABSTRACT

PhD Thesis
Post-Graduate Program in Animal Husbandry
Universidade Federal de Santa Maria

DEVELOPMENT AND SURVIVAL OF JUVENILE SILVER CATFISH (*Rhamdia quelen*) CHALLENGED BY ACIDIC pH AT DIFFERENT CONCENTRATIONS OF HUMIC ACID.

Author: Sílvio Teixeira da Costa
Adviser: Bernardo Baldisserotto
Data and Place of Defense: July 27th, 2012, Santa Maria, RS, Brazil

Humic acid is an organic substance identified in black waters, used as growth promoter and confers protection against environmental contamination by metals. The objective of this study was to evaluate survival and growth of juvenile silver catfish – *Rhamdia quelen* exposed to different concentrations of humic acid at acidic pH, observing the morphological effects in the gills and erythrocytes. In the first experiment, juveniles were exposed to two pH (5.5 and 6.5) with concentrations (0, 10, 25 and 50 $mg.L^{-1}$) of humic acid (CAT: H1 0.675-2 Aldrich® - humic acid sodium salt) for 40 days. In the second study the histological and morphometric parameters of gills of juveniles exposed to these treatments were analyzed. The third study evaluated the effect of different pH (3.8, 4.0, 4.2 and 7.0) and the concentrations of humic acid previously described on survival and erythroid morphology of juvenile silver catfish. Silver catfish showed greater weight, length, biomass, feed intake, and specific growth rate when exposed to pH 6.5 without the presence of humic acid. The gills of silver catfish exposed to pH 5.5 at 50 $mg.L^{-1}$ of humic acid presented higher filament length, width of the lamellae, thickness of the epithelium of gill filament, lower distance between the lamellae, cell proliferation and increase in chloride gill respiratory surface area GRSA. There was also proliferation of chloride cells and increase in chloride cells fractional area (CCFA) of juveniles exposed to pH 5.5 at 50 mg L^{-1} humic acid. Survival of juveniles was impaired at pH 3.8 and pH 4.0, and the higher the concentration of humic acid, the lower the survival. Silver catfish exposed to pH 7.0 showed higher erythrocyte are than those exposed to pH 3.8, 4.0 or 4.2. The presence of humic acid caused an opposite effect, increasing red cells diameter in fish exposed to higher concentrations (50 $mg.L^{-1}$). No changes in plasma Na^+, K^+, but at pH 3.8 the plasma levels of Cl^- were lower than at neutral pH and this effect was potentiated by the increase of humic acid levels. Thus, it is concluded that the presence of synthetic humic acid in water is harmful to the developing juvenile catfish, the higher the concentration of humic acid in water, the higher is the water barrier and blood-cell proliferation chloride, changing the gill morphology of juvenile catfish, hematological parameters of juvenile silver catfish are also altered by the presence of synthetic humic acid, which could compromise adaptation and survival of this species in acidic pH, the humic acid protects against the juvenile catfish ionorregulatórios negative effects of exposure the acidic pH.

Keywords: Humic substance. Histology. Fish culture.

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

Manuscrito 02 - Morphometry of Silver Catfish juveniles Gills Exposed to Different Levels of Humic Acid and Acidic pH

Manuscrito 03: Impact of humic acid and acidic pH exposure on silver catfish juveniles (*Rhamdia quelen*) ionoregulation and hematimetric parameters

SUMÁRIO

INTRODUÇÃO

As alterações climáticas estão trazendo mudanças substanciais no setor de pesca em âmbito mundial, que já se encontra sob pressão de sobrepesca e outras influências antropogênicas. Existem evidências de que as águas continentais também estão em aquecimento e que existem diferentes impactos da mudança climática sobre o escoamento dos rios que alimentam estas águas. Nos sistemas de água doce em geral, também existem preocupações específicas relativas a mudanças na intensidade, tempo e duração das cheias, o que pode alterar a desova de muitas espécies de peixes. (FAO, 2010).

A pesca e a aquicultura, direta ou indiretamente, desempenham um papel essencial no sustento de milhões de pessoas em todo o mundo, desde os pequenos pescadores de águas interiores que pescam o peixe em lagos e brejos até os homens e mulheres que trabalham nas grandes fábricas de processamento dos produtos pesqueiros. Considerando os agregados familiares, não menos do que 560 milhões de pessoas podem depender do setor, representando mais de 8% da população mundial. A pesca de captura e a aquicultura forneceram ao mundo cerca de 142 milhões de toneladas de peixe em 2008. E dessas, aproximadamente 115 milhões de toneladas foram utilizadas como alimento humano, proporcionando um fornecimento per capita aparente estimado em cerca de 17 kg (peso vivo) por pessoa, que é mais o alto de todos os tempos, e ainda estima-se que em 2030 esse consumo esteja em 20 kg por pessoa. Nesse contexto, a aquicultura representa 46% da oferta total de pescado (FAO, 2010).

O Brasil, com um alto potencial para o desenvolvimento da aquicultura, não figura nem entre os dez maiores produtores, nem entre os maiores importadores mundiais. O Brasil possui 8.400 km de Zona Econômica Exclusiva (ZEE), equivalente ao tamanho da Amazônia, 5.500.000 hectares de reservatórios de águas doces, com aproximadamente 12% da água doce disponível no planeta, clima extremamente favorável para o crescimento dos organismos cultivados, terras disponíveis e ainda relativamente baratas na maior parte do país, mão de obra abundante e crescente demanda por pescado no mercado interno. Em 2010, a produção aquícola nacional foi de 479.399 t, representando um incremento de 15,3% em relação à produção de 2009. Comparando-se a produção atual com o montante produzido em 2008 (365.366 t), fica evidente o crescimento do setor no país, com um incremento de 31,2% na produção durante o triênio 2008-2010. Seguindo o padrão observado nos anos anteriores, a

maior parcela da produção aquícola é oriunda da aquicultura continental, na qual se destaca a piscicultura continental, que representou 82,3% da produção total nacional (MPA, 2012).

Na região sul do Brasil, como existe o hábito de consumir espécies nativas obtidas por meio da pesca, existe a possibilidade de que a produção destas espécies melhore em função de um possível aumento na demanda (BALDISSEROTTO 2009). O mesmo autor comenta que o jundiá, *Rhamdia quelen*, ainda se apresenta como uma das espécies nativas de principal eleição para cultivo no sul do Brasil em função de sua resistência às baixas temperaturas e seu crescimento rápido, mas sua produção permanece muito abaixo das possibilidades. Na verdade, o cultivo do jundiá no Brasil apresenta decréscimo produtivo de 369,6 toneladas em 2008 para 352,1 toneladas em 2010 (MPA, 2012), já destacada por Baldisserotto (2009), o qual indicava um quadro de estagnação para a pesca e aquicultura a partir dos dados coletados no Rio Grande do Sul, para os anos de 2001 a 2006. Além disso, também destacou a redução na produção do jundiá, que mesmo representando a espécie nativa de maior presença na piscicultura continental do Estado, mostrou decréscimo de 7,6% para 1,5% do total. Provavelmente as causas dessa queda, assim como para qualquer espécie produzida, estejam relacionadas à falta de regularidade na demanda anual, uma infraestrutura de beneficiamento, armazenagem e distribuição deficientes.

Mesmo com um interesse já conhecido entre comunidade científica e o setor produtivo para o cultivo e desenvolvimento da ciência em espécies nativas, a inexistência de "pacotes tecnológicos" ainda figuram como um empecilho ao fomento da atividade. A produção de conhecimento que melhore as condições de criação e adaptação dessas espécies finalizará com um melhor desempenho produtivo autóctone, com menor risco ambiental.

Parâmetros de qualidade da água

Em condições de aquicultura, os desafios naturais e antropogênicos se somam àqueles impostos pela atividade, como por exemplo, práticas de manejo, transporte, tratamentos e altas densidades de estocagem. Desse modo, os peixes precisam encontrar meios de lidar com os desafios a fim de confrontá-los e superá-los, para garantir sua sobrevivência (LIMA et al., 2006). Tanto em condições de cultivo em tanques escavados, assim como no meio ambiente, é comum ocorrerem oscilações de variáveis ambientais tais como pH, temperatura, oxigênio dissolvido, concentrações iônicas entre outras. Estas variações ambientais podem interferir em aspectos biológicos dos peixes levando-os ao estresse, afetando sua sobrevivência, seu crescimento e possibilitando o aparecimento de enfermidades oportunistas.

No Brasil, relacionando suas dimensões continentais, se observam extensas variedades de solos de norte a sul. Esta variabilidade possibilita o aparecimento de baixas concentrações de íons em rios, assim como a elevada concentração de compostos orgânicos resultantes da decomposição da matéria orgânica vegetal e animal em lagos, mangues e viveiros escavados que possam não apresentar um manejo adequado de suas águas. Estes compostos orgânicos dissolvidos são referidos como substâncias húmicas (SH) e contribuem para uma acidez natural atribuída às águas pretas encontradas em regiões de manguezais e em alguns rios como o Rio Negro na bacia Amazônica.

As substâncias húmicas

A matéria orgânica natural representa componentes ubíquos resultantes do colapso físico e da atividade microbiana de componentes vegetais e animais, que frequentemente são designadas e quantificadas como carbono orgânico dissolvido (DOC). Estes compostos orgânicos, sob a óptica de constituição, podem estar constituídos de duas partes: uma fração não-húmica, formada de classes de compostos biomoleculares como lipídeos, carboidratos, polissacarídeos, aminoácidos, proteínas, ceras e resinas, e uma fração húmica que pode ser definida como sendo uma categoria de ocorrência natural, biogênicos, heterogêneo de substâncias orgânicas que pode geralmente ser caracterizada como sendo de cor amarela a preta e com alto peso molecular (MacARTHY et al., 1990). Na literatura ecológica, o carbono orgânico dissolvido é reconhecido como um regulador global de muitos processos bióticos e abióticos (por exemplo, o ciclo do carbono, o transporte de nutrientes, triagem ultravioleta) em ecossistemas de água doce (PETERSEN, 1991; KULLBERG et al., 1993; WILLIAMSON et al., 1999; STEINBERG et al., 2006). Na literatura toxicológica, aceita-se que DOC desempenha um papel chave na redução da toxicidade aquática de muitos metais, pela sua capacidade para ligá-los, reduzindo assim a biodisponibilidade para as superfícies alvo, tais como as brânquias. Na verdade, a concentração de DOC é agora incorporada aos modelos de ligandos bióticos (BLM) usados para prever a toxicidade sítio-especifica de metais ou para derivar em critérios de qualidade da água e do ambiente (DI TORO et al., 2001; SANTORE et al., 2001; PAQUIN et al., 2002; NIYOGI & WOOD, 2004). Esta capacidade "protetora" até uma época bem recente havia sido negligenciada, considerando que em algumas águas naturais as concentrações de DOC são bem superiores à de qualquer componente inorgânico (WOOD et al., 2011). Playle et al. (1993) foram os primeiros a perceber a importância dos testes com DOC's e de suas ligações com metais em experimentos de toxicidade brânquial para mostrar o diferencial destes efeitos de proteção.

A fração húmica, segundo McDonald et al. (2004), depende das diferentes capacidades de solubilização observadas nas substâncias húmicas. A humina é uma fração insolúvel, com reduzida capacidade de reação e com estrutura molecular estável. Os ácidos húmicos estão representados por uma fração escura, solúvel a partir de um pH 2,0, precipitando-se em um produto amorfo de alto peso molecular e quimicamente capaz de trocas catiônicas, combinando-se com metais ou permanecendo dissolvidos em dispersão coloidal como os humatos de sódio, potássio, amônio e etc. Os ácidos fúlvicos constituem a fração colorida que se mantém solúvel em meio alcalino ou em meio diluído, em qualquer pH. Podem apresentar grupos carboxílicos e têm peso molecular relativamente baixo (MALCON, 1989).

Inspirado em experimentos realizados com *Potamotrygon sp*, Wood et al. (2011) relacionam efeitos diretos do carbono orgânico dissolvido, dos ácidos húmicos naturais sobre a fisiologia dessas arraias como uma proteção osmorregulatória ao efluxo de Na^+ e Cl^-, com uma redução no influxo em meio acidificado que somente seria possível a partir da duplicação da concentração de Ca^+ no meio. É possível que as moléculas de DOC, através das suas propriedades anfifílicas, possam interferir nas concentrações de íons no micro-ambiente externo dos transportadores branquiais. Matsuo e Val (2007) também assumem as substâncias húmicas como compostos inertes ao cardinal tetra *Paracheirodon axelrodi*, mas podem se tornar extremamente reativos em função de seus grupos funcionais, principalmente as frações carboxílicas e grupos hidroxil-fenólicos. Há um crescente reconhecimento que as substâncias húmicas interagem com as membranas biológicas em organismos nativos de habitats enriquecidos com substâncias húmicas como o Rio Negro, não só pelo ponto de vista geoquímico (adsorção, quelação ou complexação de elementos), mas também do ponto de vista biológico (bioquímico, fisiológico e biológico-molecular).

Matsuo e Val (2007) concluem que, em cardinal tetra, as substâncias húmicas, especialmente em condições de baixo pH diminuem a perda de Na^+ corporal, sugerindo que há uma proteção ao efluxo deste íon, e aumento na capacidade de absorção de Ca^{2+}.

Hseu et al. (2000), ao contrário do que tem-se comentado até agora, identificaram a ocorrência da BFD (Black foot disease) na costa oeste de Taiwan, onde a população faz uso de águas pretas ricas em ácido húmico, tanto para beber, quanto para o cozimento dos alimentos. A BFD, é uma doença arterial oclusiva, onde se observam importantes alterações eritróides, tais como: hemólise, peroxidação lipídica eritróide e o desequilíbrio de Ca^{2+} na membrana do eritrócito, descrita por Tseng et al. (1961). A toxicidade atribuída ao ácido húmico parece estar relacionada a um aumento na concentração de Ca^{2+} nos eritrócitos, o que pode ativar proteases citosólicas solúveis capazes de degradar proteínas endógenas, tais como

a espectrina (HSEU et al., 2000). Esse aumento micromolar de Ca^{2+} na célula proporciona perdas de K^+ e de volume celular, determinando a hidrólise de ATP e o aumento na rigidez da célula. Esses seriam mecanismos plausíveis para a transformação de eritrócitos humanos em equinócitos induzidos pelo ácido húmico (WHATMORE et al., 1992).

Em experimentos realizados com ácido húmico comercial, com a expectativa de mimetizar o efeito do ácido húmico natural obtido nas águas pretas do Rio Negro – AM, e em pH ácido, Wood et al. (2003), verificaram que houve uma exacerbação no efluxo iônico ao invés de um efeito protetor. Os mesmos autores ainda explicam que o ácido húmico comercial pode ter uma elevada afinidade aos íons Ca^{2+}, mobilizando até mesmo o Ca^{2+} branquial, promovendo o desequilíbrio osmorregulatório observado em peixes expostos a condições extremas de pH ácido.

Potencial hidrogeniônico da água

O pH é um parâmetro muito importante a ser considerado, já que possui um efeito direto sobre o metabolismo e os processos fisiológicos. Variações sazonais e diárias do pH ambiental podem afetar a homeostase iônica e respiração, e consequentemente, prejudicar o crescimento e a reprodução dos peixes (ARIDE et al., 2007). O pH das águas superficiais na região central do Rio Grande do Sul (Brasil), onde o jundiá é o peixe nativo mais produzido, geralmente permanece dentro do intervalo de 5,4-8,4 (LOPES et al., 2001).

Juvenis de jundiá suportam pH na faixa de 4,0 a 9,0 com uma dureza de 30,0 mg L^{-1} $CaCO_3$, e a exposição a águas ácidas ou alcalinas provoca uma redução dos níveis corporais de Na^+ e K^+ (ZAIONS & BALDISSEROTTO, 2000). O estresse ácido perturba a regulação iônica branquial, com consequente efluxo de Na^+, Cl^- e Ca^{2+} (HEATH, 1995).

Das et al. (2006) observaram que no esfregaço sanguíneo de carpa indiana *Catla catla* exposta ao pH ácido de 5,5 os eritrócitos apresentavam-se mais inchados e esféricos, com o núcleo centralizado e também inchado. O aumento do volume dos eritrócitos ocorre em função do mecanismo de regulação do volume celular, o que requer uma ativação dos antiportes Na^{+}/H^+ e Cl^-/HCO_3 (WEAVER et al. 1999). Das et al (2006) acreditam que a mudança no pH da água pode causar alterações osmorregulatórias e distúrbios ácido-base originados na brânquia, os quais alteram o pH interno do sangue, assim como o balanço e o equilíbrio osmótico. Tais distúrbios, por sua vez, conduzem à mobilização de catecolaminas e perturbações na homeostase de glóbulos vermelhos, conduzindo à ativação do AMPc sensível a bomba de Na^+/H^+ na superfície da membrana do eritrócito para efluxo de H^+ (JENSEN et al., 2002). Das et al. (2006) ainda comentam que a redução do pH também proporcionou uma

diminuição na contagem total de eritrócitos bem como no conteúdo de hemoglobina total dos eritrócitos de carpas, diminuindo a capacidade sanguínea de carrear oxigênio. Neste contexto de distorção e lise dos eritrócitos, o mecanismo compensatório está centrado no aumento da afinidade da hemoglobina pelo oxigênio e/ou aumento na produção de células vermelhas e liberação de células imaturas na circulação (GILL et al., 1991).

Objetivos

Objetivos gerais:

Verificar a sobrevivência e o crescimento de juvenis de jundiá (*Rhamdia quelen*) mantidos em pH ácido, na presença de diferentes concentrações de ácido húmico.

Objetivos específicos:

Avaliar o efeito da adição de diferentes concentrações de ácido húmico e do pH ácido no crescimento do jundiá;

Determinar o efeito da adição de diferentes concentrações de ácido húmico e do pH ácido na morfologia das brânquias do jundiá;

Analisar a sobrevivência e os efeitos das diferentes concentrações de ácido húmico e do pH ácido nos níveis de íons plasmáticos e na morfometria dos eritrócitos e concentração de hemoglobina.

Hipótese

O ácido húmico protege as brânquias dos jundiás, reduzindo o efluxo iônico causado pelo pH ácido, além de melhorar a sobrevivência e o crescimento dessa espécie.

DESENVOLVIMENTO

MANUSCRITO 01 – Submetido ao periódico Revista Brasileira de Zootecnia

GROWTH OF SILVER CATFISH IN DIFFERENT LEVELS OF HUMIC SUBSTANCE AND EXPOSED TO ACIDIC pH

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Abstract

Humic substances are ubiquitous in aquatic environments. These organic compounds are classified as heterogeneous, possess high molecular weight and are formed by the decomposition of plant materials. The humic substances comprise humic acids, fulvic acids and humin. The humic acids have shown to be able to provide some protection to the biological membranes of fish in water with low pH, replacing the protective action of Ca^{2+}. Thus, this study was aimed at observing growth of silver catfish (*Rhamdia quelen*) juveniles exposed for 40 days to pHs 5.5 and 6.5 and to different levels of humic acid: 0, 10, 25 and 50 mg L^{-1}. Results show that, irrespective of the pH, humic acid was highly detrimental to silver catfish growth, since the higher the concentration of humic acid, the lower the weight gain and feed intake, resulting in lower biomass and lower specific growth rate of the juveniles. Hence, humic acid is extremely damaging to the performance of silver catfish juveniles in the tested concentrations.

Key words: Fish growth, Humic acid, Water pH, *Rhamdia quelen*

Introduction

The organic compounds of humic substances result from the decomposition of dead plants and animals and contain acidic functional groups, besides being important regulators of biogeochemical processes, such as global nutrients and carbon cycle (Wood et al., 2003; Sachse et al., 2005; Matsuo & Val, 2007; Galvez et al., 2008). The black waters of the Amazon region are known for their physicochemical characteristics, relatively uncommon to average global values, with particular respect to the acidic pH parameters (it can reach 3.0 in some situations) and low ionic concentration. In these black waters, humic substance corresponds from 60 to 90% of the dissolved organic carbon (Matsuo & Val, 2002).

In the ion poor black water rivers of Amazon, the humic substances may have a direct action on the gills, exerting a protective effect at low pH and, in so doing, it would be able to replace any protective property that Ca^{2+} could possess (Wood et al., 2003). Fish kept in natural black waters show a lower ion efflux after exposure to low pH levels than fish maintained in water without humic substance, suggesting that it would reduce gill

permeability, preventing ion loss by diffusion (Gonzalez et al., 1998; 2002; 2005; Wood et al., 2003). Furthermore, humic substances facilitate the secondary repair of lesions induced by stress in fish (Meinelt et al., 2008).

The increased ion efflux in acidic water promotes a greater energetic expenditure so that fish can adapt to this condition. This demand diverts the amount of energy otherwise destined for growth to the osmoregulatory mechanisms (Soengas et al., 2007). Acidic waters impair the growth of tambaqui *Colossoma macropomum* (Aride et al., 2007) and juveniles of silver catfish *Rhamdia quelen* (Copatti et al., 2005; 2011b), probably due to a decrease in food consumption. A study with green swordtail (*Xiphophorus helleri*) has been the only to report some beneficial effect on fish growth for the humic substances (Meinelt et al., 2004). Based on the supposed protective effect of humic acids on osmoregulation in fish exposed to acidic waters (Gonzalez et al., 1998; 2002; 2005; Wood et al., 2003), as well as the relevant commercial importance of silver catfish in the south of Brazil, this study was aimed at evaluating the effect of different concentrations of humic acid on the species performance when exposed to different pHs.

Materials and methods

Silver catfish juveniles were obtained from a commercial fish farm near Santa Maria city, southern Brazil. They were acclimated for a minimum of 4 days in 250 L tanks with continuously aerated fresh water, devoid of humic acid, at controlled temperature of. They were fed to satiation once a day with commercial diet for juveniles with 42% extruded crude protein (Alisul / Supra - São Leopoldo, RS, Brazil).

For the growth experiments with silver catfish, 480 juveniles (3 ± 1 g) were maintained for 40 days in 40 L polyethylene boxes, at a density of 2.60 g L^{-1}. To evaluate the effect of the

combination pH + humic acid on growth, the pHs 5.5 and 6.5 were combined with 0, 10, 25 and 50 mg L^{-1} humic acid, in triplicate for each treatment.

The animals were fed daily to satiation at 8 am with the same commercial feed provided in the acclimation period. The remains of food, as well as residues and feces, were removed 30 min after feeding, followed by an average 20% replacement of tank water previously prepared with the adequate pH and humic acid concentration.

Juveniles were anesthetized with 50 µL L^{-1} eugenol (Cunha et al., 2010). They were weighed and measured in the middle of the experiment (with replacement) and at the end of the experiment so that specific growth rate, feed intake and biomass could be estimated.

Water parameters:

Levels of dissolved oxygen and temperature were measured daily with Orion 810 oxygen meter (Thermo Electro Corporation, Waltham, Al, USA). Water samples were collected every 2-3 days to verify total ammonia nitrogen (TAN) levels by the method of Eaton et al. (2005). Un-ionized ammonia (NH_3) levels were calculated according to Colt (2002). The levels of Ca^{2+}, Na^{+} and K^{+} were determined with photometer Micronal B286 and Cl^{-} as outlined in Zall et al. (1956). Nitrite was analyzed by spectrophotometry (Boyd & Tucker, 1992).

Humic acid and pH:

The synthetic humic acid used in the trials (CAT: H1 0.675-2 Aldrich® - humic acid sodium salt) corresponded to 44% of dissolved organic carbon, according to Matsuo et al. (2005). In this experiment, 50 mg L^{-1} humic acid contained the nominal concentration of 20 mg C^{-1} of dissolved organic carbon (DOC). Water pHs 5.5 and 6.5 were verified three times a

day with pH meter DMPH-2 (Digimed, São Paulo, Brazil) and adjusted with sulfuric acid 1 M when necessary.

Statistical analysis:

Homogeneity of variances between treatments was assessed via Levene test and the comparison between treatments was performed with two-way ANOVA (pH x humic substance) and Tukey test (Statistica software 7.0). The relationship between levels of humic acid x growth parameters was performed with SigmaPlot 11.0. Minimum level of significance was 95%.

Results

During the experimental period, the overall waterborne levels of Na^+, Cl^-, K^+ and Ca^{2+} were 4.02 ± 0.95, 6.14 ± 0.75, 0.04 ± 0.01 and 0.021 ± 0.005 mg L^{-1}, respectively, and hardness was 26.3 ± 5.1 mg $CaCO_3$ L^{-1}. Nitrite levels were kept at 0.362 ± 0.037 mg L^{-1}. Levels of dissolved oxygen were 6.17 ± 0.55 mg L^{-1} and temperature was 22 ± 1.5ºC. Total ammonia levels were 0,68 mg $L^{-1}$and non-ionized ammonia at 0.055 ± 0.00073 mg L^{-1}.

The first biometric evaluation was performed on day 20 of experiment. It was found that the acidic environment of the test (pH 5.5 or 6.5) was not sufficient to affect the means of some of the assessed parameters: body weight, length and biomass. However, the distinct concentrations of humic acid and their association with both pH levels were responsible for the differences obtained. The results registered for the weight of the fish demonstrated the deleterious effects of humic acid, especially the low means observed at the highest concentrations of the humic acid when compared to 6.5 pH and 0 mg L^{-1} humic acid level. Significant differences in length, biomass, feed intake and SGR were found at the highest concentration of humic acid comparing with control without humic acid. After 20 days of

exposure, it was also observed that the higher the humic acid concentration, the lower the means of all the investigated parameters in the fish exposed to pH 6.5 (Fig 1.).

A second biometric assessment was conducted on day 40 of experiment. It was observed that the acidification of the water (without humic acid) promoted significant differences in almost all variables, characterizing the detrimental effects of an acidic pH. All the parameters had significantly lower means at pH 5.5 ± 0.31 than at 6.5 ± 0.25. Additionally, when the effect of humic acid alone was assessed, the lowest means of body weight were observed at its highest concentrations. Similarly, the length of the fish also decreased as the concentration of humic acid increased. Biomass, feed intake and SGR had three levels of significance across the four levels of humic acid tested. There was no difference in biomass and SGR between the groups subjected to 0 and 10 mg L^{-1} humic acid, but there was in feed intake. As humic acid concentration increased, the means of these three parameters significantly decreased. The association between pH and humic acid promoted the following results: biomass, feed intake, body weight and length presented lower means at pH 6.5 compared to pH 5.5 at all humic acid concentrations; and SGR means were lower at pH 5.5 (Fig. 2).

Discussion

The results obtained for ammonia and nitrite were within the limits reported earlier as appropriate for silver catfish growth (Lima et al., 2011; Miron et al., 2011). Periodic exchange of 20 % of the water volume in the experimental tanks was probably responsible for maintaining these parameters at suitable levels.

Water pH is an important element for the maintenance of fish homeostasis, and exposure to acidic water increases efflux of ions through the gill epithelium, forcing the fish to seek alternatives for adaptation (McDonald & Wood, 1981). Survival in an acidic

environment appears to be primarily related to the ability to prevent Na^+ loss (Freda & McDonald, 1988). The search for homeostasis at acidic pH apparently interferes with the energy demand in silver catfish, which can cause a delay in development (Copatti et al., 2005, 2011b) and the death of fish due to excessive loss of ions at pH < 4 in 96 hours (Zaions & Baldisserotto, 2000). In agreement with the above-mentioned, the present study demonstrated that the development of silver catfish juveniles after 40 days of exposure to acidic pH was considerably lower at pH 5.5 than at pH 6.5. These results are also consistent with previous findings: silver catfish exposed to pH 5.5 showed lower growth (Copatti et al., 2005, 2011b); and no significant effect was observed at pH 6.0 compared to pH 7.0-7.5 (Copatti et al., 2011a).

There is only one previous study relating synthetic humic acid to fish growth (Meinelt et al., 2004). It showed a greater development of green swordtail exposed to 180 mg L^{-1} humic acid compared to control (0 mg L^{-1} humic acid). However, the results of the current study are at variance with these authors, since silver catfish presented better development at the lowest concentrations of humic acid or in its absence.

The initial hypothesis that humic acids would serve as stimulant and protector of paracellular junctions of the gill epithelium, thus preventing ion efflux and sparing energy to be used in fish growth, was not corroborated by the results obtained in this study. The deleterious effects of humic acids were more evident at pH 6.5 than at pH 5.5 (Figures 1 and 2). Acidification of humic acid molecules positively charged reduce the load and increase lipophilicity, thereby increasing toxicity (Petersen Jr & Persson, 1987). Internally, humic acids can migrate to organs or organelles and cause the most diverse biological responses, such as stress and lipid peroxidation (Meinelt et al., 2008).

The present zootechnical evaluation did not support the protective effect previously observed for humic acids against ionoregulatory disturbances induced by low pH in fish

(Steinberg et al., 2006; Val & Matsuo, 2007). The poor development of silver catfish exposed to humic acids was probably a result of the increased energy demand to maintain homeostasis.

Conclusions

Humic acids display a detrimental effect on the development of silver catfish juveniles. The higher the concentration of humic acids, the greater the yield deficit of silver catfish. Water pH 5.5 has its deleterious effects exacerbated when in association with humic acids, even though it is situated within the limits of survival and tolerance for this species.

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Figure Legend

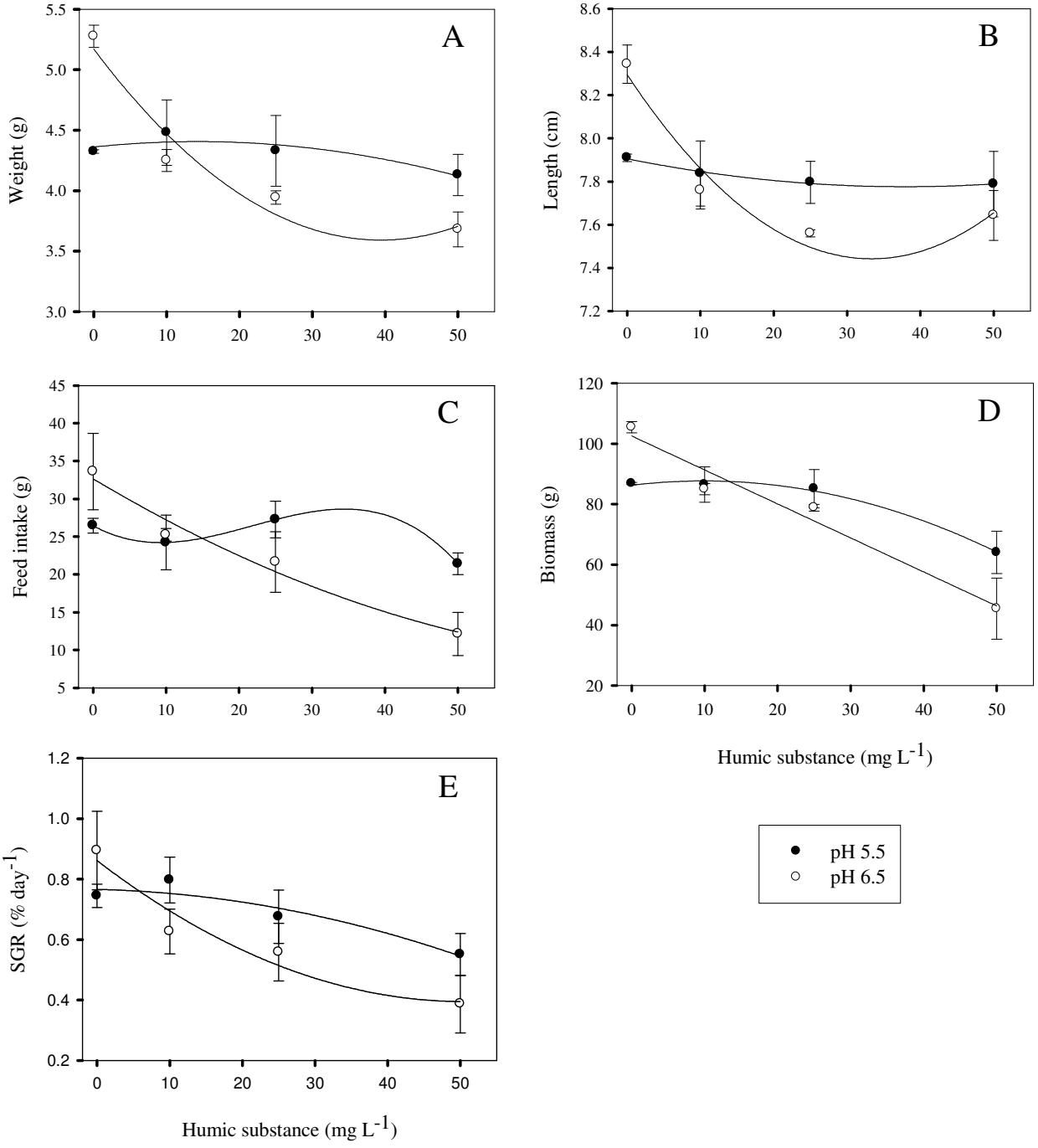
FIGURE 1 - Performance of silver catfish juveniles after 20 days of exposure to different levels of humic substance (HS) associated with pH 5.5 and 6.5.

The curves can be represented by the following equations: (A) pH 6.5 $y=5.1708-0.0802x+0.0010x^2$ ($r^2=0.9451$), pH 5.5 $y=4.3608+0.0063x-0.0002x^2$ ($r^2=0.8366$), (B) pH 6.5 $y=8.2952-0.0512x+0.0008x^2$ ($r^2=0.9567$), pH 5.5 $y=7.9059-0.0068x+0.00009026x^2$ ($r^2=0.9871$), (C) pH 6.5 $y=32.6588-0.5778x+0.034x^2$ ($r^2=0.9723$), pH 5.5 $y=26.4533-0.5318x+0.0365x^2-0.0006x^3$ ($r^2=1.0$), (D) pH 6.5 $y=102.5903-1.1248x$ ($r^2=0.9632$), pH 5.5 $y=86.2761+0.2894x-0.0146x^2$ ($r^2=0.9937$), (E) pH 6.5 $y=0.8613-0.0184x+0.0002x^2$ ($r^2=0.9415$), pH 5.5 $y=0.7660-0.0006x-0.000077x^2$ ($r^2=0.9050$), where x = level of humic substances (mg L^{-1}) and y = weight (g) (A), length (cm) (B), feed intake (g) (C), biomass (g) (D), Specific growth rate - SGR (% day^{-1}) (E).

FIGURE 2 – Performance of silver catfish juveniles after 40 days of exposure to different levels of humic substance (HS) associated with pH 5.5 and 6.5.

The curves can be represented by the following equations: (A) pH 6.5 $y=3.6503+3.7528e^{(-0.0446x)}$ ($r^2=0.9907$), pH 5.5 $y=5.4133-0.0271x$ ($r^2=0.9876$), (B) pH 6.5 $y=7.9332+1.7154e^{(-0.0668x)}$ ($r^2=0.9992$), pH 5.5 $y=8.8575-0.0331x+0.0004x^2$ ($r^2=0.9972$), (C) pH 6.5 $y=54.410-1.2202+0.0085x^2$ ($r^2=0,9986$), pH 5.5 $y=37.9135-0.4377x+0.0015x^2$ ($r^2=0.9161$), (D) pH 6.5 $y= 146.8219-2.2908x+0.0072x^2$ ($r^2=1.0$), pH 5.5 $y=106.6192-0.3068x-0.0112x^2$ ($r^2=0.9983$), (E) pH 6.5 $y=1.7739-0.0339x+0.0002x^2$ ($r^2=0.9712$), pH 5.5 $y=1.29833-0.0159x$ ($r^2=0.9962$), where x = level of humic substances (mg L^{-1}) and y = weight (g) (A) length (cm) (B), feed intake (g) (C), biomass (g) (D), Specific growth rate - SGR (% day^{-1}) (E).

* significantly different from the group exposed to pH 6.5 at the same level of humic acid ($P < 0.05$)


A
B
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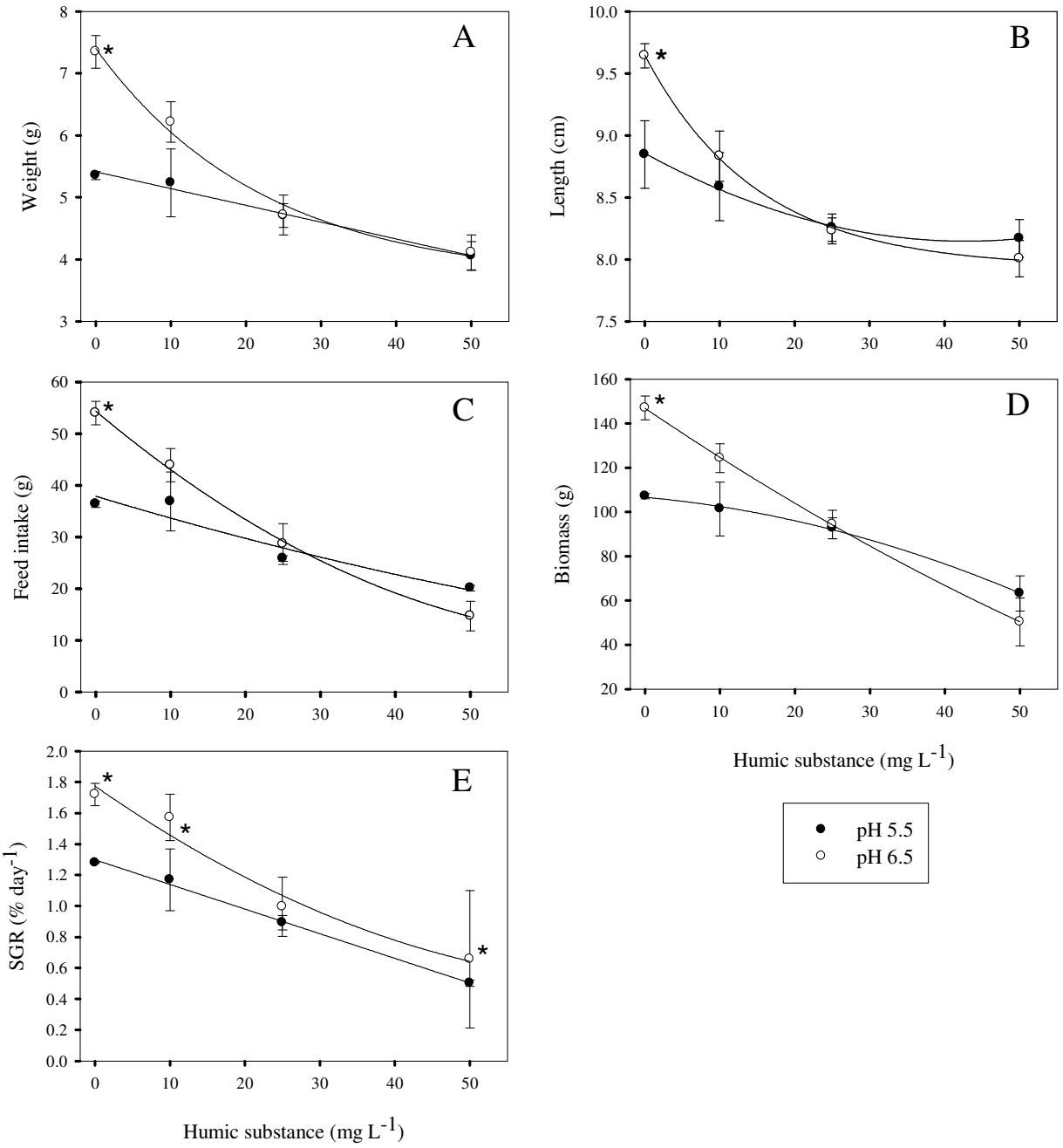
Weight (g)
Length (cm)
Feed intake (g)
Biomass (g)
SGR (% day-1)
Humic substance (mg L-1)
Humic substance (mg L-1)
pH 5.5
pH 6.5

Figure 1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Weight (g)
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Feed intake (g)
Biomass (g)
SGR (% day-1)
Humic substance (mg L-1)
Humic substance (mg L-1)
pH 5.5
pH 6.5

Figure 2

MANUSCRITO 02 – A ser submetido ao periódico Journal of Morphology

Morphometry of Silver Catfish Gills Exposed to Different Levels of Humic Acid and Acidic pH

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ABSTRACT

Humic acid is one of the main organic substances found in black waters. This study aimed at observing gill morphology of silver catfish juveniles (*Rhamdia quelen*) challenged with water at pH 5.5 and 6.5 and different levels of humic acid: 0, 10, 25 and 50 mg L^{-1}. The gills were collected at the 40th day of exposure. Humic acid may cause respiratory distress, by promoting thickening of the respiratory tissues, and also proliferation of chloride cells. Therefore, humic acid in the tested concentrations was extremely damaging to the respiratory performance of silver catfish juveniles. The negative effects caused by the acidic pH were not minimized or controlled by humic acid in juveniles of this species.

Key Words: gill histology, chloride cell, fish, acidic water

INTRODUCTION

In aquatic environments, the water may contain a multitude of dissolved or colloidal organic matter (DOM) resulting from leaching and decomposition of organic matter characteristic of the region. These substances have been shown to interact with organic chemicals by various modes of binding and adsorption. Such interactions can affect the bioavailability of organic compounds in aquatic organisms, since only freely dissolved compounds are taken up to accumulate in them (Haitzer et al., 1998). In Amazonian black waters, humic acid represents from 60 to 90% of these organic substances. These waters are recognized for their uncommon physicochemical characteristics compared to average global values, with particular respect to the acidic pH levels (it can reach 3.0) and low ionic concentration (Matsuo and Val, 2002).

Humic substances may have a direct action on the gills, exerting a protective effect at low pH and, in doing so, it would be able to replace any protective properties that Ca^{2+} could present (Wood et al., 2003). Fish kept in natural black waters showed lower ion effluxes after

exposure to acidic pH than those maintained in water without humic substance, suggesting that it would reduce gill permeability and thus prevention loss by diffusion (Gonzalez et al., 1998, 2002, 2005; Wood et al., 2003). In addition, humic substances facilitate the secondary repair of lesions induced by stress in fish (Meinelt et al., 2008).

Acidic waters hamper growth and may even be lethal to larvae of silver catfish *Rhamdia quelen* (Lopes et al., 2001) due to a decrease in food consumption. A similar response was observed in juveniles of the same species by Copatti et al. (2005). To date, no studies related gill morphology to humic acid levels. Based on the supposed protective effect of humic substances on osmoregulation in fish exposed to acidic waters (Gonzalez et al., 1998, 2002, 2005; Wood et al., 2003), this study was aimed at evaluating the effect of different humic acid levels on gill morphology of silver catfish exposed to different pH.

MATERIALS AND METHODS

Animals

Silver catfish juveniles (3 ± 1g) were acclimated in LAFIPE (Laboratory of Phisiology of Fish) in Universidade Federal de Santa Maria, southern of Brazil in December 2011 for a minimum of four days in tanks with continuous aeration (minimum dissolved oxygen levels 6.00 mg L^{-1}) and controlled temperature (24 ± 0.5 ºC). They were fed to satiation once a day with a commercial concentrate for juveniles with 42 % extruded crude protein (Alisul/Supra - São Leopoldo, RS, Brazil).

After acclimation the juveniles were maintained in 40 L polyethylene boxes for 40 days (stocking density 2.60 g L^{-1}). To evaluate the effect of the combination pH + humic acid on gill morphometry, the pHs 5.5 and 6.5 were combined with 0, 10, 25 and 50 mg L^{-1} humic acid (three replicates of each treatment). Eugenol 50 µL L^{-1} was used to anesthetize the juveniles (Cunha et al., 2010) prior to euthanasia by section of the spinal cord.

Water quality

Dissolved oxygen levels and temperature were measured four times a day with Orion 810 oxygen meter (Thermo Electron Corporation, Waltham, Al, USA). Water samples were collected every two days to verify total ammonia nitrogen (TAN) levels by Nessler technique (Eaton et al., 2005). Un-ionized ammonia (NH_3) levels were calculated according to Colt (2002). Water hardness was analyzed by the EDTA titrimetric method. The levels of Ca^{2+}, Na^{-} and K^{+} were measured in appropriate diluted samples against known standards using flame photometry (Micronal B262) and Cl^{-} concentrations were determined according to Zall et al. (1956). Nitrite was analyzed by spectrophotometry (Boyd and Tucker, 1992).

The animals were feed daily to satiation at 08:00 h with the same commercial feed provided in the acclimation period. The remains of food, as well as residues and feces, were removed 30 min after feeding, followed by an average 20% replacement of tank water previously prepared with the adequate pH and humic acid concentration.

Humic acid and pH

The synthetic humic acid used (CAT: H1 0.675-2 Aldrich® - humic acid sodium salt) corresponded to 44% of dissolved organic carbon (DOC) according to Matsuo et al. (2005). In this experiment, treatment with 50 mg L^{-1} humic acid contains a nominal concentration of 20 mg C^{-1} DOC. Water pH 5.5 and 6.5 were verified three times a day with pH meter DMPH-2 (Digimed, São Paulo, Brazil) and adjusted with sulfuric acid 1 M when necessary.

Light microscopy (LM)

The four gill arches from the left side of the branchial apparatus were carefully excised, washed with saline solution and fixed in Bouin's fluid, preserved in 8% formaldehyde, dehydrated in crescent series of ethanol and embedded in historesin. Sagittal

sections (5 µm in thickness) were stained with toluidine blue and observed in a light microscope.

Gill morphometry

Three filaments from each gill arch were randomly chosen and measured. The average number of secondary lamellae in each filament was determined. The examined morphometric variables are depicted in Figure 1. Counting of chloride cells was performed at 1 mm of filament. These cells were easily distinguished from the other cells by the fading blue (Sakuragui et al., 2003).

Gill respiratory surface area (GRSA) was estimated using the method developed by Hughes (1984) and calculated as follows: $GRSA = Lnb$, where L is the total length of all gill filaments, n is number of lamellae on both sides of the filament, and b is an estimative of bilateral area of lamellae which can be taken as representative of all lamellae of a particular gill system. All morphometric analysis was performed with Axio Imager A1-ZEISS Axion Vision System with remote capture Rel 4.7 DC – Cannon Power shot G9.

Scanning electronic microscopy (SEM)

The four gill arches from the right side of the branchial apparatus were carefully excised, washed with saline solution and fixed in 4 % glutaraldehyde buffered to pH 7.4 with 0.1 M phosphate buffer at 4 ºC. Under a stereo microscope, several pairs of filaments were separated from the gill arch tissue. They were submerged in a buffer and dehydrated in a graded ethanol series (60, 70, 80, 90, 95 and 2 x 100%) followed by consecutive 2 min baths in 1,1,1,3,3,3-hexylmethyl-disilizan (Sigma Co., Steinheim, Germany). Tissue was then allowed to dry overnight. Dried filaments were mounted using silver paint (EMS, Fort Washington, PA) on SEM specimen stubs suitable for a FEI QUANTA 250 Scanning

Electron Microscope. The filament pairs were oriented so that the area of interest, namely the inner (trailing) edge of filament close to septum, was parallel with the stub plate. The sample was then covered with gold dust for morphometric analysis under SEM.

The microscope was focused on the trailing edge of the filament epithelium near the base of the lamellae. Random areas on posterior filaments were photographed at a magnification of 6.000 X. Ten to fifteen pictures per fish were randomly selected for a subsequent measurement. Apical CC area was determined by tracing the cell perimeter on a calibrated digitizer tablet that was linked to a microcomputer utilizing specialized software (MOTIC Images Plus 2.0 ML). In addition, the chloride cell per area (μm^2) and the chloride cell fractional area (CCFA), which represents the fraction of the gill filament epithelial surface occupied by CCs, and cell density, were calculated using the following equations (Bindon et al., 1994):

$$CCFA = \frac{\text{Area of whole and partial CCs in photograph}}{\text{Area of epithelium in photograph}}$$

$$\text{Cell density} = CCFA / \text{average whole cell area}$$

Statistical analysis

Homogeneity of variances between treatments was assessed via Levene test and the comparison between treatments was carried out by two-way ANOVA (pH x humic substance) and Tukey test (Statistica software 7.0). Minimum level of significance was 95%.

RESULTS

Water quality and pH profile

During the experimental period, the overall waterborne levels of Na^+, Cl^-, K^+ and Ca^{2+} were 4.02 ± 0.95, 6.14 ± 0.75, 0.04 ± 0.01 and 0.021 ± 0.005 mg L^{-1}, respectively, and hardness 26.3 ± 5.1 mg $CaCO_3$ L^{-1}. Levels of dissolved oxygen were 6.17 ± 0.55 mg L^{-1} and temperature was 22 ± 1.5ºC. Nitrite levels were kept at 0.362 ± 0.037 mg L^{-1} and non-ionized ammonia at 0.055 ± 0.00073 mg L^{-1}.

Gill microscopy and morphometry

Fish exposed to humic acid and pH 5.5 showed greater filament length than those that were not exposed to humic acid. This parameter was not significantly altered by exposure to humic acid and pH 6.5. The GRSA means of fish exposed to 10 and 50 mg L^{-1} humic acid at pH 5.5 were significantly higher than those of the fish that were not exposed to humic acid. Exposure to humic acid did not affect GRSA in fish maintained at pH 6.5 (Table 1).

The higher the humic acid concentration, the smaller the distance between lamellae according to the equations $y=31.348-0.8932x+0.009x^2$ $(r^2=0.8336)$ (pH 5.5) and $y=68.265-2.555x+0.0266x^2$ $(r^2=0.9834)$ (pH 6.5) (Fig. 2A). Treatment with 0 and 10 mg L^{-1} humic acid induced a greater distance between the lamellae at pH 6.5 than at pH 5.5 (Fig. 2A). There was a correlation between the variables distance between lamellae (Fig. 2A) and width of lamella (Fig. 2B), expressed by $y=100.01-1.055.x$ ($r^2=0.878$).

Width of lamella increased significantly with increasing concentrations of humic acid, characterized by the regressions $y=41.545+2.804x-0.0352x^2$ $(r^2=0.9586)$ (pH 5.5) and$y=35.613+2.2864x-0.0186x^2$ $(r^2=1.000)$ (pH 6.5) (Fig. 2B). Total height of lamella was not influenced by exposure to humic acid, but a significant difference was observed between pHs at 10 and 25 mg L^{-1} humic acid concentrations (Fig. 2C). Height of potentially functional

lamella reduced when the concentration of humic acid increased, expressed by the relation $y=241.01+0.0582x-0.0221x^2$ ($r^2=0.7506$) (pH 5.5) (Fig. 2D).

Filament epithelium thickness and number of chloride cells increased with increasing levels of humic acid, as demonstrated in the equations $y=51.926+2.0843x-0.0188$ ($r^2=0.9661$) (pH 6.5) for thickness (Fig. 2E), and $y=16.2534-0.1128x+0.0171x^2$ ($r^2=0.9974$) (pH 5.5) and $y=9.8312-0.2209x+0.0123x^2$ ($r^2=0.9383$) (pH 6.5) (for) number of chloride cells (Fig. 2F).

In addition to the adaptive morphological changes, significant lesions were identified in lamellae of fish exposed to humic acid. Lamellar aneurysm, which is the detachment of the lamellar epithelium and edema of the lamellae, is among the observed alterations (Fig. 3 and 4).

Chloride cell proliferation was observed in treatments with humic acid, expressed in $y=4370.773+374.40x-5.38x^2$ ($r^2=0.7324$) (pH5.5) and $y=6240.7615+241.68x-3.3047x^2$ ($r^2=0.9935$) (pH 6.5) (Fig. 5A). Fish exposed to pH 6.5 showed a higher number of chloride cells than those maintained at pH 5.5. The fractional area of the chloride cells increased as the levels of humic acid were raised, demonstrated in the equations $y=0.0133+0.0013x-0.00001474x^2$ ($r^2=0.7908$) (pH 5.5) and $y=0.0112+0.0021-0.0000313x^2$ ($r^2=0.9988$) (pH 6.5) (Fig. 5B).

The area of contact between the chloride cells and the water was greater in fish exposed to humic acid than in the non-exposed (Fig. 5C). In the fish subjected to 10-25 mg L^{-1} humic acid, this measurement was larger at pH 6.5 than at pH 5.5.

DISCUSSION

Humic substances from the black water environment have the ability to react with many metals. This reduces the bioavailability of such compounds to interact with target surfaces such as the gills, decreasing, thus, their aquatic toxicity (Wood et al., 2011). In the

present study, exposure to synthetic humic acid induced an increase in length of filament and GRSA at pH 5.5, providing a higher bioavailability of gill tissue for interaction with the environment (Table 1).

The gill epithelium is in close contact with the water and is therefore exposed to numerous chemical compounds that are dissolved in it. Such compounds can determine the appearance of morphological changes (Roberts and Bullock, 1980; Perry and Laurent, 1993). For that reason, morphometric analysis of gill lamellae is used to assess the relationship between the fish and the environment. It is believed that the lamellar area can be greater when the environmental conditions are favorable. The opposite should occur when the animal is in the presence of irritating substances, whether chemical or biological agents. The results of this study demonstrated that silver catfish seeks to maintain its homeostasis through characteristic adaptive reactions towards the environmental aggressor, reducing the distance between the lamellae and increasing the thickness of the filament epithelium and of the lamella when exposed to humic acid (Figures 3B to 3H).

Wood et al. (2003) did not perform histologic and morphometric analyzes of the gills in *Potamotrygon*, but they exposed the elasmobranchs to the same type of humic acid as the one used in the present study at a concentration of 15 mg L^{-1} and pH 4.0. This caused the rays to exhibit a marked increase in passive efflux and a partial inhibition of Na^+ and Cl^- influx through the gills. Humic acid is very reactive because its functional groups carboxyl and hydroxy-phenol can prevent the outflow and stimulate the influx of Na^+ and Ca^{2+} in acidic pH (Matsuo and Val 2007). The addition of humic acid as biotic ligand models (BLM) for the prevention of site-specific toxicity of metals may be used as a criteria for assessing water quality, as suggested by Niyogi and Wood (2004). It cannot be used with the same purpose when there is an increase in H^+ ions availability your when the humic acid is synthetic, since morphometry indicated the proliferation of chloride cells in this study (Fig. 2F and 5A). These

cells, located in the gill epithelium, are probably the most important osmorregulatory site for Ca^{2+} uptake in fish (Evans, 2002).

In silver catfish, the thickness of the lamellae increased with increasing concentrations of humic acid, which probably makes gas exchange difficult by increasing the blood-water barrier (Figure 3H). Ion flux was not measured over the course of the present study, but histology showed results which are consistent with the observations by Perry and Laurent (1993) and Bindon et al. (1994), revealing the proliferation of these cells in the epithelium of the filaments and lamellae (Figure 2F and 5A). Bindon et al. (1993) argued that this lamellar chloride cell proliferation increases the benefits of ion transport in the gill, but it can also affect the transfer of gas in that epithelium since the thin epithelium covering the secondary lamellae is the preferred site for gas exchange.

Variations in the physical characteristics of the epithelium, histologically observed in this experiment, may indicate more than one adjustment to environment. Silver catfish exposed to higher concentrations of humic acid at pH 5.5 showed cellular changes such as hypertrophy, aneurysm lamellae, hyperplasia and edema, all of which are well characterized in gill photomicrographs (Fig. 4A and 4B). Furthermore, figures 2A and 3 show the reduced space between the lamellae. It is understood that all such alterations and/or adjustments reduce water circulation and hence gas perfusion, causing a delay in physical development of juveniles, as commented by Bindon et al. (1994).

Under SEM, the interference of humic acid is explicit and independent of the acidic pH, promoting excessive proliferation of chloride cells, with the consequent increase in contact and fractional area of chloride cells (Figure 5A and 5B). It was expected that the addition of humic acid to the water promoted welfare and set up a protective effect in silver catfish in the current study, as mentioned by Meinelt et al. (2008). The results were also surprising due to the fact that Steinberg et al. (2006) referred to the humic substances as non-

competitive when stimulating Na^+ influx, but considered likely a change in the mechanism of membrane permeability. The authors claimed that it would happen due to a better binding of the humic substances in acidic pH, despite not knowing the direct effects of the humic substances.

CONCLUSIONS

Humic acid displays a detrimental effect on the development of livestock silver catfish. The higher the concentration of humic acid, the greater the production deficit of silver catfish. Even though situated within the limits of survival and tolerance for this species, pH 5.5 has its deleterious effects enhanced when in association with humic acid. The humic acid affects the respiratory capacity of the fish, since it induces morphologic alterations that interfere with its normal physiology.

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Figure legends

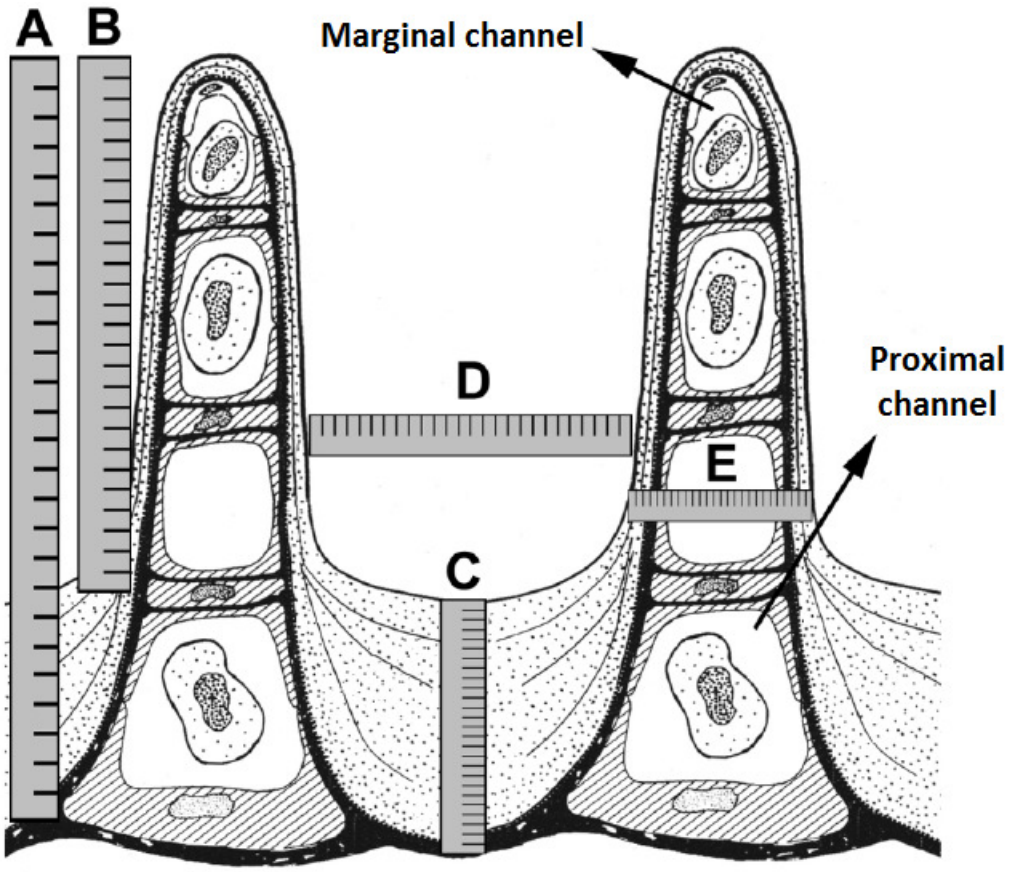
Fig. 1. Parameters measured in the gills of silver catfish juveniles *Rhamdia quelen*. **A:** Total height of lamella; **B:** Height of potentially functional lamella; **C:** Filament epithelium thickness; **D:** Distance between lamellae; **E:** width of lamella (modified from Hughes,1984).

Fig. 2. Morphological changes observed in silver catfish juveniles after 40 days of exposure to different levels of humic substance (HS) associated with pH 5.5 and 6.5. The different lower case letters express significant differences between treatments with pH 5.5. The various capital letters show no significant differences between treatments with pH 6.5. The (*) indicate the differences between pH's in each concentration of humic acid.

Fig. 3. Light photomicrographs of the silver catfish juveniles gill epithelium. **A:** exposed at pH 6.5 and 0 mg $L^{-1}$of humic acid; **B:** exposed at pH 6.5 and 10 mg $L^{-1}$of humic acid; **C:** exposed at pH 6.5 and 25 mg L^{-1} of humic acid; **D:** exposed at pH 6.5 and 50 mg $L^{-1}$of humic acid; **E:** exposed at pH 5.5 and 0 mg L^{-1} of humic acid; **F:** exposed at pH 5.5 and 10 mg L^{-1} of humic acid; **G:** exposed at pH 5.5 and 25 mg L^{-1} of humic acid; **H:** exposed at pH 5.5 and 50 mg L^{-1} of humic acid; *****interlamelar space; Arrows indicates: C and D – cell hyperplasia; G and H - cellular swelling. Scale bar = 50 µm.

Fig. 4. Light photomicrographs of damage in the gill epithelium of juveniles silver catfish stained with toluidine blue. **A:** exposed at pH 5.5 and 50 mg $L^{-1}$of humic acid; **B:** exposed at pH 5.5 and 25 mg L^{-1} of humic acid; In A - Arrows indicate aneurysm lamellae In B - Arrows indicates Detachment of the lamellar epithelium. Scale bar = 50 µm.

Fig. 5. Surface morphometry in scanning electron microscopy of juvenile silver catfish after 40 days of exposure to pH 5.5 and 6.5 and the concentrations of 0, 10, 25 and 50 mg L^{-1} humic acid. The different lower case letters express significant differences between treatments with pH 5.5. The various capital letters how no significant differences between treatments with pH 6.5. The (*) indicate the differences between pH's in each concentration of humic acid.



Modificado de Huges, 1984

Fig.1

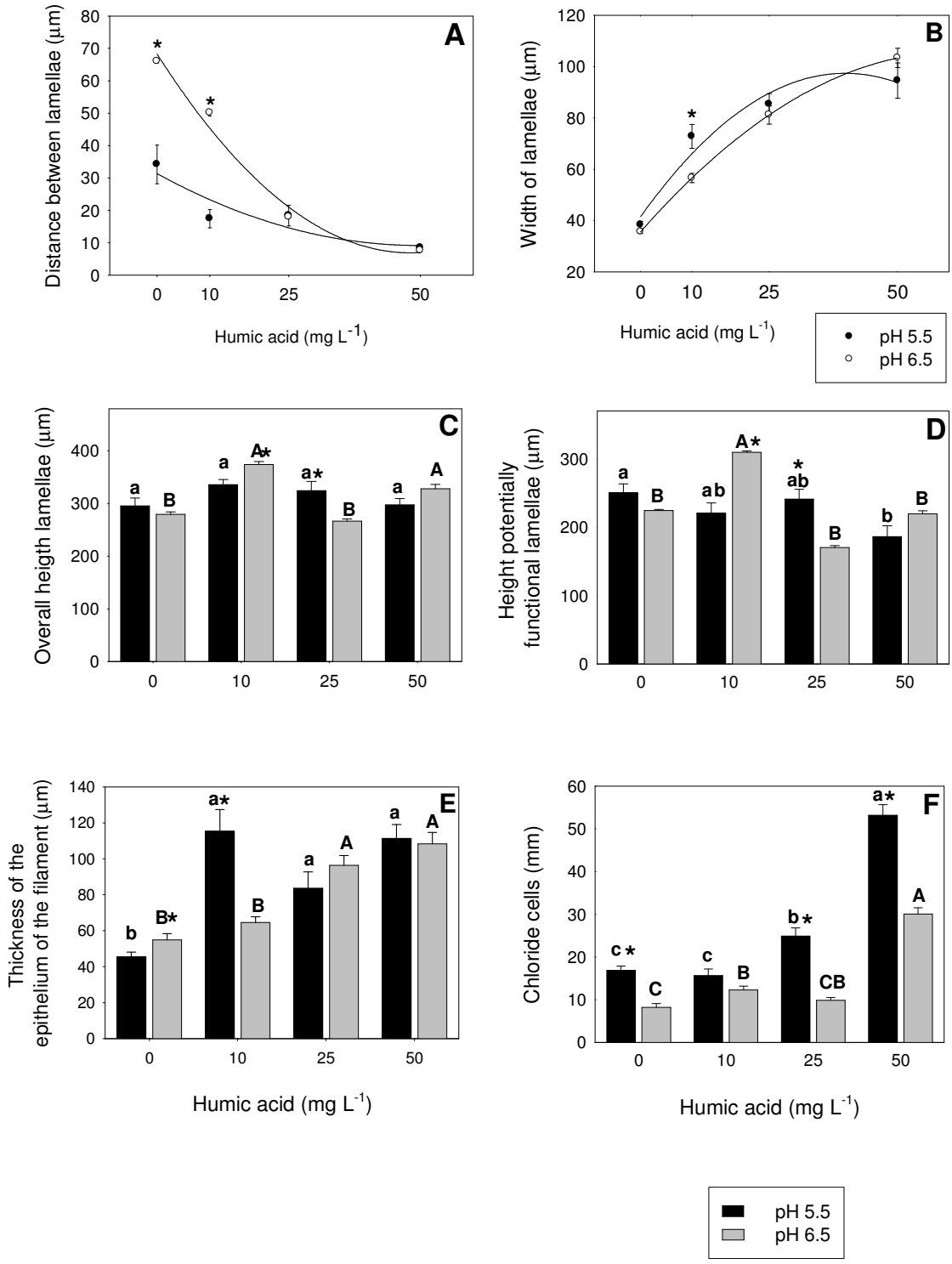

A
Distance between lamellae (µm)
Humic acid (mg L-1)
B
Width of lamellae (µm)
Humic acid (mg L-1)
pH 5.5
pH 6.5
C
Overall heigth lamellae (µm)
D
Height potentially functional lamellae (µm)
E
Thickness of the epithelium of the filament (µm)
Humic acid (mg L-1)
F
Chloride cells (mm)
Humic acid (mg L-1)
pH 5.5
pH 6.5

Fig. 2

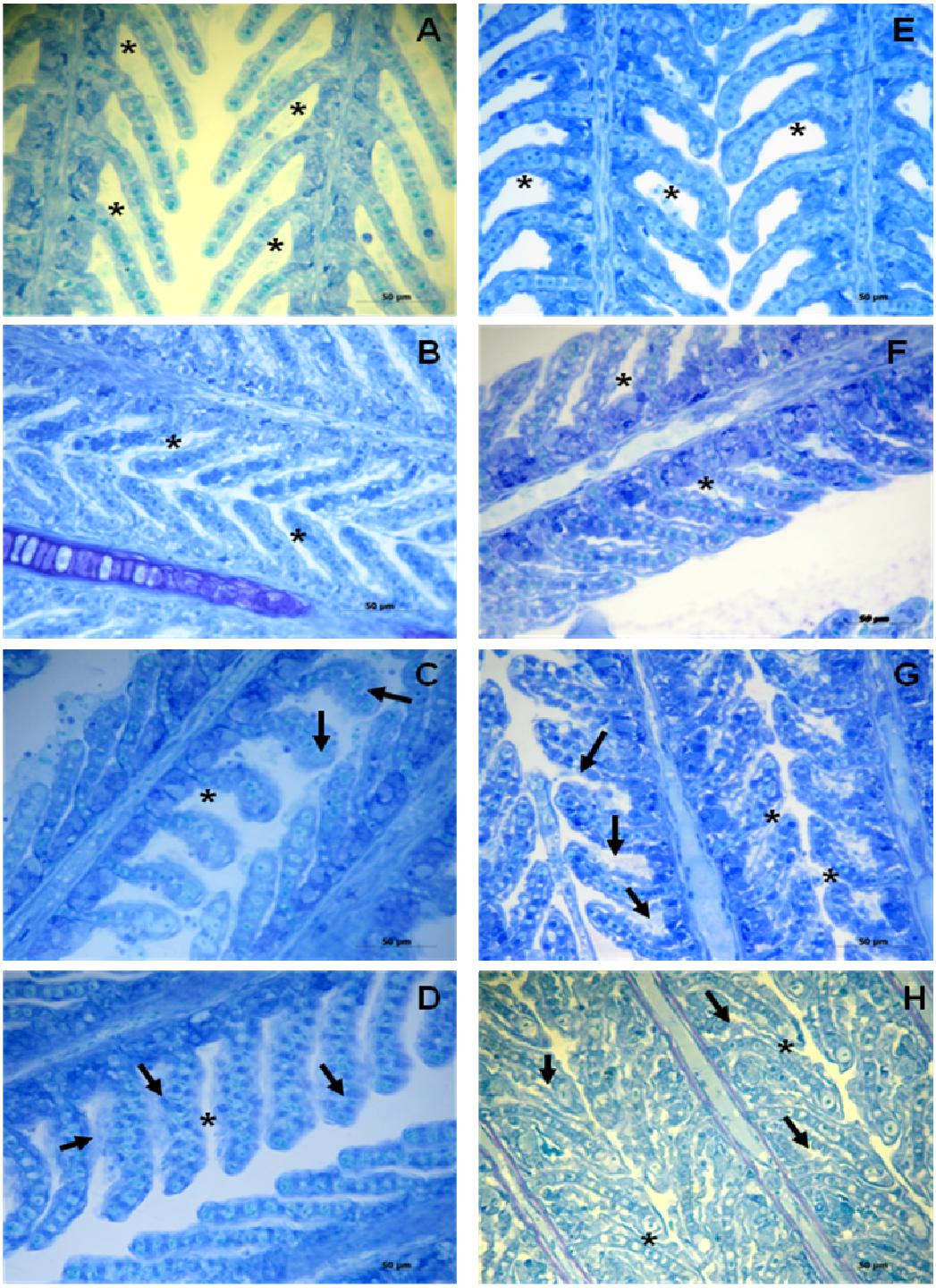


Fig. 3

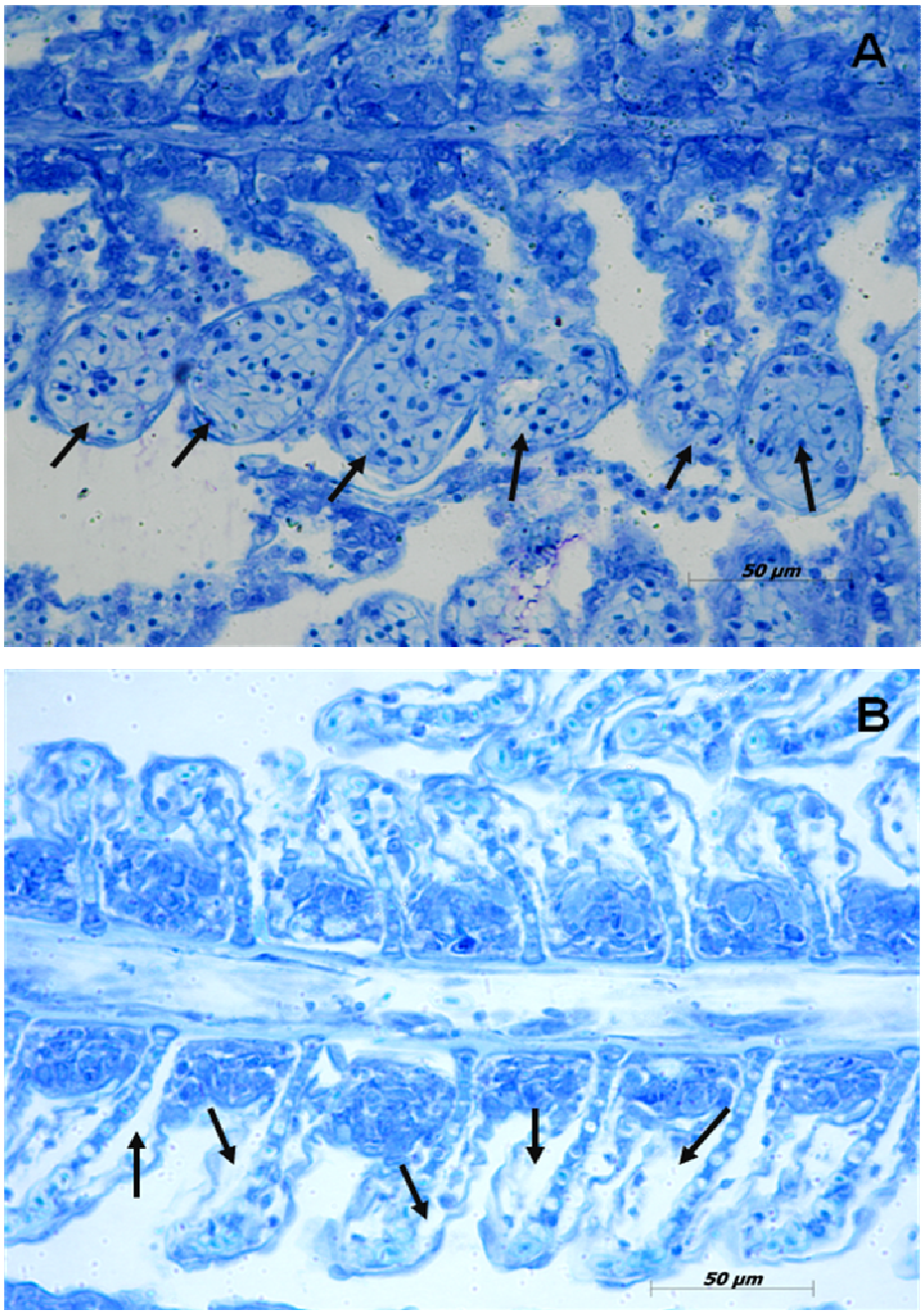


Fig. 4

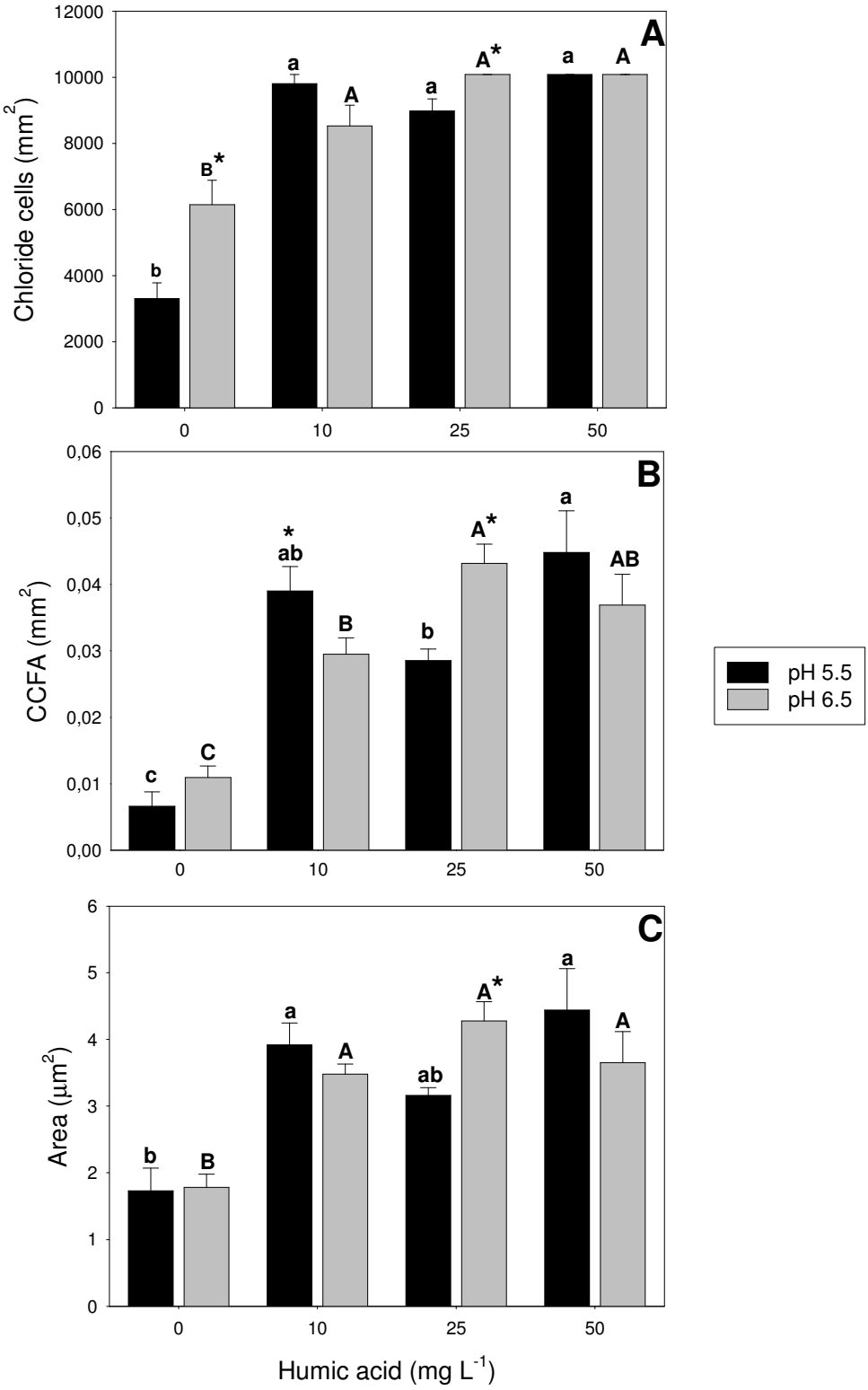

A
B
C
Chloride cells (mm2)
CCFA (mm2)
Area (µm2)
Humic acid (mg L-1)
pH 5.5
pH 6.5
0
10
25
50
12000
10000
8000
6000
4000
2000
0,06
0,05
0,04
0,03
0,02
0,01
0,00
6
5
4
3
2
1
a
A
B*
A*
b
c
C
ab
B
AB
ab

Fig. 5

TABLE 1: Summarized results of GRSA measurements in silver catfish (Rhamdia quelen). GRSA = Ln.(b)l where Ln is the total length of all gill filaments, n is the frequency of secondary lamellae on both sides of the filament, and bl is the average bilateral surface area of the secondary lamellae in humic acid (HA) and low pH about mm^2 of area:

	Length of filament(mm)		GRSA(mm^2)	
HA(mg L^{-1})	5.5	6.5	5.5	6.5
0	1.61^{b}	1.79^{a}	312.93^{b}	438.23^{a}
10	1.95^{a}	1.82^{a}	532.96^{a}	437.53^{a}
25	1.85^{a}	1.69^{a}	441.05^{ab}	415.89^{a}
50	1.86^{a}	1.72^{a}	524.73^{a}	407.12^{a}

The different letters Express significant differences between treatments with humic acid in each pH.

MANUSCRITO 03 – A ser submetido ao periódico Neotropical Icthiology

Impact of humic acid and acidic pH exposure on silver catfish (*Rhamdia quelen*) ionoregulation and hematimetric parameters

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Abstract

Humic acid, the greatest fraction of humic substances, has great reactivity due to its functional groups carboxyl and hydroxy-phenol, which act as biotic ligands protecting biomembranes. This study aimed to determine the effects of the combination of acidic pH, 3.8, 4.0, 4.2 or 7.0, and humic acid, 0, 10, 25 and 50 mg L^{-1}, on survival, erythrocyte morphology and plasma Na^+, K^+ and Cl^- levels in juvenile silver catfish (*Rhamdia quelen*) through 96h. Fish exposed to pH 3.8 showed 0% survival at all humic acid concentrations. At pH 4.0, survival decreased in the highest concentration of humic acid. Humic acid apparently had a protective effect on Na^+ and K^+ (but not Cl^-) plasma levels, hematimetric parameters and erythrocyte morphology in silver catfish exposed to pH 4.0 and 4.2. However, this supposed protective effect did not induce better survival of this species at pH 4.0, but the opposite. In addition, as well as acidic pH, humic acid induced an increase in hemoglobin levels and hematocrit of silver catfish. Therefore, the presence of humic acid in the water is deleterious for silver catfish juveniles.

Key-words: Survival, Hemoglobin, Hematocrit, Plasma ions, Erythrocyte morphometry

Introduction

Water pH constitutes an important parameter for a good productivity in fish farming (Lopes et al., 2001). Acidification of the water may occur at places where the soil contains acid cations such as aluminum or iron sulphide. Under oxygenation, the latter compound produces sulfuric acid (Zweig et al., 1999). The water may also become more acidic in the presence of humic and fulvic acids derived primarily from the decomposition of organic material formed in the soil and leached into rivers (Matsuo & Val, 2003). In addition, acid rain resulting from air pollution contributes equally to the acidity of the water (Wood, 2001).

Most species exposed to acidic waters have ionic loss through the gills affecting osmoregulation (Freda & MacDonald, 1988). Fish exposed to acidic water showed an increase in hematocrit, hemoglobin and red blood cells as a consequence of haemoconcentration caused by water displacement (MacDonald & Wood , 1981). There was also significant loss of ions and fluids with physiological disturbances in fish exposed to extremely acidic water (Wilson et al., 1999; Zaions & Baldisserotto, 2000). Apparently, there is a competition between the Ca^{2+} and H^{+} for junction sites which maintain the integrity of the gill epithelium, as well as evidence that the Ca^{2+} coupled to the sites of interaction of apical Na^{+} transporters prevents diffusive loss. Thus, the high concentration of H^{+} displaces the Ca^{2+} from the binding sites and diffusive losses occur (Wood, 2001).

Acidic waters with low ionic concentration, found in the Amazonian blackwater rivers, may induce a marked ion efflux in fish, including severe loss of K^{+} through epithelial cells (Matsuo & Val, 2002). The humic substances are complex organic molecules of the blackwater, which account for the majority of organic material dissolved in freshwater ecosystems (50-80%). In oligotrophic systems, with dissolved organic carbon (DOC) concentrations ranging from 1 to 100 mg L^{-1}, humic substance exceeds the organic carbon in all living organisms by one order of magnitude (Steinberg et al., 2006).

The objective of this study was to verify if waterborne humic acid at different concentrations may protect silver catfish (*Rhamdia quelen*) exposed to acidic pH by analyzing erythrocyte morphometry, hematological parameters (hemoglobin and hematocrit) and plasma ion levels. To date, no other study analyzed the effect of humic acid associated with acidic pH upon fish hematimetry and red blood cells morphometry.

Materials and Methods

Experimental design

Juvenile silver catfish with 73 ± 3g and 20 ± 1cm size were acquired from a commercial fish culture in Santa Maria, southern Brazil, and transferred to the Fish Physiology laboratory at the Universidade Federal de Santa Maria. Fish were maintained for three weeks in continuously aerated 250 L tanks (22 ± 1,5 ºC and a concentration of 6,05 ± 0,45 mg L^{-1} dissolved oxygen level) and were fed commercial food for juveniles with 42% crude protein.

For each concentration of humic acid (0, 10, 25 and 50 mg L^{-1}), four pH were tested: 3.8, 4.0, 4.2 and 7.0. Juveniles were fasted for 24 h prior transfer to 40 L aquarium (five fish per aquarium, three replicates per treatment, totaling 240 fish) in a 96 hours experiment.

Water parameters

Dissolved oxygen levels and temperature were measured daily with Orion 810 oxygen meter (Thermo Electron Corporation, Waltham, Al, USA). Water samples were collected every two days to verify total ammonia nitrogen (TAN) levels by nesslerization (Eaton et al., 2005). Un-ionized ammonia (NH_3) levels were calculated according to Colt (2002). Water hardness was analyzed by the EDTA titrimetric method. Water Na^+ and K^+ levels were measured in appropriate diluted samples against known standards using flame photometry (Micronal B262) and Cl^- concentrations were determined according to Zall et al. (1956). Nitrite was analyzed by spectrophotometry (Boyd and Tucker, 1992).

Humic acid and pH

The synthetic humic acid used (CAT: 0.675-2 Aldrich ® H1 - humic acid sodium salt) corresponded to 44% of dissolved organic carbon (DOC) according to Matsuo et al. (2005).

The highest concentration of humic acids tested was 50 mg L^{-1}, which corresponds to the nominal concentration of 20 mg C^{-1}. Water pH (3.8, 4.0, 4.2 and 7.0) was measured four times a day with pH meter DMPH-2 (Digimed, São Paulo, Brazil) and adjusted with sulfuric acid 1 M when necessary.

Hematological parameters

On the imminence of death (loss of equilibrium and absent opercular movements) fish were removed from the tanks and blood was rapidly collected from the caudal vein with heparinized syringes. Fish that did not die within the 96 h of experiment were anesthetized with eugenol 50 µL L^{-1} according to Cunha et al. (2010) prior blood withdraw. After sampling, all the fish were euthanized by section of the spinal cord.

To obtain the percentage of packed red cells, microhematocrit capillary tubes were filled with blood and centrifuged at 2500 rpm for 5 min, and the results were obtained by means of a hematocrit card reader. Blood samples were subsequently centrifuged at 3000 rpm for 10 min. The concentration of hemoglobin was determined by the cyanmethemoglobin method using a spectrophotometer (Brow, 1976). Plasma was then stored at – 25 °C pending ion analysis (as described in *Water parameters* for water ion levels).

Erythrocyte morphometry

Blood smears were prepared immediately from the whole blood, air-dried, fixed in methanol and stained with May-Grünwald (Tavares-Dias et al., 2004). The surface area and the major and minor axis of the erythrocyte as well as of its nucleus were determined (Benfey et al., 1984; Cogswell et al., 2002; Dorafshan et al., 2008). Ten high-power fields were randomly selected on each blood smear; morphometry of ten erythrocytes were determined in each of these fields. All morphometric analyzes were performed using the System-ZEISS

Axio Imager A1 Axion Vision System with Remote Capture 4.7 Rel DC - Cannon Power shot G9.

Statistical analysis

Homogeneity of variances between treatments was assessed via Levene test and the comparison between treatments was carried out by two-way ANOVA and Tukey test. The Kruskal-Wallis test, followed by multiple comparisons of mean ranks, was used for analysis of plasma ion levels (Statistica software 7.0). Minimum level of significance was 95% ($P<0.05$).

Results

Water parameters

During the experimental period, the overall waterborne levels of Na^+, Cl^- and K^+ were 4.02 ± 0.95, 6.14 ± 0.75 and 0.04 ± 0.01 mg L^{-1}, respectively, and hardness 26.3 ± 5.1 mg $CaCO_3$ L^{-1}. Nitrite levels were kept at 0.341 ± 0.033 mg L^{-1} and un-ionized ammonia at 0.045 ± 0.00062 mg L^{-1}. Oxygen and temerature levels were 6,17 ± 0.55 mg L^{-1} and 21 ± 2 ºC respectively.

Survival

There was no survival of fish exposed to pH 3.8, irrespective of the humic acid level. At pH 4.0, survival decreased 40, 60 and 86% with the increase of humic acid levels. The survival of silver catfish at pH 4.2 and 7.0 was 93.33% and 100% respectively, and was not affected significantly by the humic acid level (Fig. 1).

Hemoglobin and hematocrit

No difference between the humic acid treatments was observed for hemoglobin in fish maintained at pH 3.8 and 4.2. At pH 4.0 hemoglobin levels were significantly higher in fish exposed to the highest humic acid levels (25 and 50 mg L^{-1}) than the treatment without its addition. Fish kept at pH 7.0 and all humic acid levels tested presented significantly higher hemoglobin levels than those at 0 mg L^{-1} (Fig. 2a). The hematocrit levels of fish maintained at 0 and 25 mg L^{-1} humic acid at pH 3.8 were significantly higher than at 10 mg L^{-1}. No significant difference was observed between fish kept at 50 mg L^{-1} and the lower concentrations of humic acid. The lowest hematocrit levels were observed at 0 mg L^{-1} humic acid in silver catfish exposed to pH 4.0 and 4.2. Hematocrit values of fish maintained at 7.0 increased significantly from 0 to 25 mg L^{-1} humic acid, but the values found in those kept at 50 mg L^{-1} were significantly lower than at 25 mg L^{-1} (Fig 2b).

At 0 mg L^{-1} humic acid the levels of hemoglobin and hematocrit decreased as the pH increased. Exposure to 10 mg L^{-1} humic acid did not induce differences in hemoglobin values between the different pH. Hemoglobin levels at 25 mg L^{-1} humic acid were higher at pH 3.8 and 4.0 than at 4.2; the values at pH 7.0 did not differ from the means obtained at the remaining pH. At 50 mg L^{-1} humic acid the hemoglobin at pH 4.0 was significantly higher than at pH 3.8 and 4.2; the latter two pH levels presented significantly higher hemoglobin values than pH 7.0 (Fig. 2a). Exposure to 10 mg L^{-1} humic acid induced significantly higher hematocrit at pH 4.0 and 4.2 than at 3.8 and 7.0. No significant difference in hematocrit was observed between the pH at 25 mg L^{-1} humic acid. Treatment with 50 mg L^{-1} humic acid caused a significantly decrease in hematocrit levels at pH 7.0 compared with pH 4.0 and 4.2 (Fig. 2b).

Plasma ion levels

At pH 7.0 Na^+ levels were significantly higher than the values obtained at the other pH. At pH 4.2 fish exposed to 10, 25 and 50 mg L^{-1} humic acid presented greater Na^+ levels than at 0 mg L^{-1} humic acid. No significant changes were detected in fish exposed to different humic acid levels at pH 3.8 and 4.0 (Fig. 3a).

The levels of K^+ at pH 4.0 and 4.2 were higher at 0 mg L^{-1} humic acid than at the remaining concentrations. Fish maintained at pH 3.8 and 7.0 did not present significant differences in K^+ levels between the humic acid treatments. At 0 mg L^{-1} humic acid K^+ levels were significantly higher at pH 4 and 4.2 than at 7.0; the levels at pH 3.8 did not differ from those found at the other pH. The levels of K^+ at 10 mg L^{-1} humic acid were significantly highest at pH 3.8. Similar K^+ levels were obtained for all pH levels at 25 and 50 mg L^{-1} humic acid (Fig. 3b).

The levels of Cl^- did not vary between humic acid treatments at pH 7.0. At 0 mg L^{-1} humic acid Cl^- levels were highest at pH 7.0. At 10 mg L^{-1} Cl^- levels at pH 7.0 were lower than at pH 4.0 and higher than at pH 3.8 and 4.2. At 25 and 50 mg L^{-1} humic acid the levels of Cl^- at pH 4.2 were lower than at 7.0 and higher than at 3.8 and 4.0 At pH 3.8 Cl^- levels at 10 mg L^{-1} humic acid were lower than at 0 mg L^{-1} and higher than at 25 and 50 mg L^{-1}. At pH 4.0 Cl^- levels were significantly highest in fish kept at 10 mg L^{-1} humic acid. The highest Cl^- plasma levels were at 25 mg L^{-1} humic acid in fish maintained at pH 4.2 (Fig. 3c).

Erythrocyte morphometry

None of the parameters analyzed in the erythrocytes differ between the humic acid treatments at pH 3.8 and 7.0. At pH 4.0, cell area and its minor and major axis were significantly smaller at 0 mg L^{-1} than at all of the other humic acid concentrations. At pH 4.2 erythrocyte area was significantly smaller at 25 mg L^{-1} humic acid than at 10 and 50 mg L^{-1}

and significantly greater than 0 mg L^{-1}; at the same pH level, minor cell axis at 10 mg L^{-1} humic acid did not differ from the values at 25 or 50 mg L^{-1} but were significantly greater than at 0 mg L^{-1}; the latter humic acid concentration presented similar minor cell axis size to that observed at 25 mg L^{-1} humic acid (Table 1).

At 0 mg L^{-1} humic acid erythrocyte area was significantly larger at pH 3.8, 4.2 and 7.0 than at 4.0. When 10 mg L^{-1} humic acid was tested, erythrocyte area did not differ between pH levels. At 25 mg L^{-1} cell area was greater at pH 7.0 than at the remaining pH. At 50 mg L^{-1} exposure to pH 7.0 induced an increase in cell area when compared to pH 3.8 and 4.0; erythrocyte size at 4.2 did not differ from the values obtained at the other pH levels (Table 1).

Discussion

Water quality analysis indicated that parameters other than pH and acid humic levels were within limits that permit normal growth and survival of silver catfish (Miron et al., 2011; Lima et al., 2011).

According to Zaions and Baldisserotto (2000), the acid pH threshold for silver catfish survival is 4.0, what was confirmed by the 0% survival of the silver catfish exposed to pH 3.8 at all humic acid concentrations. The expected protective effect of the humic acid at pH 4.0 was not observed in this study; survival was progressively compromised as the concentration of humic acid increased.

The hematological variables of farmed fish may be influenced by variations in temperature, dissolved oxygen and food (Seibert et al., 2001; Tavares-Dias et al., 2002; 2004). The increase of the humic acid concentration induced changes in hematimetric parameters in silver catfish within the 96 h of experiment. Although the red cells were not quantified, it is clear that the presence of humic acid and the reduction of the environmental pH caused an increase in hemoglobin and hematocrit.

Water acidification may affect the oxygenation capacity of the hemoglobin, thus reducing PO_2 (Houston, 1997). Increased hematocrit and hemoglobin result from a decrease in PO_2 and blood pH (Kirk, 1973). Dheer et al. (1987) also claimed that reduced environmental pH stimulates erythropoiesis in order to maintain homeostasis. The presence of humic acid induced proliferation of chloride cells in gill lamellae of silver catfish, thus reducing the respiratory epithelium (Costa et al., in preparation). Bindon et al. (1994) had previously reported the reduction in lamellar epithelium as a consequence of chloride cell proliferation. Limited gas exchange also leads to a reduction in PO_2 and induces rises in hematimetric parameters, as proposed by Kirk (1973). Therefore, a combination of factors may have triggered the changes observed in hemoglobin and hematocrit in the present study.

Evaluation of morphologic and morphometric characteristics of red blood cells may indicate health condition and physiological status of fish (Stoskopf, 1993). The present findings suggest that pH reduction may trigger a reduction in erythrocyte size, and that the presence of humic acid in the water protected against this change.

The levels of Na^+ were higher in the plasma of silver catfish exposed to pH 7.0 than at the remaining pH tested, regardless the humic acid concentration. The same general pattern was seen in plasma Cl^- levels (except fish kept at pH 4.0 and 4.2 and 10 and 25 mg L^{-1}, respectively). As in this study, some authors demonstrated that acidic pH induced Na^+ and/or Cl^- loss and lower uptake of these ions and consequently a decrease of their plasma or body levels (MacDonald, 1983; Zaions and Baldisserotto, 2000; Wood, 2001; Aride et al., 2007). Higher concentrations of Na^+ were observed in fish exposed to pH 4.2 in the presence of humic acid than in its absence. In agreement with these results, cardinal tetras (*Paracheirodon axelrodi*) exposed to humic acid and *Geophagus sp*. and *Pimelodes sp*. maintained in water with humic substances presented lower Na^+ efflux at pH 3.72-375 than those kept in water without this substance (Gonzalez et al., 2002; Matsuo & Val, 2007).

Silver catfish exposed to pH 4.0 and 4.2 presented higher plasma K^+ levels than those kept at neutral pH. Similar effects of acidic pH on body K^+ levels were observed in this species by Zaions and Baldisserotto (2000). Silver catfish exposed to humic acid at pH 4.0 and 4.2 presented plasma K^+ levels similar to those kept at pH 7.0, and therefore this may be indicative of a protective effect of humic acid.

Conclusion

Humic acid apparently had a protective effect on Na^+ and K^+ (but not Cl^-) plasma levels, hematimetric parameters and erythrocyte morphology in silver catfish exposed to pH 4.0 and 4.2. However, this supposed protective effect did not induce better survival of this species at pH 4.0, but the opposite. In addition, as well as acidic pH, humic acid induced an increase in hemoglobin levels and hematocrit of silver catfish. Therefore, the presence of humic acid in the water is deleterious for silver catfish.

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Table 1: Erythrocyte morphology in juveniles silver catfish (*Rhamdia quelen*) exposed to different pH and humic acid levels.

	Humic acid (mg L^{-1})			
	0	**10**	**25**	**50**
		pH 3.8		
Erythrocyte area (µm²)	138.07 ± 11.0^{Aa}	142.76 ± 8.15^{Aa}	132.92 ± 12.8^{Ab}	134.51 ± 8.72^{Ab}
Minor cell axis (µm)	11.55 ± 0.37^{Aa}	11.71 ± 0.58^{Aa}	11.47 ± 0.59^{Aab}	11.20 ± 0.46^{Ac}
Major cell axis (µm)	15.59 ± 1.10^{Aa}	15.86 ± 0.27^{Aa}	15.00 ± 0.70^{Ab}	15.56 ± 0.45^{Ab}
Erythrocyte nucleus area (µm²)	17.28 ± 2.18^{Aa}	18.45 ± 2.09^{Aa}	17.43 ± 2.07^{Ab}	18.78 ± 2.26^{Aa}
Minor nuclear axis (µm)	4.17 ± 0.18^{Aa}	4.28 ± 0.27^{Aa}	4.23 ± 0.28^{Ab}	4.32 ± 0.20^{Aa}
Major nuclear axis (µm)	5.46 ± 0.48^{Aa}	5.64 ± 0.31^{Ab}	5.40 ± 0.32^{Aa}	5.71 ± 0.40^{Aa}
		pH 4.0		
Erythrocyte area (µm²)	98.73 ± 15.9^{Bb}	146.82 ± 20.3^{Aa}	135.92 ± 13.3^{Ab}	136.30 ± 6.48^{Ab}
Minor cell axis (µm)	9.55 ± 1.00^{Bb}	11.75 ± 0.75^{Aa}	11.26 ± 0.70^{Aab}	11.32 ± 0.31^{Abc}
Major cell axis (µm)	13.32 ± 0.92^{Bb}	16.13 ± 1.11^{Aa}	15.67 ± 0.73^{Aab}	15.60 ± 0.51^{Ab}
Erythrocyte nucleus area (µm²)	18.34 ± 3.62^{Aa}	20.62 ± 4.37^{Aa}	21.57 ± 2.09^{Aa}	20.41 ± 3.84^{Aa}
Minor nuclear axis (µm)	4.16 ± 0.47^{Aa}	4.42 ± 0.29^{Aa}	4.58 ± 0.19^{Aab}	5.77 ± 2.81^{Aa}
Major nuclear axis (µm)	5.79 ± 0.51^{Aa}	6.12 ± 0.80^{Aab}	6.21 ± 0.37^{Aa}	7.70 ± 3.66^{Aa}
		pH 4.2		
Erythrocyte area (µm²)	122.70 ± 6.15^{Ca}	139.88 ± 7.38^{Aa}	132.82 ± 4.57^{Bb}	144.73 ± 6.47^{Aab}
Minor cell axis (µm)	10.52 ± 0.36^{Cba}	11.45 ± 0.27^{ABa}	11.09 ± 0.53^{BCb}	11.94 ± 0.39^{Aa}
Major cell axis (µm)	15.11 ± 0.50^{Aa}	15.85 ± 0.84^{Aa}	15.53 ± 0.52^{Aab}	15.74 ± 0.27^{Aab}
Erythrocyte nucleus area (µm²)	20.07 ± 1.60^{Aa}	20.23 ± 2.61^{Aa}	20.87 ± 1.31^{Aa}	18.45 ± 1.47^{Aa}
Minor nuclear axis (µm)	4.34 ± 0.18^{Aa}	4.43 ± 0.32^{Aa}	4.48 ± 0.15^{Aab}	4.27 ± 0.26^{Aa}
Major nuclear axis (µm)	6.03 ± 0.28^{Aa}	5.99 ± 0.33^{Aab}	6.30 ± 0.39^{Aa}	5.73 ± 0.27^{Aa}
		pH 7.0		
Erythrocyte area (µm²)	135.11 ± 19.63^{Aa}	151.22 ± 20.83^{Aa}	155.43 ± 12.71^{Aa}	153.34 ± 14.78^{Aa}
Minor cell axis (µm)	11.21 ± 0.95^{Aa}	11.88 ± 0.79^{Aa}	12.21 ± 0.57^{Aa}	11.81 ± 0.23^{Aab}
Major cell axis (µm)	15.52 ± 1.08^{Aa}	16.45 ± 1.19^{Aa}	16.51 ± 0.66^{Aa}	16.85 ± 1.30^{Aa}
Erythrocyte nucleus area (µm²)	20.63 ± 3.53^{Aa}	23.73 ± 3.63^{Aa}	22.43 ± 2.40^{Aa}	22.02 ± 1.78^{Aa}
Minor nuclear axis (µm)	4.48 ± 0.35^{Aa}	4.67 ± 0.38^{Aa}	4.67 ± 0.29^{Aa}	4.55 ± 0.09^{Aa}
Major nuclear axis (µm)	6.04 ± 0.55^{Aa}	6.66 ± 0.51^{Aa}	6.31 ± 0.32^{Aa}	6.38 ± 0.40^{Aa}

Capital letters indicate significant difference between humic acid concentrations in the same pH. Lowercase letters indicate difference between pH in the same humic acid concentration. ($P<0.05$).

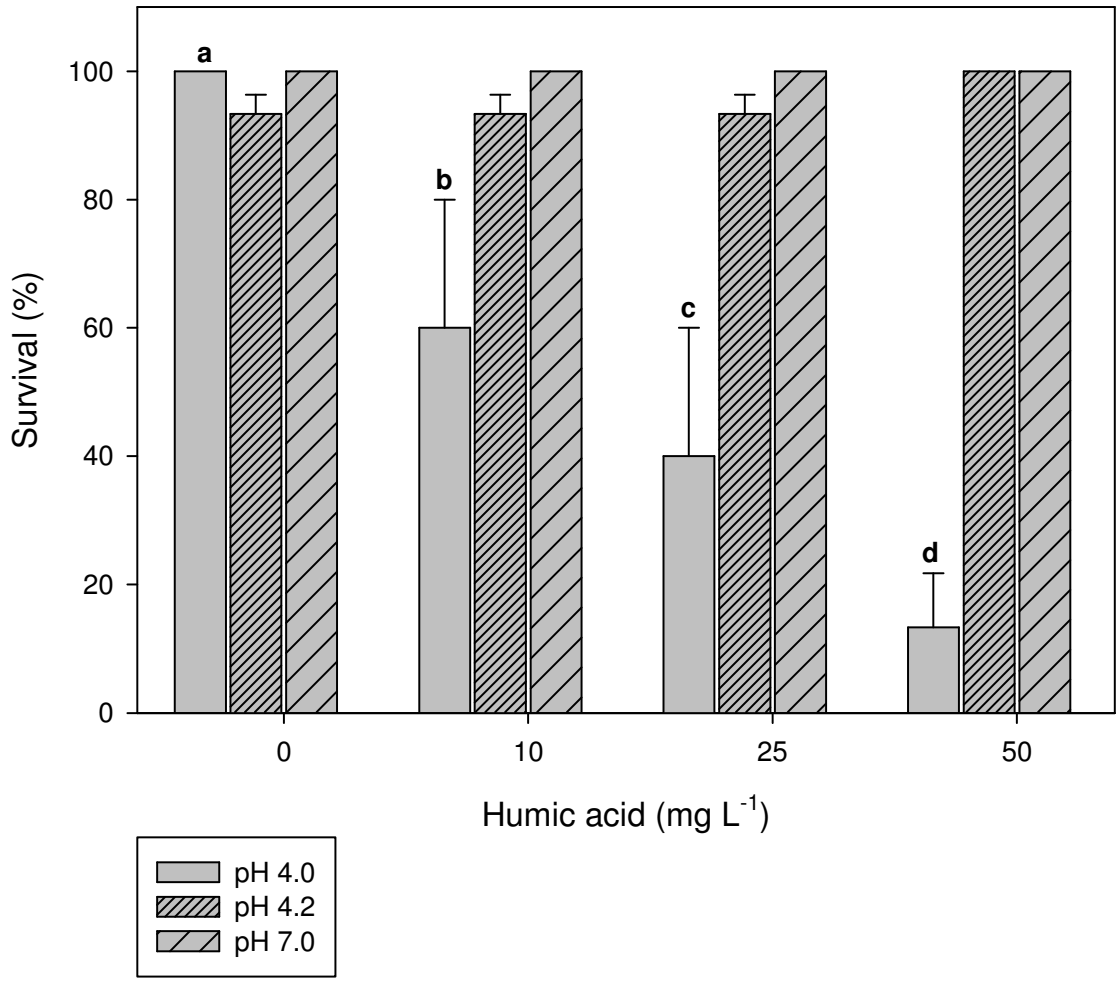

Fig. 1: Effect of pH and humic acid on survival of silver catfish (*Rhamdia quelen*). Different letters indicates significant difference between humic acid concentrations in the same pH ($P<0.05$).

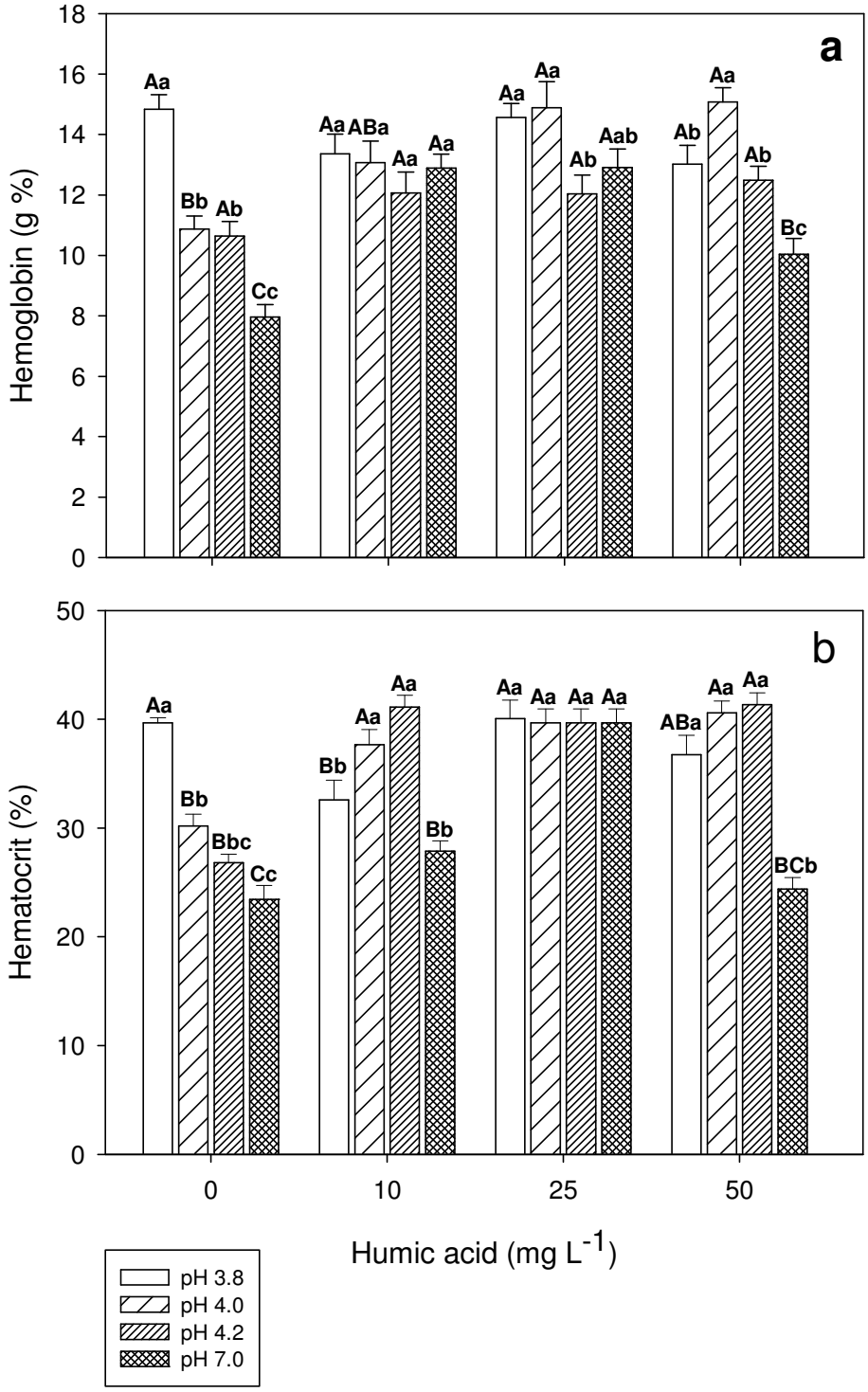

Fig. 2. Effect of humic acid and the pH of the hemoglobin (a) and hematocrit (b) of silver catfish (*Rhamdia quelen*). Capital letters indicate significant difference between humic acid concentrations in the same pH. Lowercase letters indicate difference between pH in the same humic acid concentration. ($P<0.05$).

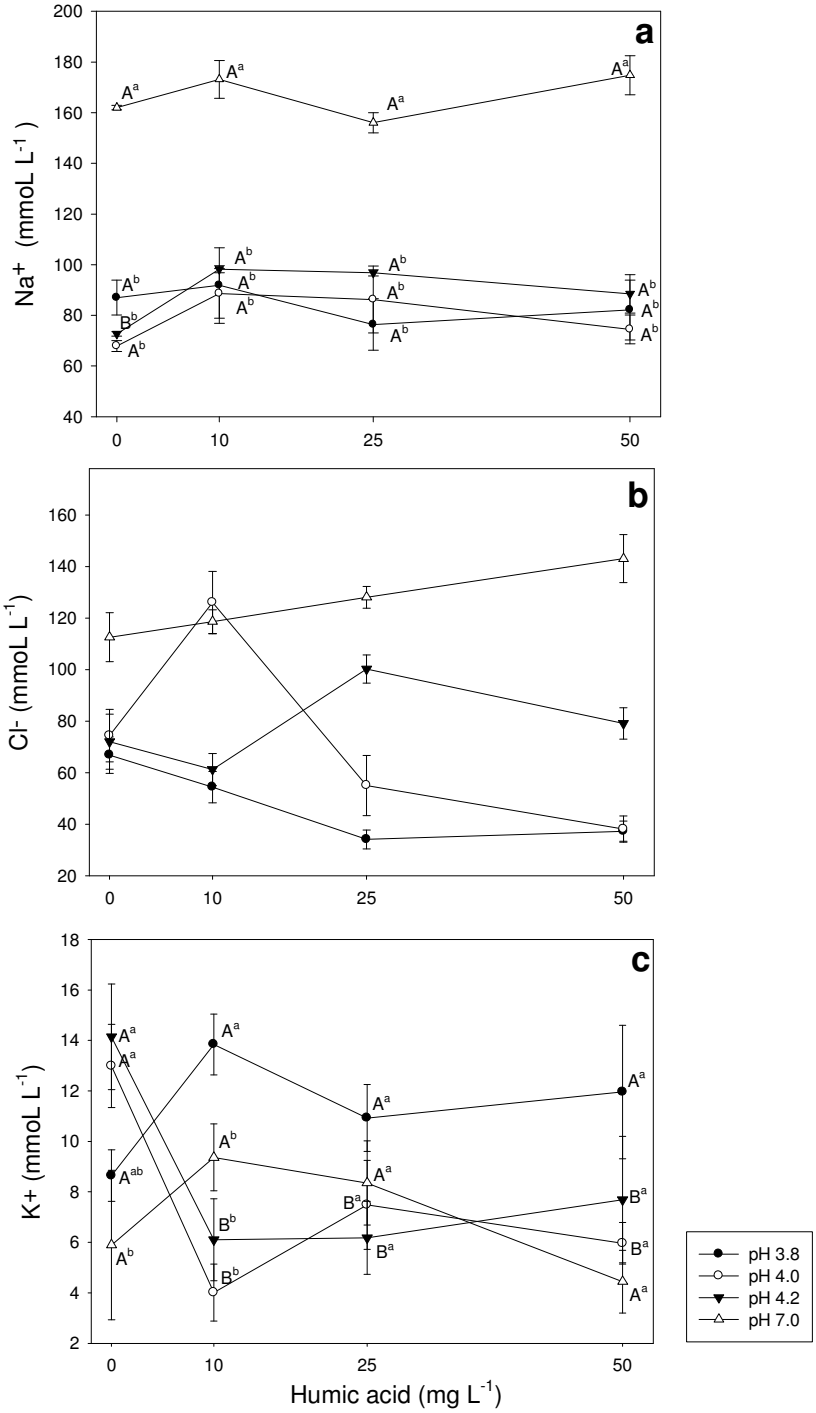

Fig. 3: Plasma Na^+ (a), Cl^- (b) and K^+ (c) in silver catfish (*Rhamdia quelen*) exposed to different pH and humic acid levels. Capital letters indicate significant difference between humic acid levels at the same pH. Lowercase letters indicate difference between pH in the same humic acid level ($P<0.05$).

DISCUSSÃO GERAL

Os peixes, assim como a maioria das espécies do planeta, são indivíduos que submetem ao arbítrio das atividades antrópicas e seus efeitos colaterais. A modificação da qualidade do ambiente em que vivem pode resultar em adaptações, lesões e até mesmo na mortalidade desses indivíduos. A chuva ácida, capaz de alterar o pH da água de rios, lagos e açudes, é um exemplo que ilustra essa condição a que os peixes são submetidos.

Para a piscicultura, o pH se constitui em um dos parâmetros fundamentais para um bom desenvolvimento da atividade produtiva (LOPES et al., 2001). Parra e Baldisserotto (2007) afirmam que a maioria das espécies de teleósteos sobrevivem às mudanças agudas de pH até a água ácida com pH 4,0. Esta informação foi confirmada no conjunto desses três experimentos, onde se identificou 100% de mortalidade de juvenis de jundiás expostos ao pH 3,8 e um índice de sobrevivência de 100% nos expostos ao pH 4,0. Ao término de 40 dias de exposição ao pH ácido de 5.5, há uma redução no crescimento do jundiá, que segundo McDonald e Wood (1981), ocorre pela utilização de boa parte da energia destinada ao crescimento para processos osmorregulatórios. Adaptações morfológicas relacionadas ao pH ambiental, nestas mesmas condições de exposição (40 dias em pH 5,5), pela primeira vez foram identificadas na estrutura branquial de jundiás, onde se verifica uma redução no fluxo de água no ambiente lamelar e aumento da barreira água-sangue. Isto, obviamente ocasiona maior dificuldade à perfusão de O_2, causando aumento no hematócrito e na concentração de hemoglobina. Este conjunto de alterações hematimétricas observadas nos experimentos com jundiás já foi identificada anteriormente por MacDonald e Wood (1981) e Aride et al.(2007), em peixes como a truta arco-iris e o tambaqui, respectivamente e confirmam a condição de hemoconcentração em espécies expostas a pH ácido. Também, pela primeira vez, foram realizadas medidas morfométricas nos eritrócitos dos jundiás, e se observou uma diminuição

de seu tamanho relacionado à exposição ao pH ácido, como já foi observado em salmão do Atlântico – *Salmo salar* (Cogswell et al., 2002).

Os níveis plasmáticos de Na^+ do jundiá não foram alterados pela exposição às diferentes concentrações de ácido húmico. A exposição aos pHs mais ácidos diminuiu os níveis plasmáticos de Na^+ e Cl^- (de um modo geral). Os níveis plasmáticos de K^+ foram maiores nos exemplares expostos a pH 3,8 em relação aos mantidos em pH neutro, quando nas menores concentrações de ácido húmico. Aride et al. (2007) encontraram menores concentrações de Na^+ e K^+ plasmático em tambaquis expostos a pH ácido. Em função dessa observação, Wood et al. (1988) sugerem que possa existir uma competição dos íons Na^+ com os íons H^+ para os transportadores branquiais, o que reduziria a captação de Na^+ da água.

Na Amazônia, peixes são encontrados em ambientes naturalmente ácidos, com águas pobres em íons (WOOD et al., 1998). A lixiviação de resíduos da decomposição da vegetação da selva forma as substâncias húmicas, constituídas por ácidos húmicos e fúlvicos (frações solúveis) e huminas (fração insolúvel) (MALCON, 1989).

Meinelt et al. (2008) citam os benefícios da utilização de substâncias húmicas no tratamento e fortalecimento de peixes. Peixes da espécie *Xilophophorus helleri* ao serem submetidos a ácido húmico comercial demonstraram melhor desenvolvimento e recuperação mais rápida ao estresse que os peixes do grupo controle. Este fato não foi observado neste estudo com jundiá, pois quanto maior a concentração de ácido húmico, maiores os efeitos deletérios encontrados.

A observação histológica do tecido branquial dos jundiás expostos ao ácido húmico demonstrou adaptações tais como: proliferação de células de cloreto, aumento na espessura do epitélio do filamento, edema e descolamento do epitélio lamelar. Características essas, que Evans et al. (1999) e Perry (1997) julgam como prejudiciais à respiração do peixe, por diminuir a superfície respiratória e aumentar a barreira água-sangue. Estas observações

encontradas na histologia branquial mostraram reflexos na morfometria dos eritrócitos, proporcionando um aumento da área da célula vermelha dos peixes expostos ao ácido húmico, como resposta a uma possível diminuição da perfusão de oxigênio. Consequentemente no desenvolvimento desses juvenis, como observado no primeiro manuscrito.

CONSIDERAÇÕES FINAIS

A formação das substâncias húmicas é extremamente dependente do tipo de solo e vegetação encontrada na margem dos fluxos d'água, podendo variar enormemente sua composição em função destas características ambientais. Nestes estudos foi utilizado apenas um composto de ácido húmico sintético, que não permite distorções quanto a sua composição química, mantendo-se constante durante todos os experimentos. Talvez essa característica, associada à concentração utilizada, tenham sido responsáveis pelos resultados deletérios encontrados em todos os trabalhos.

Como reflexão: a utilização de substâncias húmicas extraídas do meio ambiente levaria a resultados diferentes? Essas substâncias húmicas teriam a resposta protetora esperada nestes trabalhos?

Considerando os resultados obtidos, a concentração de carbono orgânico dissolvido (DOC) poderia ser acrescida como mais um parâmetro de análise físico-química para se avaliar a qualidade da água para os peixes.

CONCLUSÕES

- A presença do ácido húmico sintético na água é prejudicial ao crescimento de juvenis de jundiá;
- Quanto maior a concentração de ácido húmico na água, maior a barreira água-sangue e assim como a proliferação de células de cloreto, alterando a morfologia branquial de juvenis de jundiá;
- Os parâmetros hematimétricos de juvenis de jundiás também são alterados pela presença do ácido húmico sintético, podendo comprometer a adaptação e sobrevivência desta espécie em pH ácido;
- O ácido húmico protege juvenis de jundiá contra os efeitos ionorregulatórios negativos da exposição ao pH ácido.

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