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EFEITO DO pH E DUREZA DA ÁGUA EM JUVENIS DE *Rhamdia quelen* INFECTADOS COM *Ichthyophthirius multifiliis* (Fouquet, 1876)

TESE DE DOUTORADO

Luciano de Oliveira Garcia

Santa Maria, RS, Brasil
2009

EFEITO DO pH E DUREZA DA ÁGUA EM JUVENIS DE *Rhamdia quelen* INFECTADOS COM *Ichthyophthirius multifiliis* (Fouquet, 1876)

por

Luciano de Oliveira Garcia

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Zootecnia, Área de Concentração em Produção Animal – Qualidade da água e Fisiologia de Peixes, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de
Doutor em Zootecnia.

Orientador: Bernardo Baldisserotto

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como requisito parcial para obtenção do grau de

Doutor em Zootecnia

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RESUMO

Tese de Doutorado
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EFEITO DO pH E DUREZA DA ÁGUA EM JUVENIS DE *Rhamdia quelen* INFECTADOS COM *Ichthyophthirius multifiliis* (Fouquet, 1876)

AUTOR: LUCIANO DE OLIVEIRA GARCIA
ORIENTADOR: BERNARDO BALDISSEROTTO
Santa Maria, 20 de fevereiro de 2009.

O objetivo deste estudo foi determinar a intensidade da infecção pelo *Ichthyophthirius multifiliis*, assim como o fluxo líquido de íons (Na^+, K^+ e Cl^-), em juvenis de jundiá expostos a diferentes pHs (5,0; 6,0; 7,0; 8,0 e 9,0 por dezesseis dias), pH (5,0 e 7,0) e dureza (20, 60 e 120 mg $CaCO_3$/L por dezesseis dias) e os parâmetros de estresse oxidativo no fígado, brânquias e músculo nesta espécie e submetida a diferentes pHs (5,0 e 7,0 por 3 dias). O fluxo dos íons Na^+, K^+ e Cl^- foi determinado em diferentes tempos, o número de trofontes na pele e nas brânquias foi contado e a mortalidade foi registrada diariamente. Após seis dias os peixes submetidos aos pHs 6,0; 7,0; 8,0 e 9,0-dureza de 20 mg $CaCO_3$/L apresentaram mortalidade cumulativa (100% após oito dias) e numero de trofontes na pele e nas brânquias significativamente maior que os mantidos em pH 5,0-dureza de 20 mg $CaCO_3$/L. Jundiás infectados apresentaram efluxo de Na+ e K+ significativamente maior no primeiro dia, havendo uma recuperação (influxo) a partir do segundo dia em relação aos juvenis assintomáticos. Juvenis de jundiá infectados com *I.multifiliis* e expostos aos pHs 5,0 e 7,0 apresentaram significativo aumento dos níveis de TBARS no fígado e nas brânquias em relação aos juvenis assintomáticos. A atividade da catalase no fígado dos juvenis de jundiás infectados e expostos a ambos pHs foi significativamente maior e menor (1º e 3º dia), em relação aos juvenis assintomáticos. A atividade da GST no fígado e nas brânquias aumentou durante todo o período experimental em relação aos juvenis assintomáticos. O músculo dos juvenis infectados e mantidos em pH 5,0 apresentou significativa diminuição nos níveis de TBARS no terceiro dia comparado aos juvenis assintomáticos. A atividade da catalase foi significativamente menor no músculo dos juvenis infestados e submetidos ao pH 5,0 e 7,0 em todos os dias experimentais, exceto no primeiro dia em pH 7,0 quando comparada aos juvenis assintomáticos. O músculo dos juvenis infectados apresentou atividade da GST significativamente menor em todo o período experimental em ambos pH 5,0 e 7,0 quando comparados aos juvenis assintomáticos. Estes resultados nos permitem concluir que a infecção pelo *I. multifiliis* é menos severa em jundiás mantidos em pH 5,0-dureza de 20 mg $CaCO_3$/L. O aumento da dureza da água aumenta a infecção pelos trofontes e afeta a sobrevivência dos jundiás mantidos em pH 5,0, mas o oposto é observado quando os juvenis estão no pH 7,0. Não houve uma evidência clara da relação entre a mortalidade e o número de trofontes nos juvenis de jundiá infectados com o fluxo líquido de íons. A infecção por *I. multifiliis* induz danos no fígado e brânquias, via produtos da peroxidação lipídica, o mesmo não ficando evidenciado no músculo.

Palavras chaves: fluxo iônico, número de trofontes, TBARS, Catalase, Glutationa Transferase, doença dos pontos brancos.

ABSTRACT

PhD Thesis
Programa de Pós-Graduação em Zootecnia
Universidade Federal de Santa Maria, RS, Brasil.

EFFECT OF WATER pH AND HARDNESS IN *Rhamdia quelen* JUVENILES INFECTED WITH *Ichthyophthirius multifiliis* (Fouquet, 1876)

AUTHOR: LUCIANO DE OLIVEIRA GARCIA
ADVISER: BERNARDO BALDISSEROTTO
Santa Maria, February 20th, 2009.

The aim of this study was to determine the intensity of *Ichthyophthirius multifiliis* infection, as well as net ion fluxes (Na^+, K^+ and Cl^-), in silver catfish juveniles exposed to different pHs (5, 6, 7, 8, and 9 for sixteen days), pH (5.0 and 7.0) and hardness (20, 60 and 120 mg $CaCO_3.L^{-1}$ for sixteen days) and the oxidative stress parameters in liver, gill and muscle of this species and submitted to different pH (5.0 and 7.0 for three days). Net Na^+, K^+, and Cl^- fluxes were determined at different times, trophonts in the skin and gill were counted, and mortality was registered daily. After six days fish kept at pH 6.0, 7.0, 8.0 and 9.0-hardness 20 mg $CaCO_3.L^{-1}$ showed significantly higher cumulative mortality (100% after eight days) and number of trophonts on the skin and gill compared to pH 5.0-hardness 20 mg $CaCO_3.L^{-1}$. Infected silver catfish showed significantly higher Na^+ and K^+ effluxes in the first day, and there was a recovery (influx) after the second day compared to asymptomatic juveniles. Silver catfish juveniles infected with *I.multifiliis* and exposed to pHs 5.0 and 7.0 presented significantly higher TBARS levels in the liver and gills compared to asymptomatic juveniles. The activity of catalase in the liver of silver catfish juveniles infected and exposed to both pHs was significantly lower (1st and 3rd day) than in asymptomatic juveniles. The GST activity in the liver and gills of infected juveniles increased throughout all experimental period compared to asymptomatic juveniles. The muscle of infected juveniles maintained at pH 5.0 showed significantly lower TBARS levels at day three compared to asymptomatic juveniles. The CAT activity was significantly lower in the muscle of infected juveniles at pH 5.0 and 7.0 at all experimental days except day 1 at pH 7.0 compared to asymptomatic juveniles. The muscle of infected juveniles presented significantly lower GST activity in all experimental period at both pH 5.0 and 7.0 compared to asymptomatic juveniles. These results allowed us to conclude that infection by *I. multifiliis* is less severe in silver catfish maintained at pH 5.0-hardness 20 mg $CaCO_3.L^{-1}$. Increase of water hardness increases trophonts infection and impairs survival in silver catfish kept at pH 5.0, but the opposite is observed when juveniles are at pH 7.0. There was no clear evidence of a relationship between mortality and trophonts number in infected silver catfish with net ion fluxes. Infection with *I. multifiliis* induces liver and gill damage via lipid peroxidation products, but the same is not observed in the muscle.

Keywords: net ion fluxes, trophonts number, TBARS, Catalase, Glutathione transferase, white spots disease.

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CONSIDERAÇÕES INICIAIS

A presente tese está composta por:

- introdução e objetivos;

- três manuscritos formatados conforme a revista a serem encaminhados:

a) The relationship between water pH and infection of silver catfish (*Rhamdia quelen*) juveniles with *Ichthyophthirius multifiliis* (Fouquet, 1876) – submetido à revista Neotropical Ichthyology;

b) Water pH and hardness and their effect in the infection of silver catfish (*Rhamdia quelen*) juveniles with *Ichthyophthirius multifiliis* (Fouquet, 1876) – a ser submetido à revista Aquaculture;

c) Oxidative stress parameters in silver catfish (*Rhamdia quelen*) juveniles infected with *Ichthyophthirius multifiliis* (Fouquet, 1876) submitted to different water pH – a ser submetido à revista International Journal for Parasitology;

- conclusões;

- perspectivas;

- bibliografia geral.

INTRODUÇÃO

A piscicultura continental no Brasil no período de 1996 a 2006 apresentou um grande crescimento (231%), porém a partir de 2001 a produção permaneceu quase que em estagnação (BALDISSEROTTO, 2009). Aliado ao grande crescimento desta atividade, durante este período, e sem haver preocupações com a sanidade e a qualidade da água dos viveiros de criação nas pisciculturas houve uma disseminação rápida das doenças de peixes provocadas por bactérias, protozoários, metazoários entre outros patógenos. Essa disseminação de doenças ocorreu devido à introdução de espécies exóticas e compra e venda de alevinos contaminados sem a utilização, por parte dos piscicultores, do período de quarentena, o que gerou grandes perdas econômicas. Além disso, estas doenças ocorrem principalmente quando as condições físicas e químicas da água não são favoráveis ao cultivo de peixes devido a variações de alguns parâmetros como pH, temperatura, amônia, nitrito, oxigênio dissolvido e dureza da água. MEADE (1989) afirmou que o sucesso na aqüicultura intensiva requer a manutenção dos parâmetros físico-químicos de qualidade da água para que os peixes possam crescer satisfatoriamente e, não havendo estas condições ocorre o surgimento de doenças aliado a uma baixa produtividade.

Um dos mais importantes agentes patogênicos de peixes de água doce é o protozoário ciliado *Ichthyophthirius multifiliis,* que é encontrado nas mais variadas espécies de peixes de todo o mundo. Esse agente é um endoparasita que se aloja entre a derme e a epiderme e nas brânquias e provoca a ictioftiríase (também chamada de doença dos pontos brancos ou ictio) em peixes de água doce. A ictioftiríase causa irritação, prurido e grande produção de muco na pele e brânquias, afetando a excreção nitrogenada e o processo respiratório (MATTHEWS, 2005). Devido às alterações ocasionadas por este parasito pode ocorrer um desequilíbrio dos processos de oxi-redução, o qual caracteriza o desequilíbrio entre os agentes pró-oxidantes e antioxidantes (estresse oxidativo). Este desequilíbrio apresenta um potencial para produzir efeitos deletérios que podem causar danos a macromoléculas como o DNA, lipídeos e proteínas (HALLIWELL; GUTTERIDGE, 1999). A infecção por parasitos pode afetar os mecanismos antioxidantes dos peixes, induzindo à alterações no "status" oxidativo e/ou nas defesas antioxidantes dos peixes parasitados (MARTINEZ-ÁLVAREZ et al., 2005). Os antioxidantes atuam eliminando as espécies ativas de oxigênio formadas a partir da redução do oxigênio em nível tecidual, na tentativa de que o equilíbrio entre pró-oxidantes e antioxidantes retorne às condições de

normalidade após um período de tempo (LEITE; SARNI, 2003; GARCEZ et al., 2004). As causas mais comuns de estresse, nos peixes, estão diretamente relacionadas com a adoção de práticas de manejo inadequadas, como a utilização de rações com composições inadequadas às exigências nutricionais dos peixes cultivados, além da falta de monitoramento da qualidade da água nos tanques de cultivo (CASTILHO et al., 2008).

Nestes tanques de cultivo ou no ambiente natural, às condições do terreno e do fitoplâncton, influenciam nas variações do pH da água durante o dia. O pH é uma variável importante para o desenvolvimento e a sobrevivência de todo organismo aquático, sendo que uma variação mínima pode decretar a mortalidade ou mesmo servir como um estímulo capaz de garantir o cultivo e a produção. Valores de pH neutros e ligeiramente alcalinos (6,0 a 8,0) têm sido recomendados por uma série de autores como sendo apropriados para o cultivo de espécies comerciais de água doce (BOYD, 1982; MICHAELS, 1988). A dureza da água é outro fator de suma importância, pois é a partir da absorção do cálcio da água, através das brânquias, que é utilizado para o crescimento e homeostase dos peixes (FERREIRA; BALDISSEROTTO, 2007). Baixas concentrações deste íon (0,125 mmol) na água e na dieta reduzem as taxas de crescimento de *Salvelinus fontinalis* (truta de riacho), demonstrando que um mínimo de absorção de cálcio pelas brânquias e/ou intestino é necessário para o crescimento normal dos peixes (FERREIRA; BALDISSEROTTO, 2007). Além disso, a utilização de níveis mais elevados de dureza na água podem proporcionar uma melhor sobrevivência a juvenis de jundiá quando estes são submetidos a águas ácidas (pH 4,0) ou alcalinas (pH 9,0) em relação a níveis mais baixos de dureza da água (TOWNSEND; BALDISSEROTTO, 2001). Portanto, o objetivo deste estudo foi verificar a intensidade de infecção de juvenis de jundiá expostos ao protozoário ciliado *I. multifiliis* e submetidos a diferentes níveis de pH e durezas da água e as alterações iônicas, antioxidantes enzimáticas e não enzimáticas decorrentes desta infecção.

REVISÃO DA LITERATURA

Ichthyophthirius multifiliis (Fouquet, 1876)

O protozoário ciliado *Ichthyophthirius multifiliis*, comumente chamado de "ich", é provavelmente o mais comum parasito de peixes teleósteos de água doce, tendo uma distribuição geográfica de nível mundial que se estende desde os trópicos até o Círculo Ártico (MATTHEWS, 2005). É um endoparasita sem especificidade parasitária que infesta a pele, entre a derme e a epiderme, e as brânquias de diferentes espécies de peixes (CHAPMAN, 1984; MATTHEWS, 2005).

O seu ciclo de vida apresenta três fases: o trofonte, o tomonte e o teronte (HINES; SPIRA, 1974; McCARTNEY *et al*., 1985; DICKERSON; DAWE, 1995; XU *et al*., 2001; MATTHEWS, 2005). A fase de trofonte é definida por pontos brancos (parasitos obrigatórios), visíveis a olho nu, que infestam os peixes hospedeiros em porções próximas à superfície do corpo. Estes pontos brancos variam em tamanho (30 a 1000 μm) dependendo do estágio de desenvolvimento (maturidade) que se encontra o parasito. É nesta fase que se nota facilmente a estrutura macronuclear em forma de ferradura, característica deste parasito, porém também apresenta um micronúcleo. O desenvolvimento do trofonte e a duração do seu ciclo de vida estão diretamente relacionados à temperatura da água, pois a 22 e 27ºC o trofonte está maturo em 2,5 e 2 dias, respectivamente (MATTHEWS, 2005). Durante esta fase de desenvolvimento o trofonte alimenta-se de secreções, fragmentos de células epidérmicas e sangue do hospedeiro (PAVANELLI et al., 1998). Terminado o desenvolvimento o parasito perfura a pele do hospedeiro até atingir o meio externo (água). Com sua saída o parasito causa lesões na pele do hospedeiro, as quais são uma porta de entrada para infecções secundárias ocasionadas por fungos, bactérias e vírus e que debilitam ainda mais o peixe. A partir da sua saída do hospedeiro o *I. multifiliis* passa por transformações estruturais atingindo a fase de tomonte (MATTHEWS, 2005).

O tomonte é a fase em que o parasito fixa-se no substrato (pedras, plantas aquáticas submersas e fundo ou laterais do viveiro) através de uma camada de muco gelatinoso, onde a partir daí encista-se e inicia o processo de reprodução através de múltiplas divisões binárias, dando origem a um grande número de tomitos (EWING; KOCAN, 1992; MATTHEWS, 2005).

Nesta fase, o tomonte sobrevive e se reproduz em uma ampla faixa de temperatura, mas seu desenvolvimento diminui inversamente à temperatura (9 dias a 5ºC e 18 h a 25-30ºC) (AIHUA; BUCHMANN, 2001; MATTHEWS, 2005).

Os tomitos desenvolvidos por sua vez darão origem aos terontes, os quais são as formas de vida livre do parasito, com formato cilíndrico achatado e as extremidades anterior e posterior levemente dilatadas, com um comprimento aproximado de 30-50 µm, porém isto pode variar dependendo da temperatura e do tamanho do tomonte (MacLENNAM, 1942; AIHUA; BUCHMANN, 2001; MATTHEWS, 2005). Os terontes tem um curto período de tempo (12 h a 20ºC) após serem originados para infectarem novos hospedeiros, passado este período começam a perder a viabilidade (MacCALLUM, 1982; MATTHEWS, 2005). Nesta fase, o parasito é muito dependente da temperatura, sendo que a 28ºC a sobrevivência do mesmo é de apenas 10 h, e uma temperatura acima de 30ºC pode ser letal para os terontes (VAN DUIJN, 1967; MATTHEWS, 2005). Devido a este fato, a temperatura pode ser utilizada como tratamento para a ictiofitiríase nas espécies de peixes que toleram temperaturas acima dos 30ºC por um período de no mínimo 7 dias, pois a elevação da temperatura afeta diretamente a reprodução do *I. multifiliis*, dificultando a sua proliferação e consequentemente reduz a mortalidade dos hospedeiros (CARNEIRO et al., 2005).

Esta é a fase mais vulnerável do parasito aos tratamentos, pois o mesmo encontra-se na água e sem a proteção do cisto. Devido a isto vários tratamentos foram e estão sendo testados na tentativa de controlar ou eliminar o parasito do ambiente de cultivo. Entre estes tratamentos encontra-se a adição de sal comum, verde de malaquita, formalina, a combinação de verde de malaquita e formalina, cloramina-T, azul de metileno, sulfato de cobre, permanganato de potássio, aumento do fluxo de água ou temperatura nos tanques, entre outros (VAN DUIJN, 1956; CROSS; HURSEY, 1973; POST, 1987; SELOSSE; ROWLAND, 1990; CECCARELLI et al., 1994; TONGUTHAI, 1997; TIEMAN; GOODWIN, 2001; MIRON et al., 2003; SRIVASTAVA, et al., 2004; CARNEIRO et al., 2005; MATTHEWS, 2005; GARCIA et al., 2007). Porém, a utilização da maioria das substâncias mencionadas pode ser empregada somente em laboratórios e não nos tanques de cultivo, pois poderia resultar em sérios prejuízos ao meio ambiente e custos elevados ao produtor, além de algumas delas serem altamente prejudiciais à saúde humana (verde de malaquita, formalina).

Ao entrar em contato com o hospedeiro o teronte perfura a sua pele com uma estrutura denominada “perfuratum”, e com o auxílio de enzimas, penetra na pele e instala-se entre a derme e a epiderme, onde irá passar por um período de modificações estruturais, passando a ser chamado de trofonte, completando assim o ciclo de vida do parasito (MATTHEWS, 1994; MATTHEWS, 2005).

Com a infecção os hospedeiros demonstram-se agitados e realizam movimentos violentos de fricção, chocando-se contra as paredes, fundo dos aquários e viveiro, pedras, troncos e plantas aquáticas submersas e após algum tempo entram em estado de apatia (PRIETO *et al*., 1991). A parasitose é particularmente grave quando ocorre nas brânquias, pois causa sérios problemas respiratórios, e se não for combatida a tempo, pode provocar rapidamente a morte do hospedeiro (HOFFMAN; BAUER, 1971; HINES; SPIRA, 1973; NIGRELLI et al., 1976; EWING et al., 1994; PAVANELLI et al. 1998; BRANDÃO, 2004). A ictiofitiríase ocasiona perdas iônicas corporais em *Carassius auratus* (TUMBOL et al., 2001), porém no jundiá não apresenta o mesmo tipo de comportamento, não havendo relação entre a infecção e a perda de íons (GARCIA et al., 2007).

Jundiá *Rhamdia quelen*

O jundiá *Rhamdia quelen* (Heptapteridae) é uma das espécies de peixes de água doce afetadas pelo *I. multifiliis*, podendo inclusive ocorrer grandes mortalidades (Miron et al., 2003; Garcia et al., 2007). O jundiá tem distribuição neotropical desde o sudeste do México até o centro da Argentina (SILFVERGRIP, 1996). Vive em lagos e poços fundos dos rios, preferindo ambientes de águas mais calmas, com fundo de areia e lama, junto as margens e vegetação, onde encontra abrigo contra possíveis predadores. É um peixe de couro que possui hábito alimentar omnívoro, com tendência piscívora, apresentando rápido crescimento nos meses quentes e mais lento durante os meses mais frios do ano. É euritérmico e estenoalino e está bem adaptado aos meses mais frios do inverno no sul do Brasil, especialmente no Rio Grande do Sul, onde ocorrem temperaturas mais baixas (CHIPARI-GOMES et al., 1999; GOMES et al., 2000; BALDISSEROTTO & RADÜNZ NETO, 2005; GARCIA et al., 2008). Apresenta boa produtividade quando criado em cativeiro e uma boa aceitação no mercado consumidor

(CARNEIRO, 2004); tendo se destacado na comercialização brasileira de pescado, sendo a espécie nativa mais cultivada no Rio Grande do Sul (BALDISSEROTTO, 2009).

Esta espécie atinge a sua maturidade sexual em aproximadamente um ano de vida e seu período reprodutivo ocorre de agosto a março, sendo seu crescimento bastante pronunciado nos primeiros anos de vida, com uma taxa de crescimento nos machos maior que a das fêmeas, o que depois do terceiro/quarto ano de vida se inverte (WEIS & CASTELLO, 1980; NARAHARA et al., 1985; GOMES et al., 2000).

A melhor densidade de estocagem para juvenis de jundiá em viveiros oscila entre 2-4 peixes/m^2, alcançando 600-800g de peso corporal em oito meses de vida. Além disso, altas densidades de estocagem estão relacionadas com o crescimento de juvenis de jundiá quando confinados, pois com uma densidade de 100 juvenis/m^3 se obtêm um maior peso (63,7g), mas com 300 juvenis/m^3 a produção aumenta três vezes mais e com um bom rendimento (juvenis de 30 a 40 g) (BARCELLOS *et al*., 2004).

Esta espécie por ser rústica e nativa do Rio Grande do Sul, suporta grandes variações de temperaturas (acima de 3ºC e menor que 32ºC), porém o melhor crescimento desta espécie ocorre na temperatura de 24ºC (GARCIA et al., 2008). Variações bruscas da temperatura da água podem ocasionar nos peixes estresse, e devido a isso ocorre a diminuição de sua imunidade, deixando-os mais suscetíveis ao aparecimento de doenças. O jundiá é uma espécie muito suscetível principalmente a infestações por *I. multifiliis*, podendo os juvenis morrer em poucos dias se não forem tratados (MIRON et al., 2003; GARCIA et al., 2007). O principal tratamento utilizado para controlar esta parasitose em juvenis de jundiá é a utilização de 4 g/L de sal comum na água, porém outros tratamentos também são utilizados e alguns podem oferecer riscos a saúde (verde de malaquita e formalina) do criador e dos peixes (MIRON et al., 2003; MATTHEWS, 2005). Além disso, várias bactérias e outros parasitos já foram identificados no jundiá*: Aeromonas hydrophila, Yersinia ruckeri, Flavobacterium* sp., *Vibrio* sp., *Pasteurella* sp., *Staphylococcus* sp., *Micrococcus* sp., *Edwardsiella tarda, Actinobacter* sp., *Clinostomum detruncatum, Ergasilus thatcheri, Genarchella dubia e Genarchella genarchella* (BRANDÃO, 2005).

Estresse oxidativo

A infecção e a infestação dos peixes por parasitos podem levar a um estresse oxidativo, o qual irá gerar nos tecidos do hospedeiro radicais livres. Os radicais livres (RL) são estruturas químicas que possuem um elétron desemparelhado e isso o torna muito instável, extremamente reativo e com grande capacidade para combinar-se inespecificamente com as diversas moléculas da estrutura celular e derivados de cada uma delas (FERREIRA & MATSUBARA, 1997; HALLIWELL & GUTTERIDGE, 2000; GARCEZ et al., 2004). Estes compostos são formados por absorção de radiação, por reações redox ou por processos de catálise enzimática (SLATER, 1984; FERREIRA & MATSUBARA, 1997).

Sob condições normais, existe um equilíbrio entre a produção de pró-oxidantes e as defesas antioxidantes, onde um desequilíbrio a favor dos pró-oxidantes caracteriza a condição de estresse oxidativo e, em situações patológicas, essa produção pode aumentar consideravelmente, o que poderá induzir danos a nível de DNA e RNA, induzindo mutações. O ataque às proteínas e enzimas causa a oxidação de grupamentos tióis (-SH) metionila e carbonila, e a peroxidação lipídica pode causar alterações na permeabilidade da membrana, perda da função secretória e até a morte celular (HALLIWELL, 1992). No dano tecidual ou trauma, as toxinas geralmente desencadeiam uma série de eventos em nível intracelular que conduzem a uma situação de estresse oxidativo (HALLIWELL & GUTTERIDGE, 1999). Portanto, o desequilíbrio entre a formação e a remoção das espécies reativas de oxigênio (EROs) são importantes na patogênese de muitas doenças (GARCEZ et al., 2004).

O processo de transferência de elétrons ou a absorção de energia pode levar o oxigênio a gerar espécies reativas de oxigênio (EROs) (OGA, 2003). As principais EROs são o radical superóxido ($O_2^{\bullet -}$), peróxido de hidrogênio (H_2O_2), radical hidroxila ($OH^{\bullet}$) e oxigênio singlet (O_2^{1}). Além do oxigênio, o nitrogênio também participa da estrutura dos RL, em especial com o óxido nítrico ($ON^{\bullet}$), cujo precursor é a L-arginina (LEITE & SARNI, 2003; GARCEZ et al., 2004).

As espécies reativas de oxigênio e outros radicais livres podem ser produzidos por fontes endógenas (mitocôndrias, peroxissomos, citocromo P-450, fagocitose e xantina desidrogenase) ou exógenas (radiação, solventes orgânicos, herbicidas, patógenos) (RESENDE et al., 2003; LEITE & SARNI, 2003; GARCEZ et al., 2004). Semelhante aos mamíferos, os peixes possuem

mecanismo de defesa para neutralizar a ação das espécies reativas de oxigênio (EAO) (AHMAD et al., 2000). Este sistema é formado pelos anti-oxidantes enzimáticos catalase (CAT), glutationa peroxidase (GPx), superóxido dismutase (SOD) e glutationa-S-transferase (GST); e pelos anti-oxidantes não enzimáticos glutationa (GSH), α-tocoferol (vitamina E), caroteno, entre outros (FERREIRA & MATSUBARA, 1997; LEITE & SARNI, 2003).

pH e dureza da água

O solo onde é implementada uma piscicultura tem grande influência nas qualidades físico-químicas da água na qual vão ser cultivados os peixes. Devido ao solo, a água vai ter uma quantidade variável de substâncias dissolvidas (sais e componentes orgânicos) e uma acidificação pode ocorrer devido a presença de cátions ácidos como o Al^{3+} ou minério de ferro (pirita), que sob baixas condições de oxigenação, formam ácido sulfúrico (ZWEIG et al., 1999; PARRA & BALDISSEROTTO, 2007). Em algumas regiões como na Amazônia, nos rios de águas escuras, a presença dos ácidos húmico e fúlvico formados através da decomposição da matéria orgânica no solo podem também reduzir o pH da água para valores muitas vezes abaixo de 3,5 (MATSUO & VAL, 2003). Entretanto, a ocorrência de baixos pHs geralmente ocorre em níveis de 4,0 a 5,5, sendo considerados estes valores levemente ácidos, porém estes valores podem fazer com que os peixes apresentem respostas tóxicas relacionadas aos distúrbios de ionorregulação pelos níveis externos de H^+ estarem elevados (WOOD et al., 1998). Valores de pH acima de 9,0 no ambiente natural e em tanques de cultivo podem ocorrer devido ao aumento na taxa de fotossíntese realizada durante o período diurno pelo fitoplâncton e plantas aquáticas ou ainda pela presença de grandes quantidades de limo (WOOD, 2001).

Várias espécies de teleósteos sobrevivem a mudanças agudas de pH em níveis de 4,0-5,0 ou de até 9,0-10,0, mas a exposição a águas muito ácidas ou alcalinas pode tornar-se letal em poucas horas (ALABASTER & LLOYD, 1982; PARRA & BALDISSEROTTO, 2007). Esta tolerância ao baixo ou alto pH parece estar relacionada a habilidade do peixe em manter o seu balanço iônico (GONZALES, 1996), porém algumas espécies são adaptadas a suportarem tais adversidades como a Tilápia do lago Magadi (*Oreochromis alcalinus grahami*) e o cardinal tetra (*Paracheirodon axelrodi*) que vivem em ambientes com águas com pH 11,0 e 3,5 respectivamente (BALDISSEROTTO, 2002).

Na região centro do estado do Rio Grande do Sul o pH da água onde o jundiá é comumente criado permanece normalmente na faixa de 5,4 a 8,4 (LOPES et al., 2001) e para a sua sobrevivência o pH deve permanecer na faixa de 4,0 a 9,0 (dureza de 30 mg/L $CaCO_3$), sendo que fora desta faixa esta espécie começa a perder íons corporais (Na^+ e K^+), que podem prejudicar o crescimento e até mesmo levar a morte. O crescimento é reduzido quando os juvenis são submetidos aos pHs 5,5 ou 9,0 quando comparados ao pH 7,5 (ZAIONS & BALDISSEROTTO, 2000; BALDISSEROTTO, 2004; COPATTI et al., 2005).

Um outro parâmetro importante é a dureza da água, que é determinada pelo conteúdo de sais de cálcio e de magnésio ligados aos íons carbonatos (CO_3^{-2}) e bicarbonatos (HCO_3^-). Os níveis adequados de dureza da água para a piscicultura devem encontrar-se acima de 20 mg/L $CaCO_3$, pois o Ca^{++} e o Mg^{++} são essenciais para a formação do esqueleto, coagulação sanguínea e várias reações metabólicas (BALDISSEROTTO, 2002). Para o jundiá o melhor crescimento larval ocorre em níveis de dureza da água entre 30 e 70 mg/L $CaCO_3$ (pH 8,0-8,5), e em águas mais duras a mortalidade é grande (TOWNSEND et al., 2003). No entanto, o aumento nos níveis de dureza da água até 600 mg/L $CaCO_3$ beneficiam a sobrevivência de juvenis de jundiá quando estes são expostos a águas ácidas (pH 3,75) e alcalinas (pH 10,0 a 10,5) (TOWNSEND & BALDISSEROTTO, 2003).

OBJETIVOS

Objetivos gerais

O presente trabalho teve como objetivo verificar a intensidade de infecção de *I. multifiliis* em juvenis de jundiá expostos a diferentes níveis de pH e durezas da água e as alterações iônicas e antioxidantes enzimáticas decorrentes desta infecção.

Objetivos específicos

- Verificar se diferentes níveis de pH e durezas da água diminuem ou até mesmo eliminam os terontes, desempenhando um controle do *I. multifiliis;*

- Verificar em quais níveis de pH e durezas da água ocorrem os maiores índices de mortalidade cumulativa dos juvenis de jundiá infestados com o parasito;

- Determinar o fluxo líquido dos íons sódio, potássio e cloreto de juvenis de jundiá (assintomáticos ou infestados com *I. multifiliis*) em diferentes níveis de pH e durezas da água e relacioná-los com a sobrevivência frente a esta infecção;

- Determinar as alterações ocasionadas pela infecção do parasito no conteúdo das enzimas antioxidantes catalase (CAT) e glutationa-S-transferase (GST) no fígado, músculo e brânquias, assim como os níveis de lipoperoxidação (TBARS).

A seguir, são apresentados os artigos que compõem a Tese.

Manuscrito 01

Relação entre o pH da água e a infecção de juvenis de jundiá *Rhamdia quelen* com *Ichthyophthirius multifiliis* (Fouquet, 1876)

The relationship between water pH and infection of silver catfish (*Rhamdia quelen*) juveniles with *Ichthyophthirius multifiliis* (Fouquet, 1876)

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Abstract

The aim of this study was to determine the intensity of *Ichthyophthirius multifiliis* infection, as well as net ion fluxes, in silver catfish juveniles exposed to different pHs (5, 6, 7, 8, and 9). Net Na^+, K^+, and Cl^- fluxes were determined at different times, trophonts in the skin and gill were counted, and mortality was registered daily. After six days, the lowest and highest mortality rates were observed in juveniles exposed to pH 5 and pH 9, respectively; mortality was 100% after eight days for all treatments except at pH 5. The lowest number of trophonts/juvenile was observed in juveniles at pH 5. Infected silver catfish showed significantly higher net Na^+ and K^+ efflux than non-infected juveniles exposed to the same pH at the onset of the experiment, and a net Na^+ influx on the two following days. The net Cl^- loss in infected juveniles was usually significantly lower than in non-infected juveniles. These results allow concluding that juveniles infected with *I. multifiliis* and maintained at pH 5 showed lower mortality and trophont number. In spite of some differences between infected and non-infected fish, there was no evidence of a relationship that links net ion fluxes with mortality and trophont number.

Keywords: net ion fluxes, theronts, trophonts number, parasite, white spots disease.

Resumo

O objetivo deste estudo foi determinar a intensidade da infestação pelo *Ichthyophthirius multifiliis*, assim como o fluxo líquido dos íons Na^+, K^+ e Cl^- em juvenis de jundiá expostos a diferentes pH (5, 6, 7, 8 e 9). O fluxo de íons (Na^+, K^+ e Cl^-) foi determinado em diferentes tempos, o número de trofontes na pele e nas brânquias foi contado e a mortalidade foi registrada diariamente. Após seis dias, as menores e maiores taxas de mortalidade foram observadas nos juvenis expostos aos pH 5 e pH 9, respectivamente, e a mortalidade foi de 100% em 8 dias em todos os tratamentos, exceto no pH 5. O menor número de trofontes/juvenil foi observado nos juvenis mantidos no pH 5. Os jundiás infestados apresentaram maior efluxo de Na^+ e K^+ que os juvenis sadios expostos ao mesmo pH no início do experimento, e um influxo de Na^+ nos dois dias seguintes. A perda de Cl^- nos juvenis infestados foi significantemente menor que nos juvenis sadios. Estes resultados permitem concluir que os juvenis infestados com *I. multifiliis* e mantidos em pH 5 apresentaram menor mortalidade e número de trofontes. Apesar de algumas diferenças entre peixes infestados e sadios, não se encontrou evidência de uma relação entre o fluxo líquido de íons com a mortalidade e o número de trofontes.

Palavras chaves: fluxo iônico, terontes, número de trofontes, parasita, doença das manchas brancas.

Introduction

The silver catfish, *Rhamdia quelen* (Quoy & Gaimard) occurs across a range stretching from southern Mexico to Central Argentina (Gomes *et al.*, 2000). Silver catfish culture is increasing in Brazil, but data regarding production are either incomplete or lacking for most Latin American countries (Baldisserotto, 2003). The total fish culture production in Brazil from 2001 to 2002, was 150,000-176,000 tons while the silver catfish production was only 2.5 tons, or 1.4-1.7% of the total. However, it was the fifth most commonly raised native species (Crescêncio, 2005), and well adapted for cultivation even in the winter months of southern Brazil (Garcia *et al.*, 2008). Production of this species is affected by one of the most important pathogens of freshwater fishes, the ciliate protozoan *Ichthyophthirius multifiliis* (Fouquet) (Miron *et al.*, 2003; Garcia *et al.*, 2007); this pathogen is also found in other fish cultures and ornamental fishes worldwide (Ewing & Kocan 1992; Matthews, 1994; Dickerson & Dawe 1995; Nielsen & Buchmann 2000; Xu *et al.*, 2001). This ectoparasite causes ichthyophthiriasis, also known as ‘‘white spot disease’’ or ‘‘ich’’ (Traxler *et al.*, 1998; Buchmann & Nielsen, 1999; Gleeson *et al.*, 2000; Matthews, 2005). Considerable losses caused by mortality or reduced growth as a result of infections have been observed in common carp (*Cyprinus carpio* Linnaeus), rainbow trout (*Oncorhynchus mykiss* Walbaum), tilapia (*Oreochromis niloticus* L.), European eel (*Anguilla anguilla* L.), channel catfish (*Ictalurus punctatus* Rafinesque), and silver catfish cultures, as well as in ornamental fish (Ling *et al.*, 1991; Lom & Dyková, 1992; Matthews, 1994; Buchmann & Bresciani, 1997; Scholz, 1999; Miron *et al.*, 2003; Garcia *et al.*, 2007). The trophont stage of *I. multifiliis* is an obligate parasite that infests the gills, skin, eyes, and fins (Chapman, 1984; Ventura & Paperna, 1985). Ichthyophthiriasis causes damage to the host gill epithelium during the trophont lifestage, compromising osmoregulation and gas exchange in the gills, which could cause host mortality (Ewing *et al.*, 1994; Tumbol *et al.*, 2001); when fish are raised at high densities, an infection can

eradicate an entire population (Tumbol *et al.*, 2001).

The pH is a very important variable for the development and survival of aquatic organisms. The usual pH range for fish growth is 6.0 to 9.0 (Parra & Baldisserotto, 2007), but water acidification may occur in places where the soil contains acidic cations, as Al^{3+}, or iron pyrite, which, under oxygenating conditions, forms sulfuric acid (Zweig *et al.*, 1999). The presence of humic and fulvic acids, formed in the soil through the decomposition of organic matter, can also reduce water pH to 3.5, as has been observed in Amazonian blackwaters (Matsuo & Val, 2003) and some densely vegetated swamps and bogs of North America (Patrick *et al.*, 1981; Gonzalez, 1996). Alkaline water may be a consequence of phytoplankton or aquatic plant blooms, which decrease the level of CO_2 available in the water during daylight (Wood, 2001). Acute exposure of silver catfish juveniles to pHs ranging from 4.0 to 6.0 decreased plasma Na^+, Cl^- and K^+ levels (Bolner & Baldisserotto, 2007), and exposure to pH 10.1 also decreased Na^+ and Cl^- levels in rainbow trout plasma (Yesaki & Iwama, 1992).

As *I. multifiliis* has a life cycle with a free-swimming theront stage, this form could possibly be eliminated by either acid or alkaline pH variations, thus reducing fish infection or even eliminating this parasite (Alabaster & Lloyd, 1982). The aim of this study was to determine if changes in the pH of water can alter the extent of *I. multifiliis* infection in silver catfish, and hence, if varying the pH could be an effective means of controlling this parasite in fish culture. Moreover, the effect of *I. multifiliis* infection on the ionoregulation of silver catfish exposed to different pHs was also investigated through the analysis of the net flux of Na^+, K^+, and Cl^-.

Material and methods

Experimental Animals

Six hundred silver catfish juveniles (6.4 ± 0.1 cm and 4 ± 0.2 g) were obtained from fish farmers around Santa Maria, southern Brazil. These juveniles did not present any apparent illness when they arrived at the Fish Physiology Laboratory at the Universidade Federal de Santa Maria, where they were maintained for four days in twelve continuously aerated (using two 20 W air pumps) 250-L tanks.

Juvenile Infection

Three hundred juveniles were infected by adding one silver catfish juvenile infected with *I. multifiliis* (more than 800 trophonts) and 1 L of water infected with theronts (free form) to each 250-L tank. Juveniles maintained in these tanks began to present white spots after seven days (35–70 trophonts/fish). They were then transferred to fifteen continuously aerated 40-L polypropylene tanks and kept for sixteen days. There were five treatments (pH 5, 6, 7, 8, and 9; three replicates each), and twenty infected juveniles (35–70 trophonts/fish) were placed in each replicate. A closed circulation system was used, and a daily change of 10% of the water volume of the tanks performed. The same experimental procedure (except infection) was performed with the same number of asymptomatic juveniles.

Tank Management

Water pH was changed to the experimental pH by adding either H_2SO_4 or NaOH (1M) to the tanks where the fish were placed. In all treatments, juveniles were fed once a day (0800) with a commercial diet (Supra, 32% CP, Alisul Alimentos, Carazinho, Brazil) at 5.0% of their body mass. Uneaten food as well as other residues and feces were siphoned out 30 min after feeding. Dead fish were also removed daily. The feeding and swimming behavior of the juveniles were observed several times each day throughout the experimental period.

Net Ion Fluxes

Three specimens were collected from each replicate of both asymptomatic and infected fish at 1, 2, 4, 8, 12, and 16 days after the appearance of white spots in the infected fish and placed in individual chambers with 100 mL water under the same treatment conditions (pH 5, 6, 7, 8, and 9). Water samples (5 mL) were taken from each chamber at the beginning and after three hours of experiment and stored in a -20°C freezer for measurement of Na^+, Cl^-, and K^+ concentrations. After the ion fluxes experiment fish were weighed, measured and returned to their respective replicates. Sampled water was analyzed using a flame photometer (Micronal B262, São Paulo, Brazil; precision 1 mg/L) (Na^+ and K^+). The method of Zall *et al.* (1956) was used for determining the Cl^- concentration in these samples. Net ion fluxes were calculated according to Gonzalez *et al.* (1998):

$Jnet = V([ion]_1 – [ion]_2).(Mt)^{-1}$, where $[ion]_1$ and $[ion]_2$ are the bath ion concentrations at the beginning and end of the flux period, respectively, V is the bath volume (in Liters), M is the mass of the fish (in kg), and t is the duration of the flux period (in hours).

Mortality and the Number of Trophonts

Mortality rate for both asymptomatic and infected fish was calculated from the number of dead fish removed from each tank throughout the experimental period. The number of trophonts was determined by counting white spots on the skin and gills of anaesthetized juveniles (40 µg/L clove oil) with the assistance of a stereomicroscope (total magnification10 x).

Water Quality

Water pH was measured twice a day with a DMPH-2 (Digimed, São Paulo, Brazil) pH meter and adjusted according to the experimental conditions (pH 5.0, 6.0, 7.0, 8.0, and 9.0). Total ammonia levels were determined twice a week by nesslerization according to the method of Greenberg *et al.* (1976), and non-ionized ammonia levels were calculated according to the method of Piper *et al.* (1982). Dissolved oxygen and temperature were measured daily with a YSI oxygen meter (Model Y5512; YSI Inc., Yellow Springs, OH, USA), and laboratory temperature was maintained by using an air conditioner. Total alkalinity, nitrite (Boyd, 1998), and water hardness levels (Greenberg *et al.* 1976) were determined once a week.

Statistical Analysis

Data are reported here as mean ± SEM (N). The homogeneity of variances among groups was tested with the Levene test. Comparisons of net ion fluxes between different treatments were made by two-way ANOVA (time X treatment) and a Tukey test. Mortality and the number of trophonts (skin and gill) in all treatment groups were compared by one-way ANOVA and Tukey test. All tests were performed with the Software Statistica 6.0 (1997; StatSoft Inc., Tulsa, OK, USA). The relationship between trophont number on the skin and gills was determined using the software Sigma Plot. The minimum significance level was set at $p < 0.05$.

Results

The water pH in the 250-L tanks was 7.6-7.8; in the 40-L tanks, it was 4.75-5.15, 5.80-6.12, 6.80-7.20, 7.80-8.20, and 8.85-9.05. The total alkalinity changed with water pH, but remained in the 4.6-43.8 mg/L $CaCO_3$ range. Other water parameters in both the 250 and 40 L tanks were: temperature 23.0 ± 1ºC, dissolved oxygen levels 6.95 ± 0.3 mg/L, maximum total

ammonia levels 0.65 ± 0.05 mg/L, maximum non-ionized ammonia levels 0.07 ± 0.01 mg/L, maximum nitrite levels 0.05 ± 0.01 mg/L, and water hardness 18.5 ± 0.8 mg/L $CaCO_3$.

A positive relationship between the number of white spots on the skin and gills was observed, i.e., the number of trophonts on the skin showed a significant tendency to increase with the number of trophonts on the gills regardless of pH ($y = 1.61 + 3.94x$, $r^2=0.961$, where y is the number of trophonts in the skin, and x is the number of trophonts on the gills) (Fig. 1). Therefore, it is possible to calculate the total number of trophonts in silver catfish by counting only the number of trophonts on the skin based on the following equation: $y = 9.21 + 1.59x$, $r^2=0.99$, where y is the total number of trophonts in the fish, and x is the number of trophonts on the skin.

Fish mortality in most treatment groups started three to four days after the appearance of white spots on the skin. Fish kept at pH 5.0 and 9.0 showed significantly lower mortality than those maintained at pH 7.0 on the fourth day. In addition, juveniles maintained at pH 5.0 presented significantly lower mortality than those maintained at all other pHs on days six, eight and twelve (Fig. 2A). In addition, juveniles exposed to pH 5.0 presented 50% mortality at 13 days, and all others pHs showed 50% mortality at approximately five days. Total mortality (100%) in all treatments occurred after seven to eight days, except in the treatment with pH 5.0 (28% mortality at eight days and 55% mortality at the end of the experiments) (Fig. 2A). Juveniles exposed to pH 5.0 and 6.0 showed significantly lower numbers of trophonts on the skin two and four days after the appearance of the white spots compared to those exposed to the other treatments. The trophont number on the skin increased up to the eighth day in fish kept at pH 5.0 (Fig. 2B). The trophont number on the gills was significantly lower in juveniles kept at pH 5.0, compared to those maintained at pH 7.0 and 9.0, two and four days after the appearance of white spots, respectively (Fig. 2C).

Juveniles infected with *I. multifiliis* showed a significantly higher Na^+ efflux (or lower influx) at all experimental pHs up to day one after the appearance of white spots on the skin (except day one in those exposed to pH 9.0) than the asymptomatic juveniles. On day two, infected fish at all experimental pHs (except pH 6.0) presented significantly higher Na^+ influx than the asymptomatic fish, but on day four, only infected fish maintained at pH 5.0 and 6.0 still showed significantly higher Na^+ influx than the asymptomatic fish. Eight and twelve days after the appearance of the white spots, infected juveniles kept at pH 5.0 presented significantly higher Na^+ efflux than asymptomatic ones (Fig. 3). Net Na^+ fluxes were similar in juveniles maintained at the different experimental pH at days 8 and 16, but on day 12, kept at pH 5.0 and 6.0 showed significantly higher net Na^+ efflux than those exposed to more alkaline pHs (Fig. 3).

The net Cl- efflux was significantly higher in infected than asymptomatic juveniles exposed to pHs 7.0 and 8.0 on the first day of the appearance of white spots (day 0) and in those maintained at pH 5.0 on day 12. Infected juveniles at all experimental pH (except pH 6.0) presented, at some time, a significantly lower Cl^- efflux (i.e., lower Cl^- loss) than asymptomatic fish (Fig. 4). Asymptomatic juveniles maintained at pH 9.0 showed a significantly lower net Cl^- efflux than those kept at other pH up to day 12; at the end of the experiment, however, these juveniles presented a net Cl^- influx significantly higher than those kept at other pH (except pH 8.0) (Fig. 4).

Infection by *I. multifiliis* increased the net K^+ efflux significantly one (in those kept at pH 9.0), two (in those exposed to pH 5.0, 6.0 and 7.0), and four (in those kept at pH 7.0) days after the appearance of white spots. In addition, this infection also reduced K^+ efflux one day after appearance of the white spots on juveniles maintained at pH 6.0 and 7.0 (Fig. 5).

Discussion

The culture of silver catfish in southern Brazil is affected by *I. multifiliis*, which usually causes production loss due to the resulting high mortality (Miron *et al.*, 2003). The appearance of white spots on the skin can be used as an indicator of disease progression (Tumbol *et al.,* 2001) and their number is related to silver catfish mortality (Garcia *et al.*, 2007). Some treatments for *I. multifiliis* infection have been studied, but they are either toxic (addition of malachite green or copper sulfate to the water) or can only be used in a laboratory setting (addition of common salt in the water, elevation of water temperature or increasing water flux in the tanks) (Tieman & Goodwin, 2001; Miron *et al.*, 2003; Carneiro *et al.*, 2005; Matthews, 2005). Cloramine-T showed was shown to be effective against this parasite in the pH 6.0-8.0 range in soft and hard water (95% mortality of the free-living stage), but in more acidic water, this substance is toxic to fish (Cross & Hursey, 1973).

In our study, it was clear that the cumulative mortality rate caused by this parasite is higher when fish are maintained at pH 6.0, 7.0, 8.0, and 9.0, with 100% mortality within seven/eight days of exposition under these experimental conditions. However, juveniles maintained at pH 5.0 presented a smaller number of white spots on the skin and gills, and were the only ones to survive (72 %) eight days after the appearance of white spots, with 55% mortality only at the end experiment. Therefore, infection at this pH (5.0) is less lethal, which suggests that the use of acidic water may be an effective means of decreasing the mortality of infected fish.

The pH of water in fish culture tanks in the central region of the state of Rio Grande do Sul (Brazil), where silver catfish is most commonly raised, usually ranges from 5.4 to 8.4 (Lopes *et al.*, 2001). This species can survive acute changes in the pH 4.0-9.0 range without significant mortality (Zaions & Baldisserotto, 2000), but growth is reduced at both acidic (5.5) and alkaline pHs (9.0) (Copatti *et al.*, 2005). *Ichthyophthirius multifiliis* can reproduce normally in the pH 7.2

to 8.7 range, and can attach to host fish within the pH 5.5 to 10.1 range. Nevertheless, no difference in the incidence of parasites in trout living in water kept at pH 5.6 vs. pH 7.8-8.0 was found (Alabaster & Lloyd, 1982; Matthews, 2005).

Infected silver catfish juveniles present an initially higher net Na^+ efflux compared to asymptomatic juveniles, and in the two following days (days two and four) in most treatments, there was a higher net influx of this ion (also compared to asymptomatic juveniles), apparently in an attempt to restore the ionic balance. Net Cl^- and K^+ fluxes did not show any clear relationship with *I. multifiliis* infection. These results are in agreement with a previous study that reported significantly lower whole body Na^+ levels of infected silver catfish 26-36 h after the appearance of white spots on the skin and subsequent recovery of these levels at the end of two days (Garcia *et al.*, 2007). Moreover, the same authors did not detect significant changes in whole body Cl^- and K^+ levels in infected silver catfish in this period. In agreement with our results, infected goldfish, *Carassius auratus* L. (one day before the appearance of white spots), presented a higher net efflux of Na^+ and Cl^-, and similar K^+ efflux compared with control fish. Goldfish that survive infections are able to restore ion levels to normal after infection and subsequent traumas (Tumbol *et al.*, 2001). In addition, in channel catfish, plasma Cl^- levels were kept stable up to day five post-exposure to *I. multifiliis* (Ewing *et al.*, 1994). It is possible that ion loss in infected fish is lower than expected due to gill damage because of mucus cell hyperplasia, which has a beneficial effect against gill damage caused by this parasite and reduces diffusive ion loss (Handy *et al.*, 1989; Ferguson *et al.*, 1992; Tumbol *et al.*, 2001).

The results of the present study allow us to conclude that exposure to slightly acidic water (pH 5.0) reduces both mortality and the number of white spots on the skin and gills of silver catfish juveniles. This study has also demonstrated that there is a net Na^+ loss in the first days of

infection, with a subsequent rapid recovery of this loss. Therefore, the mortality caused by *I. multifiliis* appears to not be due to an ionoregulatory imbalance.

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Figure legends:

Fig. 1 – Trophont number on the skin and gills of silver catfish juveniles (data from fish exposed to all experimental pH).

Fig. 2 – Cumulative mortality (A) and number of trophonts on the skin (B) and gills (C) of silver catfish as a function of time (days after the appearance of white spots on the skin of infected juveniles) in silver catfish juveniles infected with *Ichthyophthirius multifiliis*. Values are expressed as means ± SEM.

The different letters indicate significant differences between treatments on the same day by two-way ANOVA and Tukey test ($P < 0.05$).

Fig. 3 – Net Na^+ flux as a function of time (days after the appearance of white spots on the skin of infected juveniles) in silver catfish juveniles exposed to *Ichthyophthirius multifiliis* (infected) or not (asymptomatic) exposed to pH 5.0 (A), 6.0 (B), 7.0 (C), 8.0 (D) and 9.0 (E). Values are expressed as means ± SEM.

* Significantly different from asymptomatic juveniles by two-way ANOVA and Tukey test ($P < 0.05$).

Fig. 4 – Net Cl^- flux as a function of time (days after the appearance of white spots in the skin of infected juveniles) in silver catfish juveniles exposed to *Ichthyophthirius multifiliis* (infected) or not (asymptomatic) exposed to pH 5.0 (A), 6.0 (B), 7.0 (C), 8.0 (D) and 9.0 (E). Values are expressed as means ± SEM.

* Significantly different from asymptomatic juveniles by two-way ANOVA and Tukey test ($P < 0.05$).

Fig. 5 – Net K^+ flux as a function of time (days after the appearance of white spots on the skin of infected juveniles) in silver catfish juveniles exposed to *Ichthyophthirius multifiliis* (infected) or not (asymptomatic) exposed to pH 5.0 (A), 6.0 (B), 7.0 (C), 8.0 (D) and 9.0 (E). Values are expressed as means ± SEM.

* Significantly different from asymptomatic juveniles by two-way ANOVA and Tukey test ($P < 0.05$).

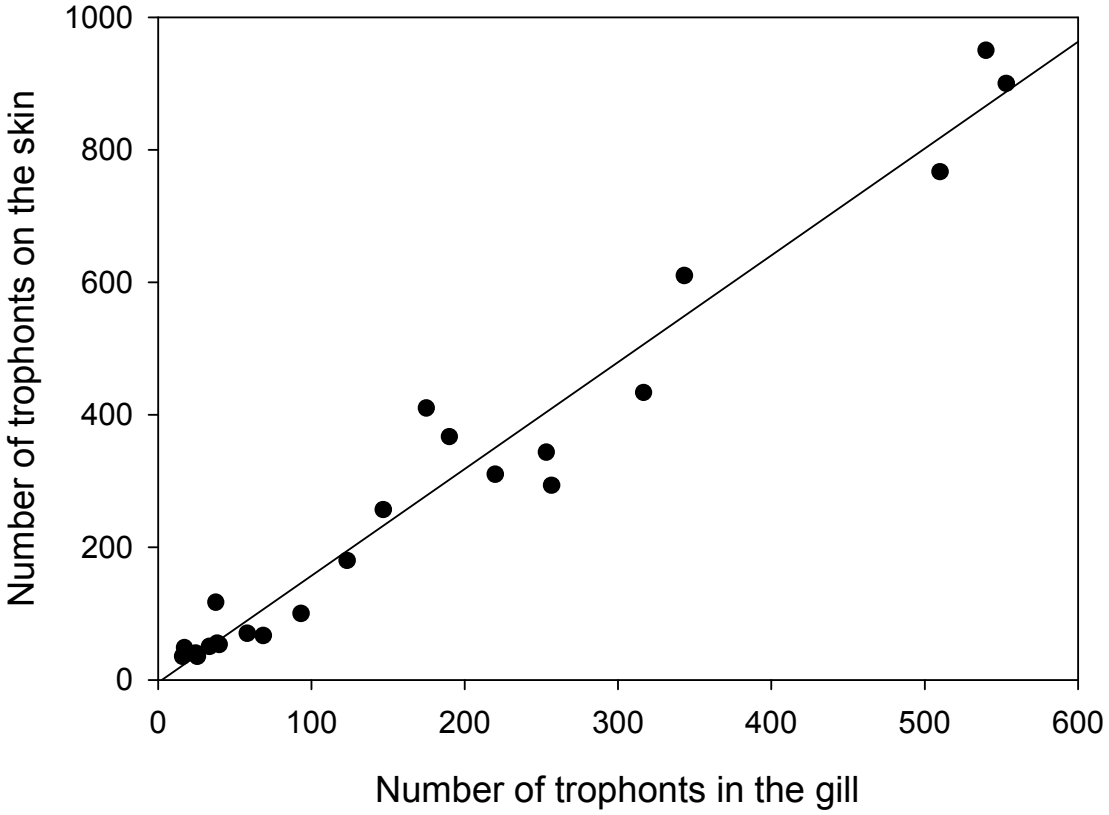
1000
800
600
400
200
0
Number of trophonts on the skin
0
100
200
300
400
500
600
Number of trophonts in the gill

Fig. 1

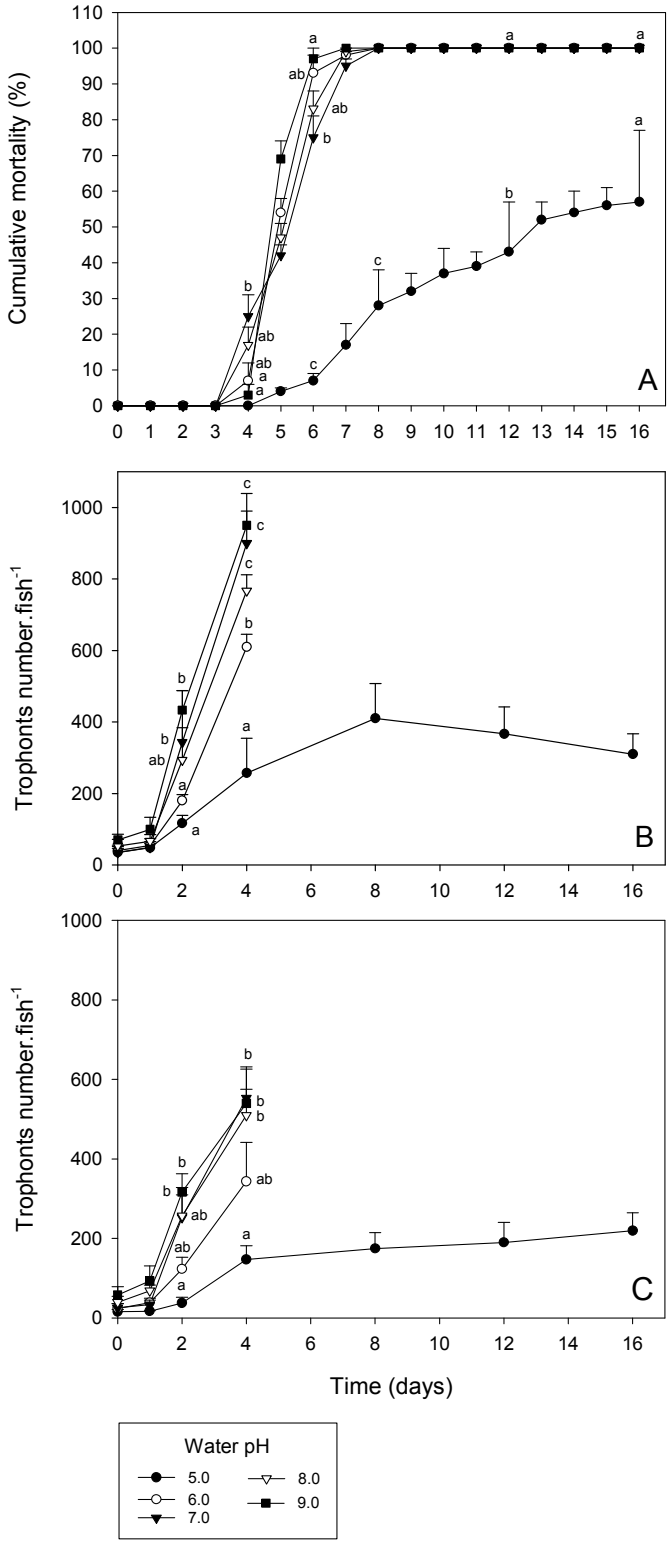
Cumulative mortality (%)
Trophonts number.fish-1
Trophonts number.fish-1
Time (days)
A
B
C
Water pH
5.0
6.0
7.0
8.0
9.0

Fig. 2

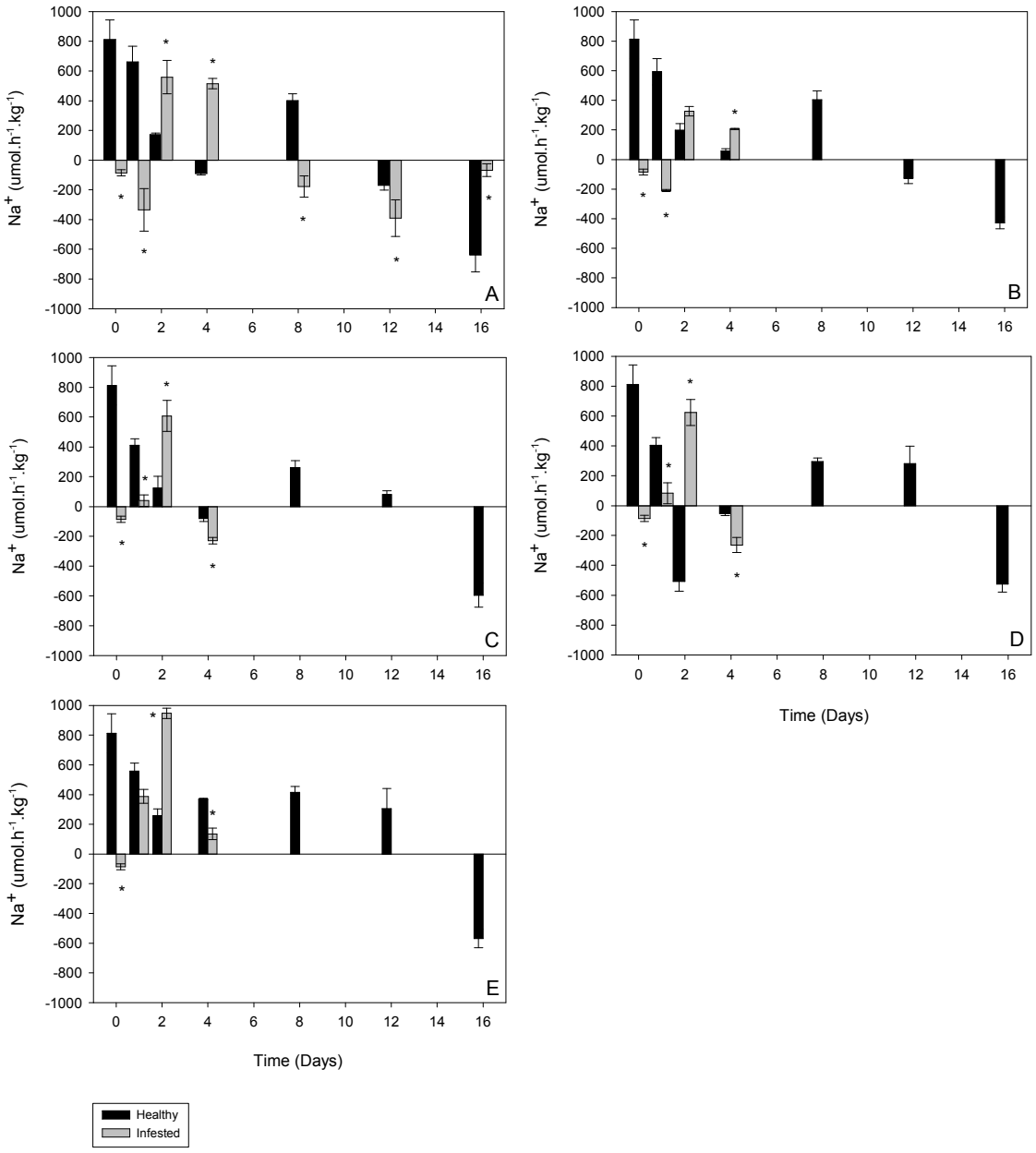

Fig. 3

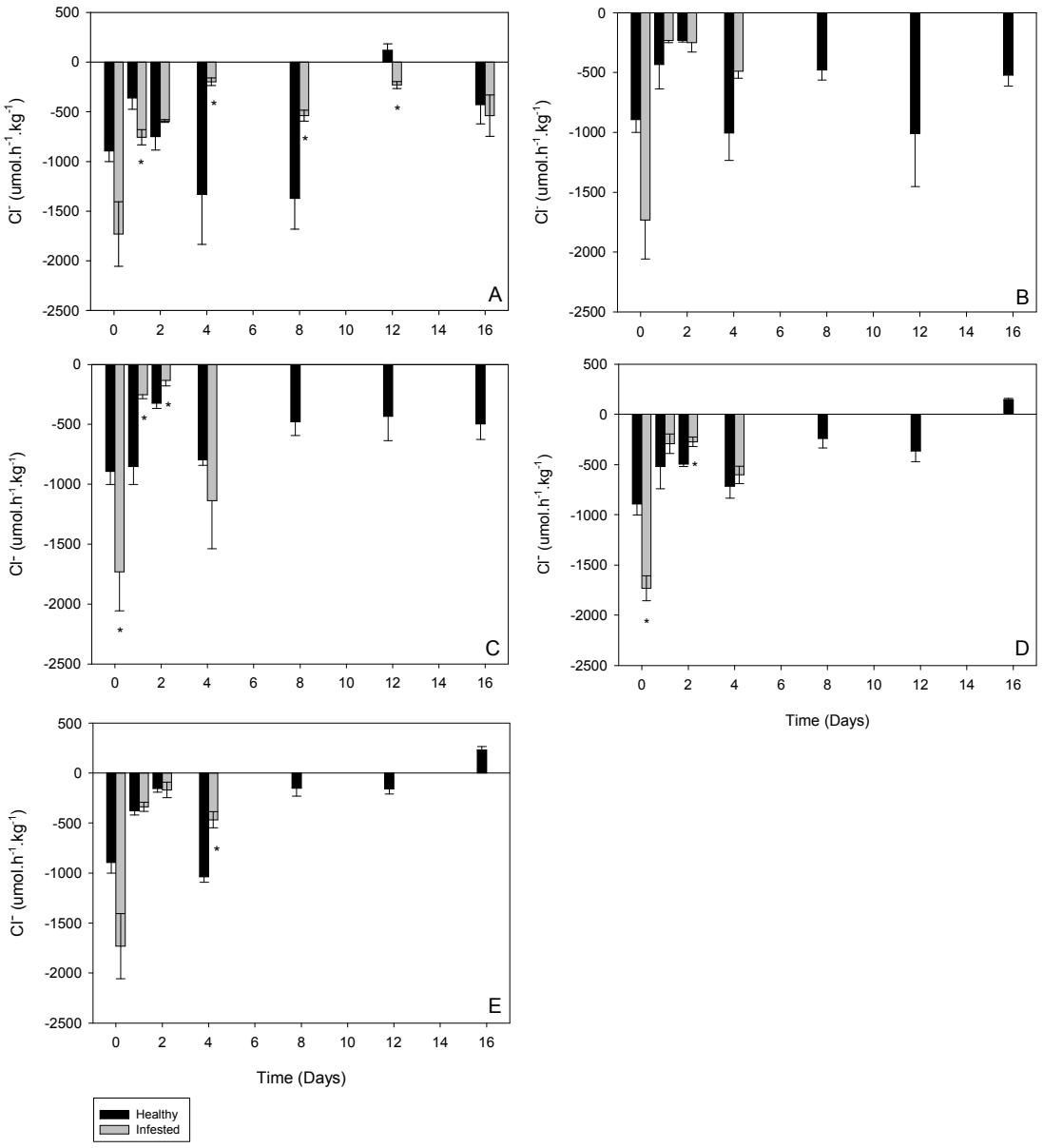

Fig. 4

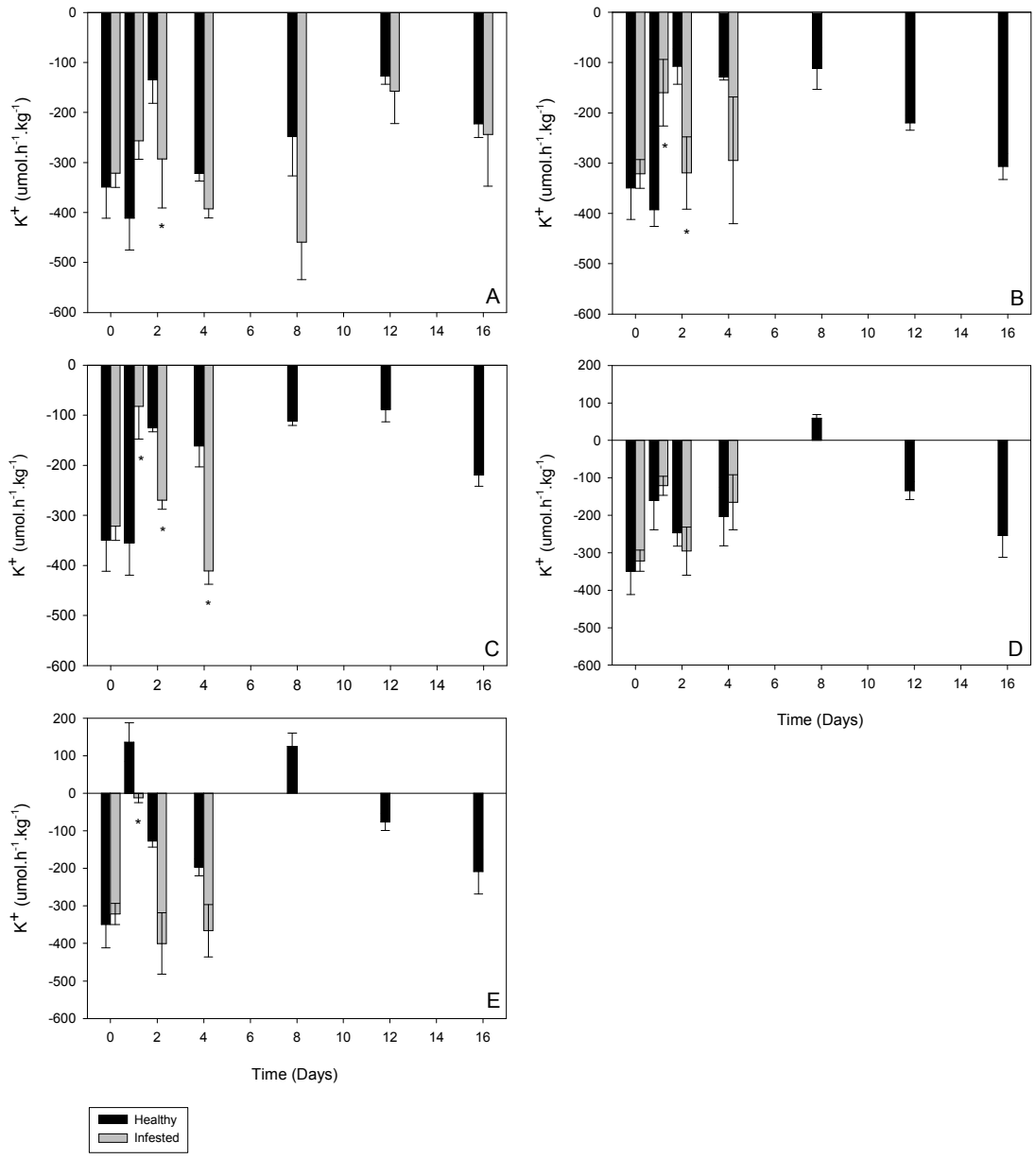

Fig. 5

Manuscrito 02

pH e dureza da água e seus efeitos na infecção de juvenis de jundiá (*Rhamdia quelen*) com *Ichthyophthirius multifiliis* (Fouquet, 1876)

Water pH and hardness and their effect in the infection of silver catfish (*Rhamdia quelen*) juveniles with *Ichthyophthirius multifiliis* (Fouquet 1876)

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Running title: Relationship between *I. multifiliis* infection and water pH and hardness.

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ABSTRACT

The aim of this study was to determine the intensity of *I. multifiliis* infection as well as net ion fluxes in silver catfish juveniles exposed to different pHs (5 and 7) and hardness (20, 60 and 120 mg $CaCO_3.L^{-1}$). Net Na^+, K^+, and Cl^- fluxes were determined at different times, trophonts in the skin and gill were counted, and mortality was registered daily. After four days, fish kept at pH 7.0-hardness 20 mg $CaCO_3.L^{-1}$ showed significantly higher cumulative mortality than those maintained in the other treatments. Juveniles exposed to pH 5.0-hardness 20 mg $CaCO_3.L^{-1}$ showed significantly lower number of trophonts on the skin throughout all experimental period compared to those exposed to the pH 7-hardness 20 mg $CaCO_3.L^{-1}$. Silver catfish exposed to pH 5.0-hardness 20 mg $CaCO_3.L^{-1}$ also showed significantly lower number of trophonts on the skin when compared to those exposed to the other treatments, except the treatment with pH 5.0-hardness 60 mg $CaCO_3.L^{-1}$ at days 1 and 2. Infected silver catfish showed significantly higher net Na^+ and K^+ effluxes at day one than asymptomatic (non-infected) juveniles exposed to pH 5.0 and 7.0, but there was a rapid recovery (influx) after two days. There was a significantly higher Cl^- influx in infected juveniles than in asymptomatic juveniles maintained at pH 5.0 in all hardness and at pH 7.0–hardness 60 mg $CaCO_3.L^{-1}$. These results allowed us to conclude that infection by *I. multifiliis* is less severe in silver catfish maintained at pH 5.0-hardness 20 mg $CaCO_3.L^{-1}$. Increase of water hardness increases trophonts infection and impairs survival in silver catfish kept at pH 5.0, but the opposite is observed when juveniles are at pH 7.0. There was no clear evidence of a relationship between mortality and trophonts number in infected silver catfish with net ion fluxes.

Keywords: net ion fluxes, theronts, trophonts number, parasite, cumulative mortality.

1. Introduction

Silver catfish, *Rhamdia quelen,* is the native species most raised in the state of Rio Grande do Sul (Baldisserotto, 2009), responds easily to induced spawning, is well adapted for cultivation even in the winter months and present fast growth in the summer of southern Brazil (Gomes et al., 2000; Barcellos et al., 2004; Garcia et al., 2008).

The ciliate *Ichthyophthirius multifiliis* is an important pathogen of freshwater teleosts, presents worldwide distribution and causes ichthyophthiriasis, also known as ‘‘white spot disease’’ or ‘‘ich’’ (Traxler et al., 1998; Buchmann and Nielsen, 1999; Gleeson et al., 2000; Matthews, 2005). Ichthyophthiriasis presents three life stages: a trophont stage which is an obligate parasite that infests fish gills, skin, eyes, and fins (Chapman 1984; Matthews, 2005), a tomont stage that shows a rapid phase of division, with the production of cells called tomites that differentiate into a theront stage, infective to the fish host (Matthews, 2005). Ichthyophthiriasis causes damages of the gill epithelium and skin during the parasite life-cycle and can cause death of the host (Ewing et al., 1994; Tumbol et al., 2001). Silver catfish is commonly affected by *I. multifiliis,* which causes high mortality rates (Miron et al., 2003; Carneiro et al., 2005; Garcia et al., 2007).

Water pH may reduce due to the presence of acidic cations, as Al^{3+}, or iron pyrite, which under oxygenating conditions forms sulfuric acid (Zweig et al., 1999; Parra and Baldisserotto, 2007) and to the presence of humic and fulvic acids in the soil (Matsuo and Val, 2003). Another very important parameter is water hardness. Soft waters have low content of Ca^{2+} and Mg^{2+} and hard water present large amounts of these ions, originated from soils that contain limestone (Baldisserotto, 2003). The pH range tolerated by silver catfish juveniles is 4.0 to 9.0 with water hardness of 30 mg $CaCO_3.L^{-1}$ without significantly mortality (Zaions and Baldisserotto, 2000).

The increase water hardness to 150-600 mg $CaCO_3.L^{-1}$ improves the survival of juveniles exposed to very acidic (pH 3.75) and alkaline (pH 10.0-10.5) (Townsend and Baldisserotto, 2001). However, the best pH and water hardness level for survival and growth of silver catfish larvae is 8.0-8.5 and 30 to 70 mg $CaCO_3.L^{-1}$, respectively (Lopes et al., 2001; Townsend et al., 2003).

Water pH and hardness can affect the survival of free-living stages of *I. multifiliis*, therefore the aim of this study was to determine if water pH and hardness could affect the infection of this parasite in silver catfish. Moreover, the effect of *I. multifiliis* infection on the ionoregulation of silver catfish maintained at different pH and hardness was also investigated through the analysis of the net Na^+, K^+ and Cl^- fluxes.

2. Material and methods

2.1. Experimental Animals

Seven hundred and twenty silver catfish juveniles (9.2 ± 1.0 cm and 6.3 ± 0.8 g) were obtained from fish farmers around Santa Maria, southern Brazil. These juveniles did not present any apparent illness when they arrived at the Fish Physiology Laboratory at the Universidade Federal de Santa Maria, where they were maintained for four days in twelve continuously aerated (using two 20 W air pumps) 250-L tanks.

2.2. Juvenile Infection

The juveniles were infected by adding one silver catfish juvenile infected with *I. multifiliis* (more than 900 trophonts) and 1 L of water infected with theronts (free form) to each 250-L tank. Juveniles maintained in these tanks began to present white spots after five days (15–32 trophonts.fish^{-1}). They were then transferred to eighteen continuously aerated 40-L polypropylene tanks and kept for sixteen days. There were six treatments pH 5.0-hardness 20, 60 and 120 mg $CaCO_3.L^{-1}$ and pH 7.0-hardness 20, 60 and 120 mg $CaCO_3.L^{-1}$ (Table 1), three replicates each, and twenty infected juveniles (15–32 trophonts.fish^{-1}) were placed in each replicate. A closed circulation system was used, with a daily change of 10% of the water volume of the tanks. The same experimental procedure was performed with asymptomatic (non-infected/control group) juveniles.

2.3. Tank Management

Water pH was changed to the experimental pH by adding H_2SO_4 (1M) and hardness by adding $CaCO_3.2H_2O$ to the tanks where the fish were placed. In all treatments, juveniles were fed once a day (0800) with a commercial diet (Supra, 32% CP, Alisul Alimentos, Carazinho, Brazil) at 5.0% of their body mass. Uneaten food as well as other residues and feces were siphoned out 30 min after feeding. Dead fish were also removed daily.

2.4. Net Ion Fluxes

Three fish were collected from each replicate at 1, 2, 4, 8, 12, and 16 days after the appearance of white spots and placed in individual chambers with 100 mL water under the same treatment conditions (pH 5.0 and pH 7.0 both with hardness 20, 60 and 120 mg $CaCO_3.L^{-1}$) of both asymptomatic and infected juveniles. Water samples (5 mL) were taken from each chamber at the beginning and after three hours and stored in a freezer (-20ºC) for posterior measurement of Na^+, Cl^- and K^+ concentrations. After the period of three hours fish were removed from the chambers and placed again in the replicates. The fish were weighed and measured at the end of every flux experiment. Sampled water was analyzed using a flame photometer (Micronal B262, São Paulo, Brazil; precision 1 $mg.L^{-1}$) (Na^+ and K^+). The method of Zall et al. (1956) was used for determining the Cl^- concentration in these samples. Net ion fluxes were calculated according to Gonzalez et al. (1998):

$Jnet = V([ion]_1 - [ion]_2).(Mt)^{-1}$, where $[ion]_1$ and $[ion]_2$ are the bath ion concentrations at the beginning and end of the flux period, respectively, V is the bath volume (in liters), M is the mass of the fish (in kg), and t is the duration of the flux period (in hours).

2.5. Mortality and the Number of Trophonts

Cumulative mortality rate was calculated from the number of dead fish removed from each tank throughout the experimental period for both asymptomatic and infected fish. The number of trophonts was determined by counting white spots on the skin of anaesthetized juveniles (40 $\mu g.L^{-1}$ clove oil) with the assistance of a stereomicroscope (total magnification 10 x).

2.6. Water Quality

Water pH was measured twice a day with a DMPH-2 (Digimed, São Paulo, Brazil) pH meter and adjusted according to the experimental conditions (pH 5.0 or 7.0), as well as water hardness levels (20, 60 and 120 $mg.L^{-1}$ $CaCO_3$) (Greenberg et al., 1976) . Total ammonia levels were determined twice a week by nesslerization according to the method of Greenberg et al. (1976), and non-ionized ammonia levels were calculated according to the method of Piper et al. (1982). Dissolved oxygen and temperature were measured daily with a YSI oxygen meter (Model Y5512; YSI Inc., Yellow Springs, OH, USA), and laboratory temperature (24.0 ± 2ºC) was maintained, throughout all experimental period, by using an air conditioner. Total alkalinity and nitrite (Boyd, 1998) were determined once a week.

2.7. Statistical Analysis

Data are reported here as mean ± SEM (N=9). The homogeneity of variances among groups was tested with the Levene test. Comparisons of net ion fluxes between different treatments were made by two-way ANOVA (time X treatment) and a Tukey test. Mortality and the number of trophonts (skin and gill) in all treatment groups were compared by one-way ANOVA and Tukey test. All tests were performed with the Software Statistica 5.1 (1997; StatSoft Inc., Tulsa, OK, USA). The minimum significance level was set at $p < 0.05$.

3. Results

The water pH and hardness in the 250-L tanks were 7.3-7.7 units and 18.0-24.0 mg $CaCO_3.L^{-1}$, respectively. Other water parameters in both the 250 and 40 L tanks were: temperature 24.0 ± 2ºC, dissolved oxygen levels 6.35 ± 0.8 $mg.L^{-1}$, maximum total ammonia levels 0.35 ± 0.02 $mg.L^{-1}$, maximum non-ionized ammonia levels 0.03 ± 0.01 $mg.L^{-1}$, maximum nitrite levels 0.05 ± 0.01 $mg.L^{-1}$.

Fish cumulative mortality in most treatment groups of infected fish started three to seven days after the appearance of white spots on the skin, while no mortality occurred in the control group. After four days, fish kept at pH 7.0-hardness 20 mg $CaCO_3.L^{-1}$ showed significantly higher cumulative mortality than those maintained in the other treatments (Fig. 1). Juveniles exposed to pH 5.0-hardness 20 mg $CaCO_3.L^{-1}$ showed significantly lower numbers of trophonts on the skin throughout all experimental period after the appearance of the white spots compared to those exposed to the pH 7.0-hardness 20 mg $CaCO_3.L^{-1}$. Silver catfish exposed to pH 5.0-hardness 20 mg $CaCO_3.L^{-1}$ also showed significantly lower number of trophonts on the skin when compared to those exposed to the other treatments, except the treatment with pH 5.0-hardness 60 mg $CaCO_3.L^{-1}$ at days 1 and 2 (Fig. 2). Increase of water hardness at pH 5.0 significantly increased number of trophonts in infected silver catfish throughout all experimental period, except at day one, but the opposite was observed in juveniles submitted to pH 7.0 (Fig. 2).

Juveniles infected with *I. multifiliis* and exposed to all treatments showed a significantly higher Na^+ efflux at days one and four after the appearance of white spots on the skin (except day one in those exposed to pH 5.0-hardness 60 and 120 mg $CaCO_3.L^{-1}$) than the asymptomatic juveniles. However, on day two, infected fish at all experimental pH and hardness presented significantly higher Na^+ influx than the asymptomatic fish (Fig. 3). Eight to sixteen days after the

appearance of the white spots, infected juveniles kept at pH 5.0-hardness 120 mg $CaCO_3.L^{-1}$ presented significantly higher Na^+ efflux than the asymptomatic ones (Fig. 3 C). Infected juveniles kept at pH 7.0-hardness 120 mg $CaCO_3.L^{-1}$ and pH 5.0-hardness 60 mg $CaCO_3.L^{-1}$ also presented significantly higher Na^+ efflux than the asymptomatic ones at day eight (Fig. 3 B and F), but the infected juveniles from the other treatments showed significantly higher Na^+ influx than asymptomatic juveniles from eight to sixteen days (Fig. 3 A, D and E).

First day after appearance of white spots there was a significantly higher net Cl^- influx in infected juveniles kept at pH 5.0-hardness 20 mg $CaCO_3.L^{-1}$ and pH 7.0-hardness 60 mg $CaCO_3.L^{-1}$ compared to asymptomatic juveniles, which showed a net efflux of this ion from one to twelve days (Fig.4 A and E). In the other treatments there was a net Cl^- influx in infected juveniles at the first day, followed by efflux with subsequent recovery, except at pH 5.0-hardness 120 mg $CaCO_3.L^{-1}$, while asymptomatic juveniles usually presented net Cl^- effluxes in all treatments (Fig. 4 B, C, D and F).

Silver catfish submitted to all treatments and infected with *I. multifiliis* presented significantly higher net K^+ efflux at days zero and one after appearance of the white spots (except those maintained at pH 5.0-hardness 60 mg $CaCO_3.L^{-1}$ at day one) than asymptomatic juveniles. There was a recovery (influx) of this ion at the second day in infected juveniles from the fourth day after the appearance of the white spots up to the end of the experiments, infected juveniles showed net K^+ effluxes in most treatments. Asymptomatic juveniles presented net K^+ effluxes throughout all experiment period in all treatments (except at day two in juveniles maintained at pH 5.0-hardness 120 mg $CaCO_3.L^{-1}$) (Fig. 5).

4. Discussion

The *I. multifiliis* is probably the most widespread parasite of freshwater teleosts with a geographical range extending from the tropics to temperate regions (Matthews, 2005) and has an important impact on fish cultures resulting in great economic loss (Traxler et al., 1998).

In our study the cumulative mortality rate caused by *I. multifiliis* was higher in fish maintained at pH 7.0–hardness 20 mg $CaCO_3.L^{-1}$ and pH 5.0 or 7.0–hardness 60 and 120 mg $CaCO_3.L^{-1}$, with 80% and 45 to 60%, respectively, compared to those kept at pH 5.0–hardness 20 mg $CaCO_3.L^{-1}$, with approximately 20% cumulative mortality in sixteen days. The number of white spots for fish at pH 5.0–hardness 20 mg $CaCO_3.L^{-1}$ was also lower through almost all experimental period when compared to other treatments.

In a previous study with *Bidyanus bidyanus* maintained at pH 5.0 and 6.0 there was a decrease in both theronts and trophonts number in twelve days, due to inhibition of theronts production, while all those exposed to pH 7.0-7.5 died within three days. However, high cumulative mortality was observed in this period (86 – 93%), and the authors supposed that was due to high initial infection and/or pH fluctuations (Mifsud and Rowland, 2008).

Silver catfish juveniles maintained at pH 5.0–hardness approximately 20 mg $CaCO_3.L^{-1}$ presented lower number of white spots on the skin and gills, and higher survival (72 %) compared to pH 6.0 to 9.0 starting from the eight days after the appearance of white spots (Garcia et al., submitted).

Silver catfish juveniles infected with the parasite presented initially net Na^+ and K^+ effluxes and a rapid recovery in the following days after infection in all treatments except at pH 5.0–hardness 120 mg $CaCO_3.L^{-1}$, compared to asymptomatic juveniles. There were usually net Cl^- influxes in infected juveniles at pH 5.0–hardness 20 and 120 mg $CaCO_3.L^{-1}$ and pH 7.0–hardness

60 mg $CaCO_3.L^{-1}$ in comparison with asymptomatic juveniles. In the other treatments there was net Cl^- influx with posterior loss and rapid recovery of these fluxes at the eighth day.

These results are in agreement with a previous study that reported higher net Na^+ efflux in the first days in silver catfish juveniles infected with *I. multifiliis* compared to asymptomatic juveniles (Garcia et al., submitted). Lower whole body Na^+ levels were detected after the appearance of white spots on the skin, and subsequent recovery of these levels at the end of two days, but this did not occur in whole body K^+ and Cl^- levels (Garcia et al., 2007). Infected *Carassius auratus* (goldfish) showed loss of whole body Na^+ and Cl^- only at two days post-exposure to theronts of *I. multifiliis* (white spots were not detected up to three days post-exposure) (Tumbol et al., 2001).

These results allowed us to conclude that infection by *I. multifiliis* is less severe in silver catfish maintained at pH 5.0-hardness 20 mg $CaCO_3.L^{-1}$. Increase of water hardness increases trophonts infection and impairs survival in silver catfish kept at pH 5.0, but the opposite is observed when juveniles are at pH 7.0. There was no clear evidence of a relationship between mortality and trophonts number in infected silver catfish with net ion fluxes.

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Table 1- Physicochemical parameters of the tanks water of different treatments.

Physicochemical parameters				
pH (Units)		Hardness (mg $CaCO_3.L^{-1}$)		Alkalinity (mg $CaCO_3.L^{-1}$)
		20	19 ± 3	3.7 ± 0.6
5.0	5.4 ± 2	60	61 ± 3	12.3 ± 2.5
		120	126 ± 6	26.1 ± 6.0
		20	20 ± 2	44.3 ± 5.5
7.0	7.1 ± 4	60	62 ± 6	63.0 ± 7
		120	125 ± 7	80.2 ± 8.7

Figure captions

Figure 1 – Cumulative mortality of silver catfish juveniles infected with *Ichthyophthirius multifiliis* and exposed to pH 5.0 (A) and pH 7.0 (B) and different water hardness as a function of time (days after the appearance of white spots on the skin of infected juveniles). Values are expressed as means ± SEM.

Different letters indicate significant difference between treatments on the same day by two-way ANOVA and Tukey test ($P < 0.05$).

Figure 2 – Trophonts number of *Ichthyophthirius multifiliis* in infected silver catfish juveniles maintained at pH 5.0 and 7.0 and different water hardness for sixteen days (A – day 1; B – day 2; C – day 4; D – day 8; E – day 12 and F – day 16). Values are expressed as means ± SEM (N=9). Different letters indicate significant difference between different water hardness at the same pH (capital letters – pH 7.0, lowercase letters – pH 5.0) ($P < 0.05$).

* Significantly different from pH 5.0 ($P < 0.05$).

Figure 3 – Net Na^+ fluxes as a function of time (days after the appearance of white spots on the skin of infected juveniles) in silver catfish juveniles exposed to *Ichthyophthirius multifiliis* (infected) or not (asymptomatic) at pH 5.0 (A, B and C) and 7.0 (D, E and F) and different water hardness. Values are expressed as means ± SEM (N=9).

* Significantly different from asymptomatic juveniles at the same time by two-way ANOVA and Tukey test ($P < 0.05$).

Figure 4 – Net Cl^- fluxes as a function of time (days after the appearance of white spots in the skin of infected juveniles) in silver catfish juveniles exposed to *Ichthyophthirius multifiliis* (infected) or not (asymptomatic) at pH 5.0 (A, B and C) and 7.0 (D, E and F) and different water hardness. Values are expressed as means ± SEM (N=9).

* Significantly different from asymptomatic juveniles at the same time by two-way ANOVA and Tukey test ($P < 0.05$).

Figure 5 – Net K^+ fluxes as a function of time (days after the appearance of white spots on the skin of infected juveniles) in silver catfish juveniles exposed to *Ichthyophthirius multifiliis* (infected) or not (asymptomatic) at pH 5.0 (A, B and C) and 7.0 (D, E and F) and different water hardness. Values are expressed as means ± SEM (N=9).

* Significantly different from asymptomatic juveniles at the same time by two-way ANOVA and Tukey test ($P < 0.05$).

Figure 1

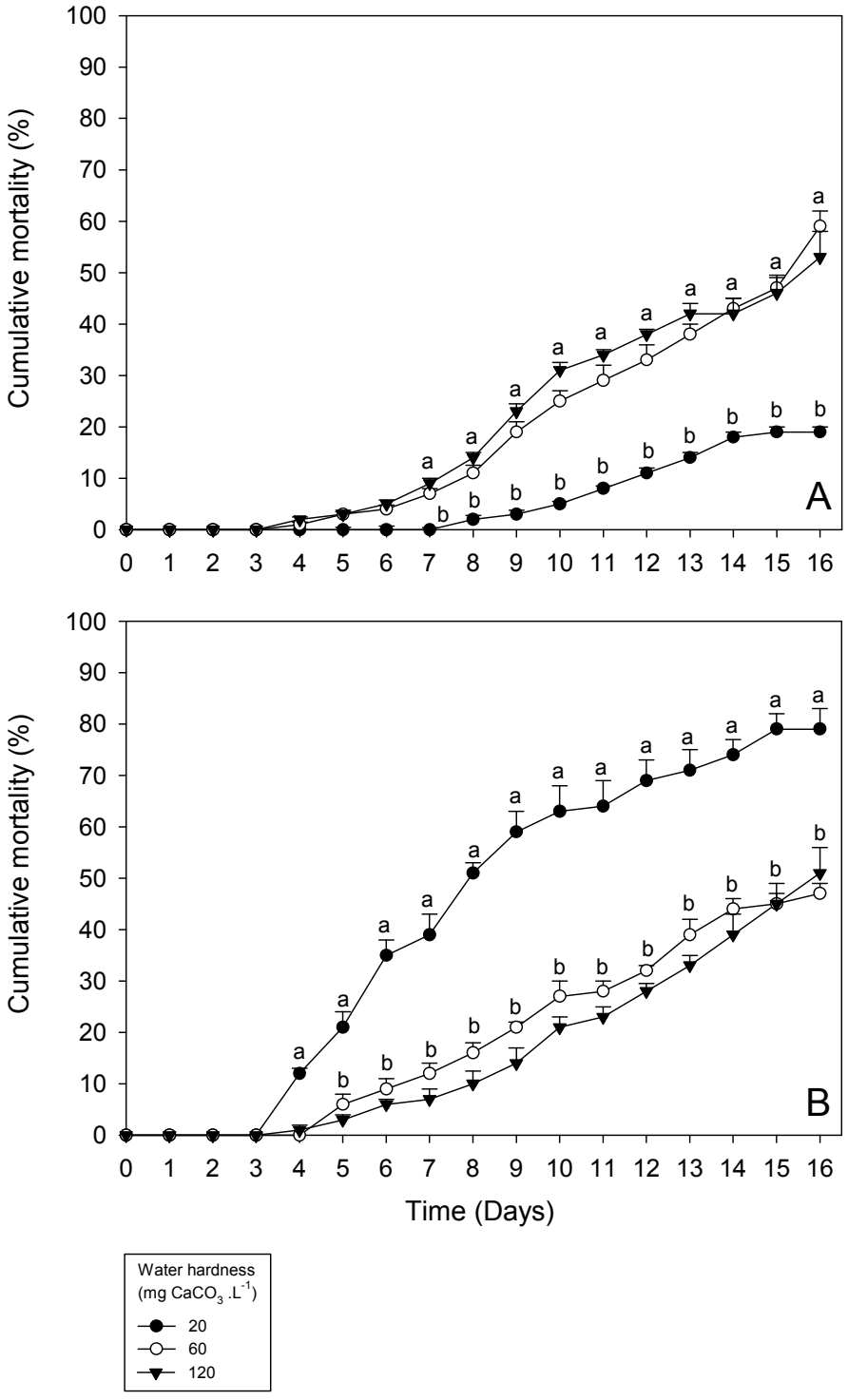


Figure 2

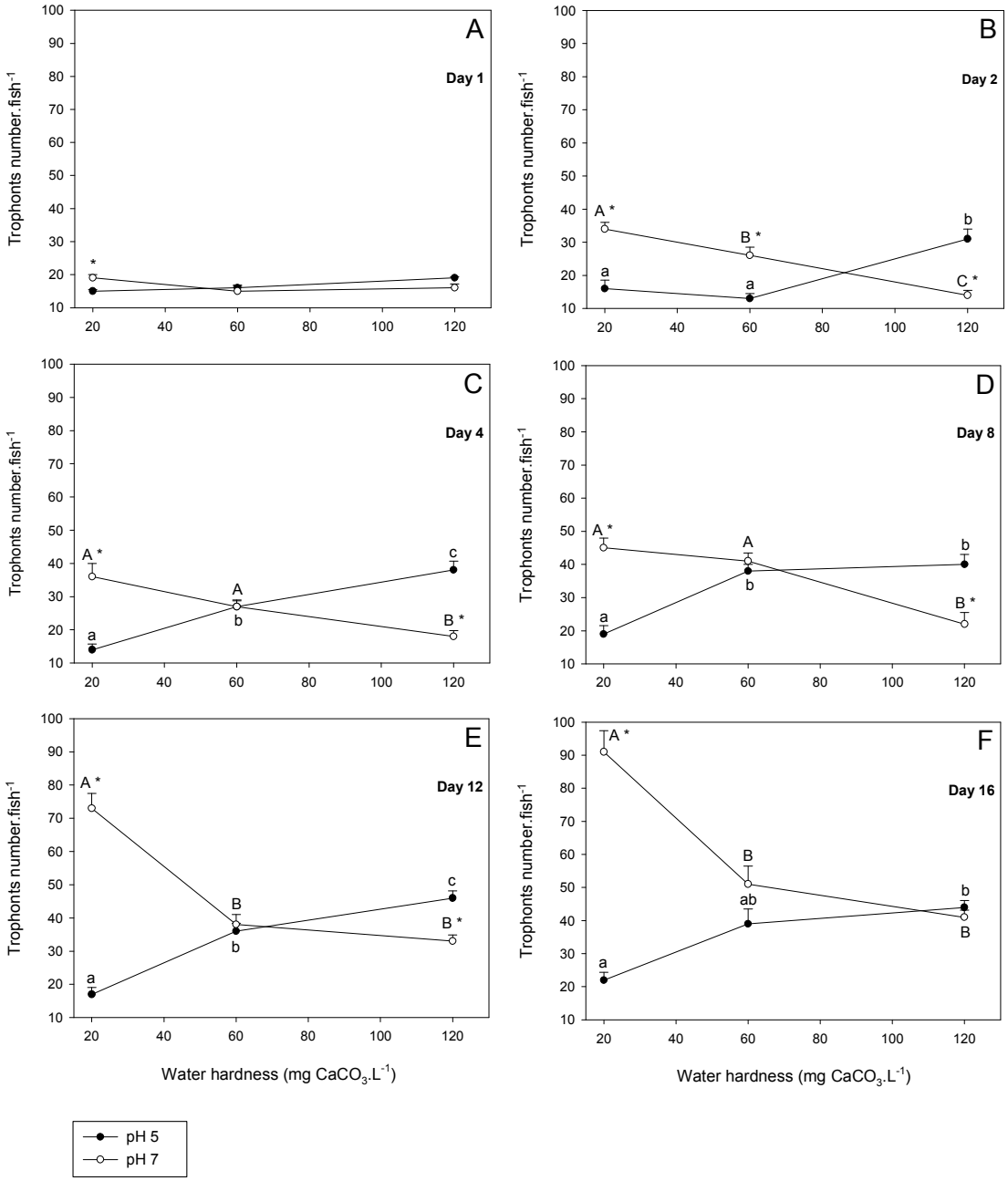

A
Day 1
B
Day 2
C
Day 4
D
Day 8
E
Day 12
F
Day 16
Trophonts number.fish-1
Water hardness (mg CaCO3.L-1)
pH 5
pH 7

Figure 3

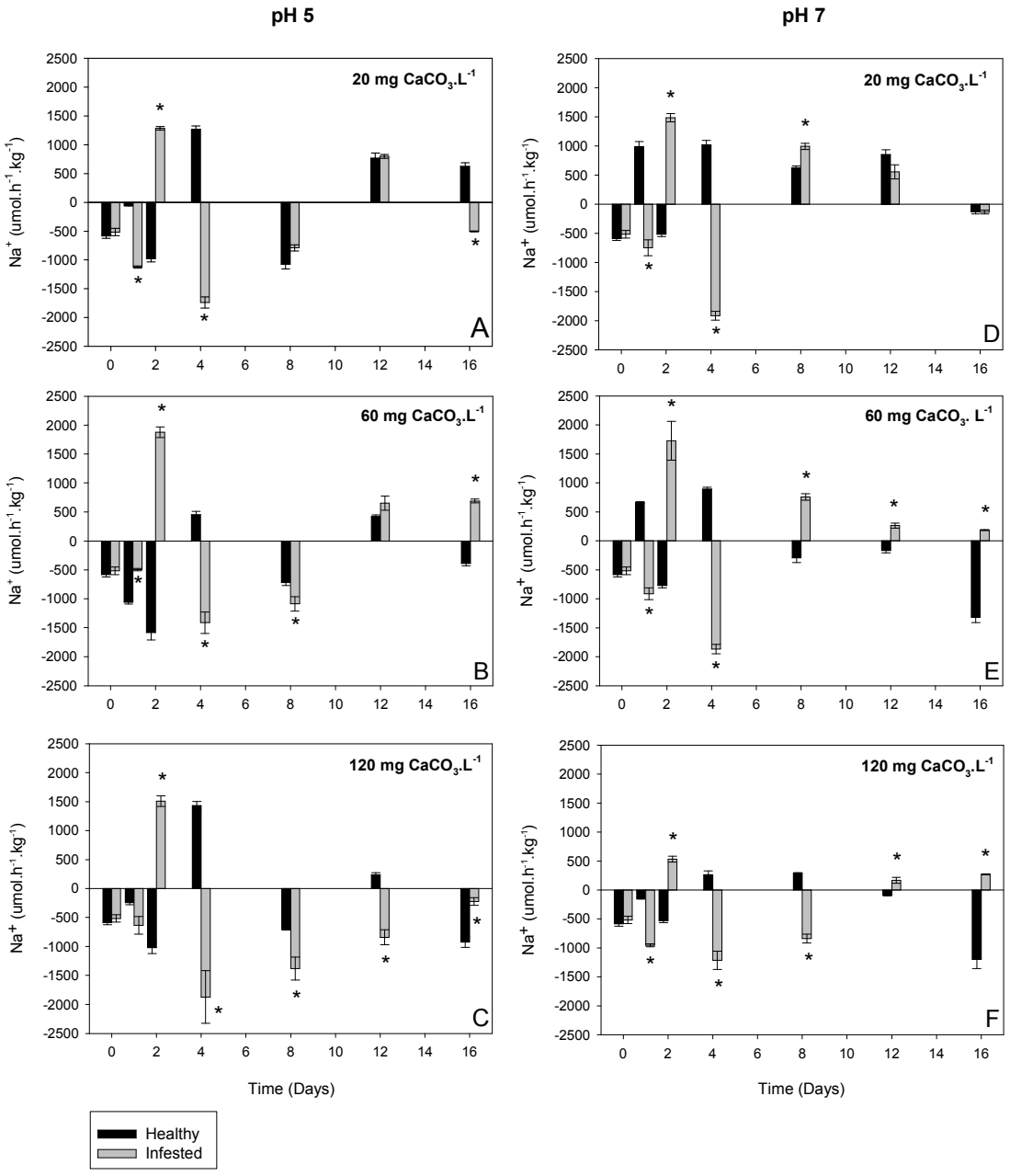

pH 5
pH 7
20 mg CaCO3.L-1
60 mg CaCO3. L-1
120 mg CaCO3.L-1
Na+ (umol.h-1.kg-1)
Time (Days)
A
B
C
D
E
F
Healthy
Infested

Figure 4

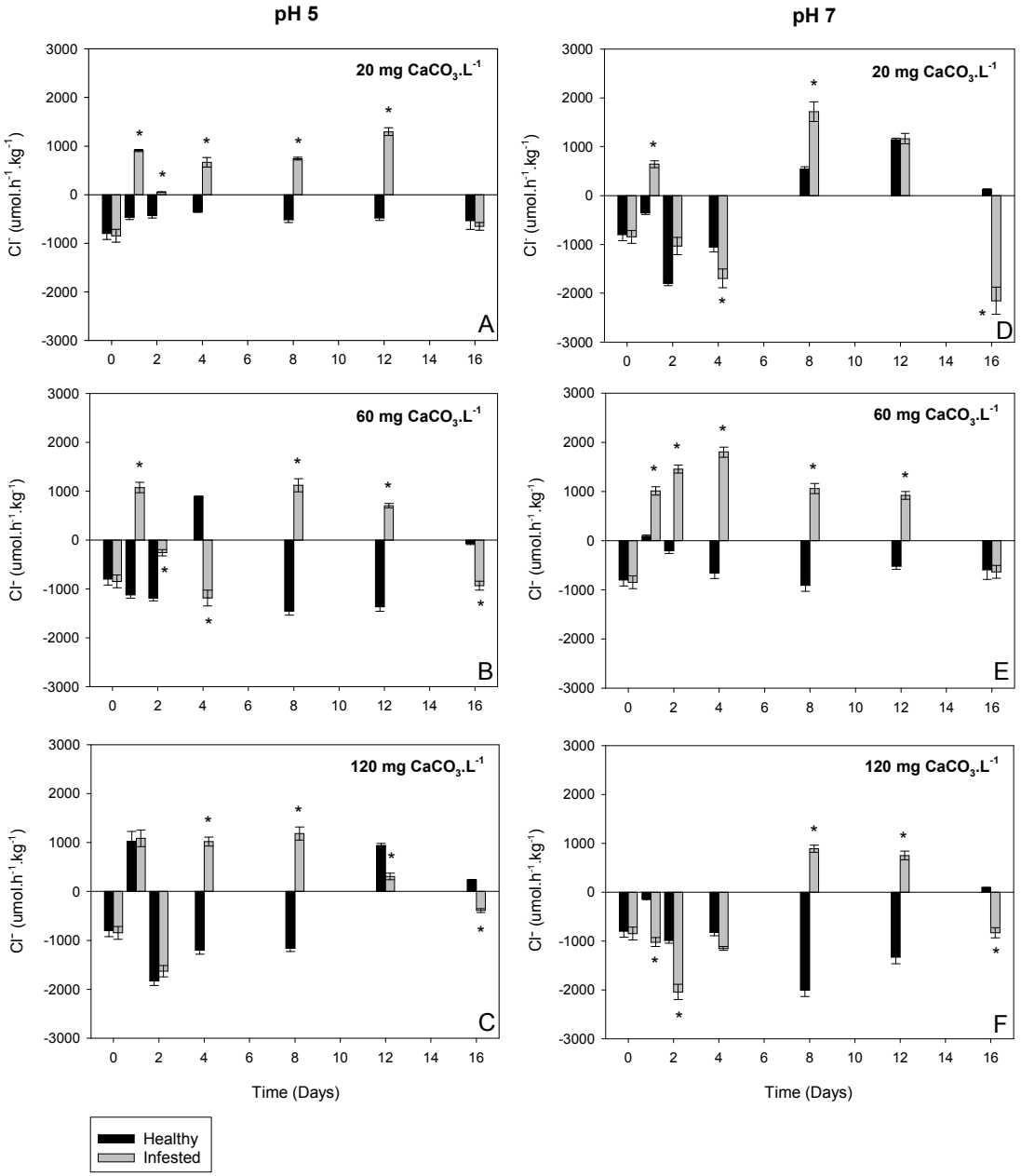
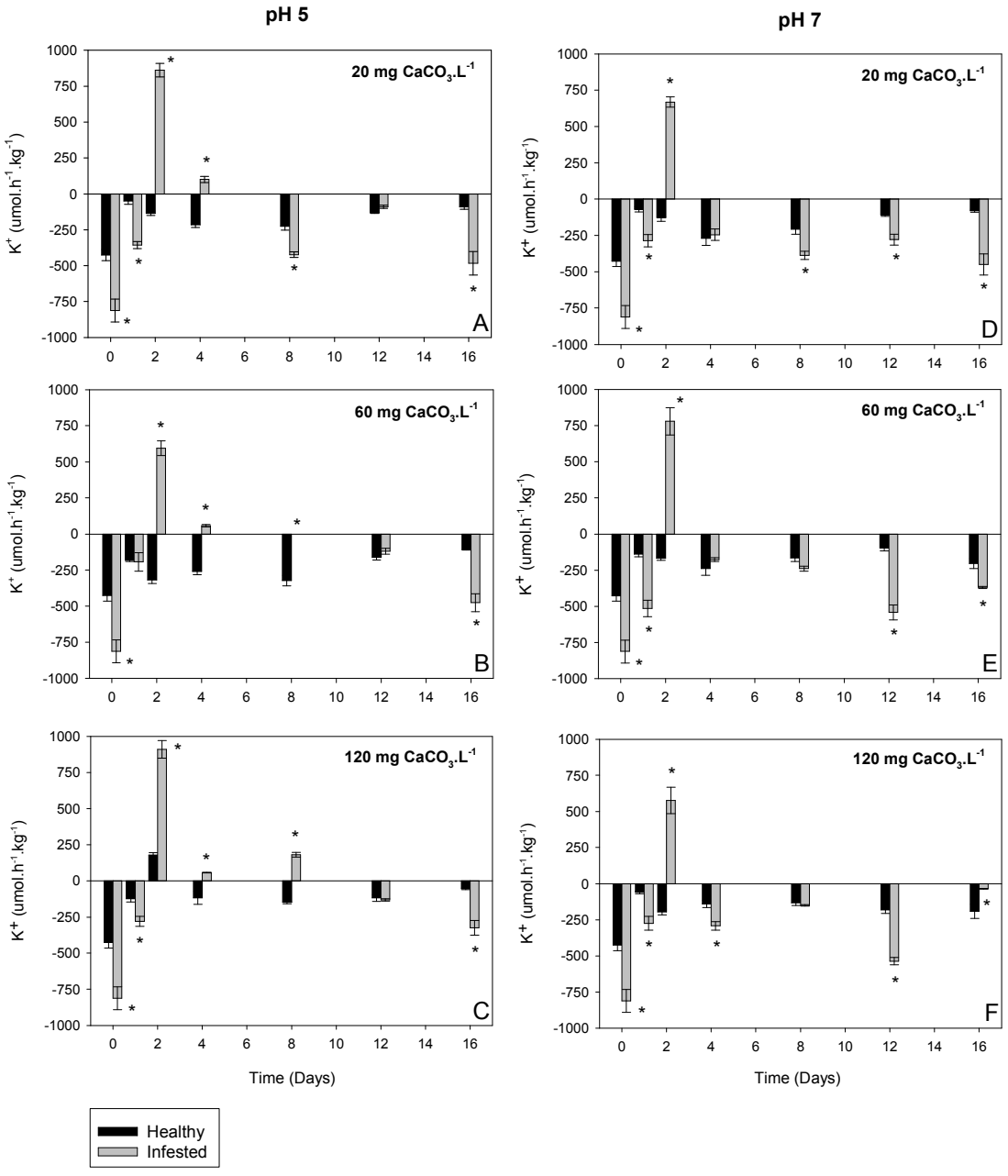

pH 5
pH 7
20 mg CaCO3.L-1
60 mg CaCO3.L-1
120 mg CaCO3.L-1
Cl- (umol.h-1.kg-1)
Time (Days)
A
B
C
D
E
F
Healthy
Infested

Figure 5


pH 5
pH 7
20 mg CaCO3.L-1
60 mg CaCO3.L-1
120 mg CaCO3.L-1
K+ (umol.h-1.kg-1)
Time (Days)
A
B
C
D
E
F
Healthy
Infested

Manuscrito 03

Parâmetros de estresse oxidativo em juvenis de jundiá (*Rhamdia quelen*) infectados com *Ichthyophthirius multifiliis* (Fouquet, 1876) e submetidos a diferentes pH da água.

Oxidative stress parameters in silver catfish (*Rhamdia quelen*) juveniles infected with *Ichthyophthirius multifiliis* (Fouquet, 1876) and submitted to different water pH

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Abstract

The aim of this study was to determine the oxidative stress parameters in liver, gill and muscle of silver catfish juveniles infected with *I. multifiliis* and submitted to different pH (5.0 and 7.0) for three days. Silver catfish juveniles infected with *I. multifiliis* (three days after the appearance of white spots on the skin) and exposed to pH 5.0 and 7.0 showed significantly higher TBARS levels in the liver (988 and 176%, respectively) , than the asymptomatic juveniles. The catalase (CAT) and glutathione-S-transferase (GST) activities in the liver of infected silver catfish exposed to pH 7.0 were significantly lower and higher respectively, throughout the three days of experiment than those of the asymptomatic juveniles (57, 50 and 61%; and 1372, 835 and 2165%), but those maintained at pH 5.0 showed significantly higher GST activity through all experimental period (1229, 823 and 732%). Infected juveniles kept at pH 5.0 showed significantly higher TBARS levels in the gill at days two and three (238 and 243%) and those at pH 7.0 at day three (216%) compared to asymptomatic juveniles. The CAT activity in the gill of infected juveniles exposed to pH 5.0 and 7.0 was significantly higher at day two (339 and 327%, respectively), but at day three it was significantly lower (39 and 11%, respectively) than asymptomatic fish. The gill of infected juveniles showed significantly higher GST activity throughout all experimental period at both pH 5.0 (350, 464 and 390%) and 7.0 (278, 495 and 213%) compared to asymptomatic juveniles. The muscle of infected juveniles maintained at pH 5.0 showed significantly lower TBARS levels (44%) at day three compared to asymptomatic juveniles. The CAT activity was significantly lower in the muscle of infected juveniles at pH 5.0 and 7.0 at all experimental days except day 1 at pH 7.0 compared to asymptomatic juveniles (59, 75 and 41%; 50 and 57%, respectively) . The muscle of infected juveniles presented significantly lower GST activity in all experimental period (days 1-3) at both pH 5.0 (25, 44 and 19%) and 7.0 (32, 43 and 73%) compared to asymptomatic juveniles. In conclusion, infection with *I. multifiliis* in silver catfish induce liver and gill damage via lipid peroxidation products, but, the higher antioxidant enzymes activity can indicate a higher protection of tissues against this parasite, the same did not being evidenced in the muscle.

Keywords: Theronts, TBARS, catalase, GST, parasite, white spots disease.

1. Introduction

In fish culture ponds the good water quality is dependent of various chemical factors dissolved in the water (dissolved oxygen, pH, NH_3, hardness, and alkalinity), as well as temperature and others physical attributes, all combined (Diana et al., 1997). The usual best water pH range for teleost reproduction and growth is 6.0 to 9.0, but the optimum range may differ for different species (Parra and Baldisserotto, 2007).

The silver catfish, *Rhamdia quelen*, is a endemic fish species from South America and is the native species most raised in southern Brazil (Gomes et al., 2000; Barcellos et al., 2003; Garcia et al., 2008; Baldisserotto, 2009). Juveniles of this species tolerate a 4.0 to 9.5 pH range without mortality (Zaions and Baldisserotto, 2000), but the best growth is at pH 7.5 (Copatti et al., 2005).

One of the main problems in the production of silver catfish is ichthyophthiriasis, "ich" or white spot disease (Baldisserotto, 2003), caused by infection by the protozoan ciliate *Ichthyophthirius multifiliis* (Matthews, 2005).

This protozoan is an endoparasite that causes economic loss in worldwide commercial and ornamental fish culture (Ling et al., 1991; Lom and Dyková, 1992; Matthews, 1994; Buchmann and Bresciani, 1997; Scholz, 1999; Miron et al., 2003; Matthews, 2005; Garcia et al., 2007; Garcia et al., submitted). The *I. multifiliis* showed a life cycle with three stages: theront (infective free form), trophonts (cyst in tissue of host) and tomont (reproduction). The last stage causes damages to host epidermis and gill epithelium during this cycle, compromising gas exchange (Ewing et al., 1994), which could lead to oxidative stress.

Oxidative stress is defined as an unbalanced state between pro-oxidants and antioxidants, resulting in elevated production of reactive oxygen species (ROS) and free radicals, agents with a potential for producing deleterious effects (Mates et al., 1999; Halliwell, 1992; Halliwell and

Gutteridge, 1999). Oxidative stress can be induced by a large variety of conditions, including nutritional imbalance, exposure to chemical and physical environmental agents, strenuous physical activities, injury, and hereditary disorders (Chow, 1991). The unbalanced state of pro-oxidant and antioxidants can induce damages to DNA and RNA, inducing mutations. The attack to proteins and enzymes causes oxidation of tiol groups (-SH) and in consequence the lipid peroxidation that could induce membrane permeability changes and loss of secretory function and even to cellular death. The tissue damage and released toxins usually unchain a series of events in intracellular level that lead to a situation of oxidative stress (Halliwell, 1992; Halliwell and Gutteridge, 1999), which can be detected through measurement of oxidative stress parameters. Parasite infections may also affect antioxidant mechanisms in fish, inducing to alterations in oxidative status and/or antioxidant defenses of parasitized fish (Martinez-Álvarez et al., 2005).

Therefore, the aim of this study is to analyze the oxidative stress parameters in the liver, gill and muscle of silver catfish juveniles infected with *I. multifiliis* and submitted to different pH.

2. Materials and methods

2.1. Experimental Animals

Eighty silver catfish juveniles (32.5 ± 5.5 cm and 72.1 ± 11.0 g) were obtained from fish farmers around Santa Maria city, southern Brazil. These juveniles did not present any apparent illness when they arrived at the Fish Physiology Laboratory at the Universidade Federal de Santa Maria, where they were maintained for seven days in six continuously aerated (using two 20 W air pumps) 250-L tanks.

2.2. Juvenile Infection

Forty juveniles were infected by adding one silver catfish juvenile infected with *I. multifiliis* (more than 1000 trophonts) and 1 L of water infected with theronts (free form) to each 250-L tank. Juveniles maintained in these tanks began to present white spots after seven days (80–120 trophonts.fish^{-1}). They were then transferred to two continuously aerated 250-L polypropylene tanks and kept for three days. There were teen treatments (pH 5 and 7; two replicates each), and teen infected juveniles (80–120 trophonts.fish^{-1}) were placed in each replicate. A closed circulation system was used, and a daily change of 10% of the water volume of the tanks performed. The same experimental procedure (except infection) was performed with asymptomatic juveniles (control group).

2.3. Tissue collection

Approximately four specimens were collected from each replicate of both asymptomatic (control – day 0) and infected fish at 1, 2 and 3 days after the appearance of white spots in the infected fish. These fish were placed in recipients with water and ice for 5 min for anesthetizing and after killed by spinal section, and the tissues (gills, liver and muscle) were removed, weighed separately, and immediately frozen in liquid argon. The tissues were then stocked in a −70 ºC freezer for subsequent analysis of TBARS and oxidative stress parameters.

2.4. Water Quality

Water pH was measured twice a day with a DMPH-2 (Digimed, São Paulo, Brazil) pH meter and adjusted according to the experimental conditions (pH 5.0 and 7.0). Total ammonia levels were determined twice a week by nesslerization according to the method of Greenberg et al. (1976), and non-ionized ammonia levels were calculated according to the method of Piper et al. (1982).

Dissolved oxygen and temperature were measured daily with an YSI oxygen meter (Model Y5512; YSI Inc., Yellow Springs, OH, USA), and laboratory temperature was maintained by using an air conditioner. Total alkalinity, nitrite (Boyd, 1998), and water hardness levels (Greenberg et al., 1976) were determined once a week.

2.5. Tank Management

Water pH was changed to the experimental pH by adding 0.1M H_2SO_4 to the tanks where the fish were placed. In all treatments, juveniles were fed once a day (08:00) with a commercial diet (Supra, 32% CP, Alisul Alimentos, Carazinho, Brazil) at 5.0% of their body mass. Uneaten food as well as other residues and feces were siphoned out 30 min after feeding.

2.6. Parameters of oxidative stress

The liver, gills and muscle tissues were homogenized in 1.15% (w/v) KCl solution containing 1mM Phenylmethylsulphonyl-Fluoride (PMSF). The homogenates were centrifuged at 600×*g* for 10 min to eliminate nuclei and cell debris, and the supernatant fraction obtained was frozen at −70 ºC for further measurements. The supernatants were used for analysis of thiobarbituric acid reactive substances (TBARS), glutathione-S-transferase (GST) and catalase (CAT). CAT activity was determined by using the method described by Boveris and Chance (1973), in which the disappearance of H_2O_2 is followed spectrophotometrically at 240 nm. The results were reported as $pmol.mg^{-1}$ protein. Glutathione-*S*-transferase activity towards CDNB (1-chloro-2,4-dinitrobenzene) was determined spectrophotometrically at 340 nm using the method described in Habig et al. (1974). Activity was calculated by changes in absorbance at 340 nm using the extinction coefficient of $9.6mmol^{-1}.cm^{-1}$. One unit of GST activity was defined as the amount of enzyme catalyzing the conjugation of 1μmol of CDNB with GSH per minute at 25 ºC. Lipid

peroxidation was measured by thiobarbituric acid reactive substances (TBARS) using the method described by Buege and Aust (1978). In this method, absorbance measurements at 535nm were used to measure the reaction between thiobarbituric acid and the lipoperoxidation (LPO) products, resulting in the formation of a chromogen (Schiff's base). The results were reported as nmol.mg^{-1} protein. The protein content of the homogenate was measured using the method described in Lowry et al. (1951) using bovine serum albumin as the standard.

2.7. Statistical analysis

Data are reported as mean ± SEM (*N*). Homogeneity of variances among groups was tested with the Levene test. Data for TBARS, CAT and GST presented homogeneous variances, and comparisons between different treatments were made by two-way analysis of variance and the Tukey test. Analysis was performed using the software Statistica (version 5.1), and the minimum significance level was set at $P < 0.05$.

3. Results

The physicochemical parameters of water in the experiments are presented in Table 1. Data control during three experimental days did not show significantly difference themselves and were united in one only data (0 day or control).

Silver catfish juveniles infected with *I. multifiliis* (three days after the appearance of white spots on the skin) and exposed to pH 5.0 and 7.0 showed significantly higher TBARS levels in the liver than the asymptomatic juveniles (988 and 176% respectively) (Fig.1A and B). The CAT activity in the liver of silver catfish exposed to pH 7.0 was significantly lower in the infected than in the asymptomatic juveniles (57, 50 and 61%, days 1, 2 and 3 respectively) (Fig. 1C). The liver of infected silver catfish juveniles showed significantly higher GST activity throughout all

experimental period (days 1-3) an both pH 5.0 (1229, 823 and 732%) and 7.0 (1372, 835 and 2165%) compared to asymptomatic juveniles (Fig. 1E and F).

Infected juveniles kept at pH 5.0 showed significantly higher TBARS levels in the gill at days two and three (238 and 243% respectively) and at pH 7.0 at day three (216%) compared to asymptomatic juveniles (Fig. 2A and B). The CAT activity in the gill of infected silver catfish juveniles exposed to pH 5.0 and 7.0 was significantly higher at day two (339 and 327%, respectively), but at day three it was significantly lower (39 and 11%, respectively) than asymptomatic fish (Fig. 2C and D). The gill of infected juveniles showed significantly higher GST activity throughout all experimental period at pH 5.0 (350, 464 and 390%) and 7.0 (278, 495 and 213%) compared to asymptomatic juveniles (Fig. 2E and F).

The muscle of infected silver catfish juveniles maintained at pH 5.0 showed significantly lower TBARS levels (44%) at day three compared to asymptomatic juveniles (Fig. 3A). The CAT activity was significantly lower in the muscle of infected juveniles at pH 5.0 and 7.0 at all experimental days except day 1 at pH 7.0 compared to asymptomatic juveniles (59, 75 and 41%; 50 and 57%, respectively) (Fig. 3C and D). The muscle of infected juveniles presented significantly lower GST activity in all experimental period (days 1-3) in both pH 5.0 (25, 44 and 19%) and 7.0 (32, 43 and 73%) compared to asymptomatic juveniles (Fig. 2E and F).

4. Discussion

In the present study changes in the lipid peroxidation levels (TBARS), CAT and GST activity in the liver, gill and muscle of silver catfish juveniles infected with *I. multifiliis* were detected. Infected juveniles of this species exposed to slightly acid water (pH 5.0) presented very high lipid peroxidation levels (988%) at day three compared to control juveniles in the liver. The same

occurred in infected juveniles maintained at pH 7.0, but in lower proportions (176%). Consequently, infection by *I. multifiliis* provoked lipid oxidative damage in the liver, and the increase of levels coincided with the progression of the infection. In *Colossoma macropomum* (tambaqui), *Leporinus elongatus* (piapara), *Carassius auratus* (goldfish) and *R. quelen* (silver catfish) submitted to hypoxia similar results were found (Marcon and Wilhelm Filho, 1999; Lushchak et al., 2001; Wilhelm Filho et al., 2005; Braun et al., 2008). However, rainbow trout, *Oncorhynchus mykiss*, exposed to hyperoxia did not show lipid peroxidation in the liver (Ritola et al., 2002). LPO levels in the liver of acará, *Geophagus brasiliensis*, exposed to polluted sites also increased compared to control fish (Wilhelm Filho et al., 2001).

Infected silver catfish juveniles exposed to pH 7.0 showed lower CAT activity in the liver than asymptomatic juveniles, probably due to its higher utilization for H_2O_2 degradation in this organ. In polluted sites the induction of liver CAT activity in acará occurred due to the increase in endogenous H_2O_2 levels compared to control fish (Wilhelm Filho et al., 2001). In another study CAT activity increased in rainbow trout submitted to hyperoxia and ozone, suggesting that this higher activity might be in response to the increased oxygen consumption in comparison to control fish (Ritola et al., 2002). The same occurred in rays and sharks that showed higher CAT activity values in the liver, corresponding to the higher oxygen consumption derived from the accelerated swimming activity of sharks (Wilhelm Filho and Boveris, 1993). However, *C. macropomum* kept in hyperoxic conditions presented lower CAT activity in the liver (Marcon and Wilhelm Filho, 1999). Infected silver catfish juveniles present higher GST activity throughout all experimental period at both pH 5.0 and 7.0 compared to asymptomatic juveniles, in the attempt of establishing the pro-oxidants and antioxidants balance in the liver and gill and remove the reactive species of oxygen, avoiding oxidative damage and reduced catalytic activity developed in muscle in function of the higher amount of free radicals due to infection. This

enzyme also has an important role in the antioxidant defenses of goldfish subjected to catalase inhibition by aminotriazole in the brain (Bagnyukova et al., 2005a,b; Lushchak et al., 2005). GST activity in the liver of piapara decreased with the increase of oxygen availability due to the higher amount of free radicals formed (Wilhelm Filho et al., 2005).

The increase of *I. multifiliis* infection throughout three experimental days increased more than two-fold the lipid peroxidation in the gills of silver catfish. Therefore, lipid peroxidation is a very sensitive marker of oxidative damage in the gills due to this parasite . Lipid peroxidation products are not only a marker of oxidative damage, they are also possibly involved in triggering the up-regulation of antioxidants enzymes (Lushchak and Bagnyukova, 2006). Goldfish and piapara exposed to acute change of temperature, and different oxygen tensions, respectively, showed increase in lipid peroxide levels which serves as signal for behavior antioxidants enzymes (Bagnyukova et al., 2007; Wilhelm Filho et al., 2005). In rainbow trout and *Salvelinus leucomaenis* (Japanese charr) submitted to ozonation the lipoperoxidation provoked damage in the gills, and after 30 min ozone (0.7 ppm) exposure there was a 120% increase of lipoperoxidation in Japanese charr (Ritola et al., 2002; Fukunaga et al., 1992). In contrast, hyperoxia did not cause lipoperoxidation in the gills of Japanese charr, which indicates adequate antioxidants defenses against reactive species of oxygen (Ritola et al., 2002).

In our study the CAT activity in the gill of infected silver catfish juveniles exposed to pH 5.0 and 7.0 increased at day two (339 and 327%, respectively), but at day three it was lower (39 and 11%, respectively) than asymptomatic fish. Rainbow trout exposed to ozone and hyperoxia, and in larvae compared to embryos of *Scophthalamus maximus* and *Dentex dentex* similar results were found, as well as in *G. brasiliensis* submitted to polluted sites compared to control (Ritola et al., 2002; Peters and Livingstone, 1996; Mourent et al., 1999; Wilhelm Filho et al., 2001). In contrast, in another study no changes in CAT activity in the gills of rainbow trout exposed to 5 h

ozone exposure (Morita et al., 1995), and *Leiostomus xanthurus* exposed to different concentrations of dissolved oxygen (Cooper et al., 2002) were detected. In our study, the increase of CAT activity in the gills coincided with the increase in the muscle, possibly indicating simultaneous accumulation of H_2O_2 in both tissues.

GST activity in the gills of infected juveniles increased their rate throughout all experimental period at pH 5.0 (350, 464 and 390%) and 7.0 (278, 495 and 213%) compared to asymptomatic juveniles. The increase in the number of parasites throughout three experimental days justify the increase in the rates of antioxidant enzymes, because the oxygen capture by the gills probably reduced due to the presence of the higher amount of parasites in the gills. These higher levels of antioxidants enzymes demonstrate that a response occurred in an attempt of neutralizing the generated reactive oxygen species (Monteiro et al., 2009).

Silver catfish juveniles infected with the parasite *I. multifiliis* and maintained at pH 5.0 and 7.0 showed lower lipoperoxidation levels (44%), CAT and GST activity in the muscle when compared to asymptomatic juveniles. These results demonstrate that damage in the muscle is lower when compared to liver and gills, but there was an unbalance in pro-oxidants and antioxidants products, causing an oxidative stress in this tissue as occurred in the liver and gills. Silver catfish infected by the parasite *Clinostomum detruncatum* presented reduction of non-enzymatic antioxidants defenses with respect to pro-oxidants production, which led to oxidative stress and consequently muscle damage via products from lipid peroxidation (Belló et al., 2000). Exposure of rainbow trout blood “in vitro” to ozone increased lipid peroxidation in both red blood cells and plasma compared to control group (Ritola et al., 2003). The peroxidation levels were higher in the muscle of silver catfish, matrinxã and goldfish exposed to hypoxia compared to those maintained in normoxia (Braun et al., 2008; Marcon and Wilhelm Filho, 1999; Lushchak et al., 2001). However, apparently lipoperoxidation response changes according to species,

because in piapara the lipoperoxidation levels increased in juveniles exposed to moderate hypoxia and normoxia compared to those maintained in severe hypoxia (Wilhelm Filho et al., 2005).

Our results demonstrated that there is an unbalance in the pro-oxidants and antioxidants levels in silver catfish juveniles exposed to *I. multifiliis* compared to asymptomatic fish. This infection induces liver and gill damage via lipid peroxidation products, the same did not being evidenced in the muscle. However, the higher antioxidant enzymes activity can indicate a higher protection to tissues against *I. multifiliis*.

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Table 1 – Physicochemical parameters of the water in the experimental tanks .

Physicochemical Parameters	Experimental pH	
	5.0 (5.2 ± 0.4)	7.0 (6.7 ± 0.5)
Dissolved oxygen ($mg.L^{-1}$)	6.4 ± 0.6	6.5 ± 0.2
Temperature (ºC)	24.1 ± 2.0	23.5 ± 1.0
Hardness (mg $CaCO_3.L^{-1}$)	18.0 ± 6.0	20.0 ± 4.0
Alkalinity (mg $CaCO_3.L^{-1}$)	7.6 ± 2.0	32.0 ± 2
Total ammonia ($mg.L^{-1}$)	0.4 ± 0.01	0.3 ± 0.01
Non-ionized ammonia ($mg.L^{-1}$)	0.04 ± 0.01	0.03 ± 0.01
Nitrite ($mg.L^{-1}$)	0.05 ± 0.02	0.05 ± 0.02

List of figures

Figure 1

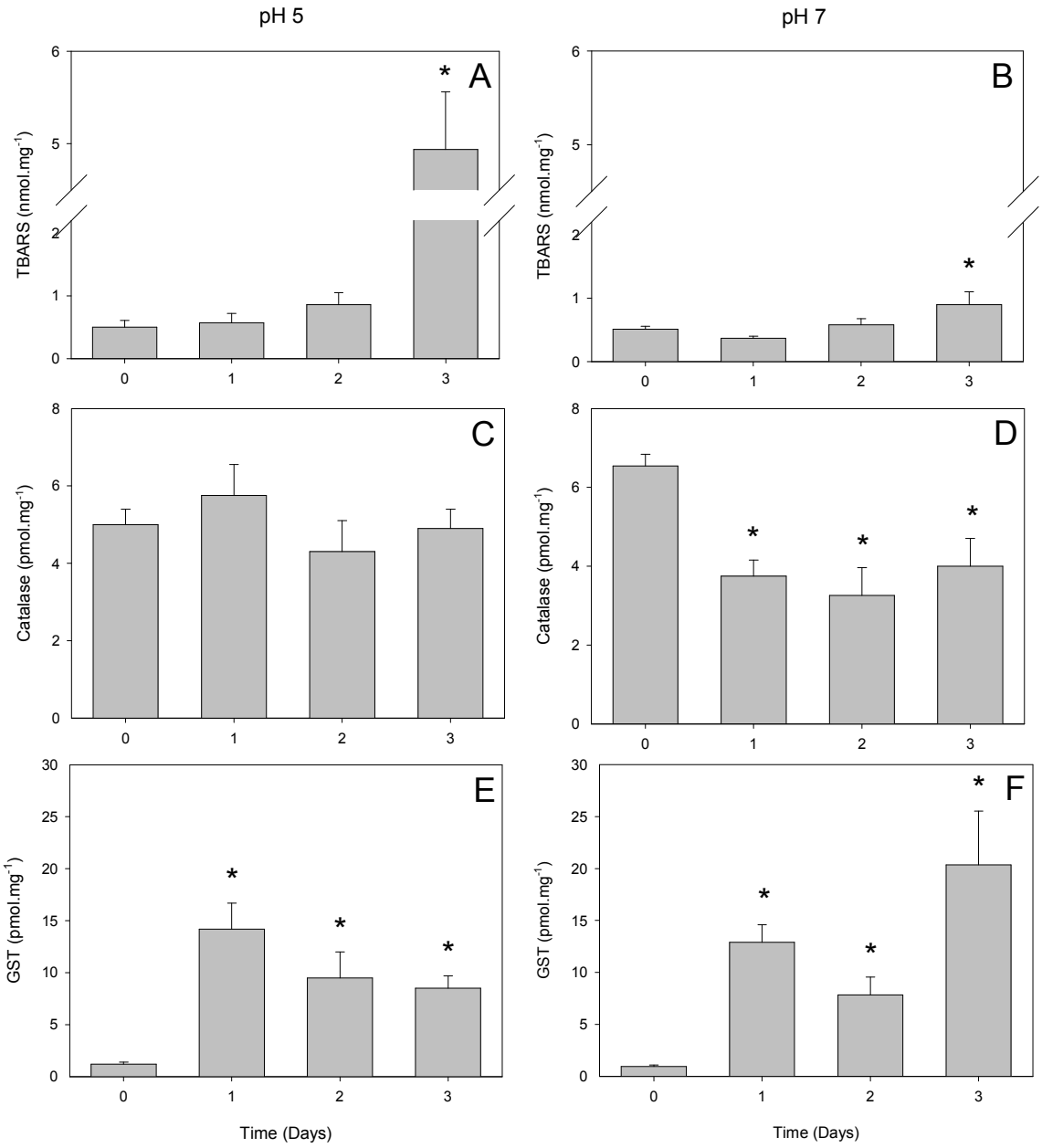
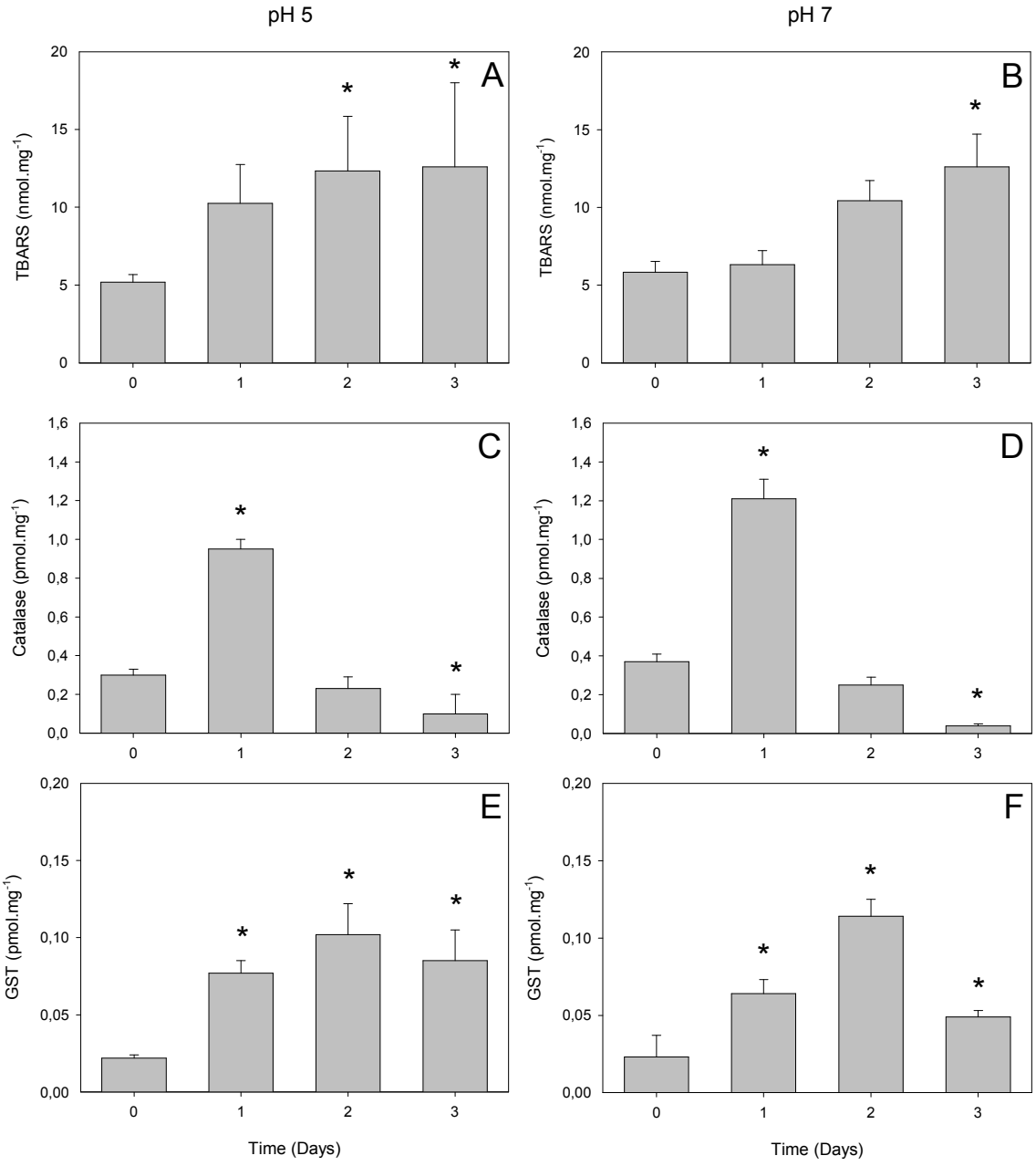

pH 5
pH 7
A
B
C
D
E
F
TBARS (nmol.mg-1)
Catalase (pmol.mg-1)
GST (pmol.mg-1)
Time (Days)
*

Figure 2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pH 7
A
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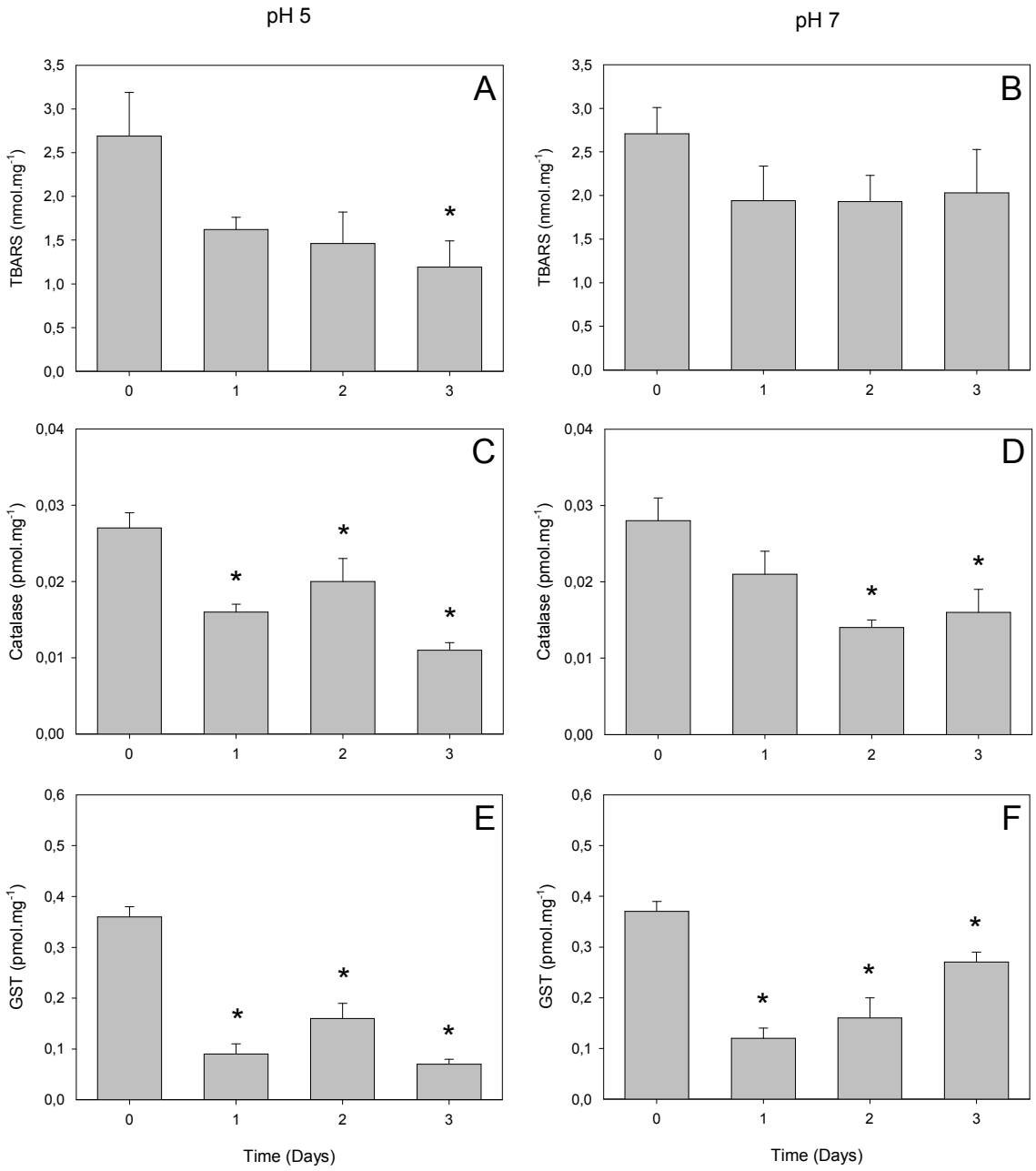
TBARS (nmol.mg-1)
Catalase (pmol.mg-1)
GST (pmol.mg-1)
Time (Days)
Time (Days)

Figure 3


pH 5
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TBARS (nmol.mg-1)
Catalase (pmol.mg-1)
GST (pmol.mg-1)
Time (Days)
Time (Days)

CONCLUSÕES

Verificamos durante a fase de experimentação deste trabalho se diferentes níveis de pH e durezas da água diminuem ou até mesmo eliminam os terontes, desempenhando um controle do *I. multifiliis*. Além disso, verificamos também em quais níveis de pH e durezas da água ocorrem os maiores índices de mortalidade cumulativa dos juvenis de jundiá infestados com o parasito. Com os resultados podemos constatar que a exposição a águas levemente ácidas, pH 5,0 e com dureza de 20 mg/L $CaCO_3$ podemos reduzir ambos, a mortalidade cumulativa e o número de manchas brancas na pele e nas brânquias de juvenis de jundiá, tornando a infecção pelo *I. multifiliis* menos severa. Também constamos que o aumento dos níveis de dureza da água em pH 5,0 aumenta os níveis de infecção e prejudicou a sobrevivência dos juvenis de jundiá, porém quando estes juvenis são submetidos ao pH 7,0 ocorre o inverso. Deste modo, quando juvenis infectados com este parasito estão em água com pH neutro é recomendável aumentar a dureza.

Neste trabalho também determinamos o fluxo líquido dos íons sódio, potássio e cloreto de juvenis de jundiá (assintomáticos ou infestados com *I. multifiliis*) em diferentes níveis de pH e durezas da água e relacionamos com a sobrevivência frente a esta infecção e podemos determinar que ocorrem perdas iônicas de Na^+, K^+ e Cl^- em alguns dias durante a infecção, mas as mesmas são reestabelecidas, e não se encontrou uma relação entre a mortalidade causada pelo protozoário ciliado *I. multifiliis* e a perda de íons, de modo que a mortalidade parece não ocorrer devido a um desbalanço ionorregulatório.

Determinamos também as alterações ocasionadas pela infecção do parasito na atividade das enzimas antioxidantes catalase (CAT) e glutationa-S-transferase (GST) no fígado, músculo e brânquias, assim como os níveis de lipoperoxidação (TBARS) e podemos concluir que a infecção pelo protozoário ciliado *I. multifiliis* em juvenis de jundiá induz danos no fígado e brânquias, via produtos da peroxidação lipídica, o mesmo não ocorrendo no músculo.

PERSPECTIVAS

O desenvolvimento de pesquisas sobre espécies de interesse comercial e nativas no Brasil é um fator determinante para o desenvolvimento da piscicultura no nosso país. Espécies como o jundiá tem despertado o interesse dos criadores no sul do Brasil (Paraná, Santa Catarina e Rio Grande do Sul) por ser uma espécie nativa, rústica e por apresentar bom desenvolvimento em cativeiro. Porém, um dos principais entraves a um melhor desenvolvimento desta espécie, quando cultivada em sistema intensivo, são as infecções e infestações por parasitos, principalmente pela espécie *Ichthyophthirius multifiliis*.

Nos ambientes naturais normalmente existe um grande número de patógenos na água, mas que não causam sintomas ou lesões nos peixes, devido a estes apresentam um estado nutricional e fisiológico ajustado ao ambiente, evitando a manifestação da doença. Quando, porém, ocorrem alterações ambientais devido aos mais variados fatores, com reflexo nos mecanismos de defesa dos peixes, imediatamente poderão elas levar esses animais a manifestarem os sintomas de certas enfermidades, já que passam a se tornar sujeitos a ação dos patógenos.

Outro fator que merece atenção especial na criação de peixes é a qualidade da água do ambiente de cultivo, pois é o ambiente ao qual eles estão diretamente em contato e sujeitos as suas modificações. Na água encontramos diversos microorganismos que convivem em equilíbrio com os peixes, porém devido a má conservações do ambiente (água), como a utilização de arraçoamento demasiado e a não correção dos parâmetros de qualidade da água, os peixes passam a apresentar modificações comportamentais e fisiológicas as quais irão gerar estresse nestes animais. Isto irá fazer com que estes fiquem expostos aos agentes infecciosos, devido a uma diminuição da atuação do seu sistema de defesa, deixando-os mais vulneráveis aos parasitos. Sendo assim, as características físico-químicas de qualidade da água são de fundamental importância para um maior desenvolvimento da piscicultura no Brasil. O pH, a dureza, a temperatura, a alcalinidade, o nitrito, a amônia e a quantidade de oxigênio dissolvido na água são fatores importantes e que muitas vezes não recebem a atenção devida nos sistemas de cultivo de peixes. Estes fatores quando não controlados geram estresse nos peixes e suas condições fisiológicas são afetadas de tal forma que o crescimento e a sua sanidade ficam prejudicadas.

As principais infestações pelo parasito *I. multifiliis* (ictio) ocorrem no início da primavera, outono e inverno com as variações bruscas de temperatura ambiente e em conseqüência na água dos tanques de cultivo. Um fato importante de se salientar é que os peixes que são infectados uma vez adquirem resistência à infecção, podendo estes serem infectados novamente mas de uma forma mais branda e por um período de tempo mais curto. Sendo assim, a infecção torna-se menos comum em peixes adultos, pois geralmente estes já passaram por uma infecção e sobreviveram, adquirindo defesa contra os parasitos. Devido a isto uma área de estudo bastante promissora e que está começando a despontar é a área de imunologia de peixes. Tratamentos realizados com base em pesquisas com vacinas tem se destacado nos últimos anos, visando a partir do parasito morto ou atenuado buscar uma solução para minimizar os impactos ocasionados por esta doença.

Outra área que tem apresentado bons resultados e que tem muito a ser explorada em futuros estudos é a de nutrição dos peixes cultivados. Estudos recentes, tem demonstrado, que a dieta influencia o comportamento, a integridade estrutural, a saúde, as funções fisiológicas, a reprodução e o crescimento dos peixes. Devido a isto é fundamental que as necessidades qualitativas e quantitativas dos nutrientes essenciais para determinada espécie sejam elucidadas para que todas estas influências sejam sanadas. Experimentos realizados com uma dieta, contendo adição de cerca de 1,0 % de sal (31 mmol/kg de Na^+ e 145 mmol/kg de Cl^-) nos mostrou que ocorre um aumento de peso e biomassa dos juvenis de jundiá em relação ao tratamento com níveis mais elevados (6 % de sal na ração) em um período de 30 dias.

Uma alimentação adequada evita vários problemas que acometem os peixes, fazendo com que o equilíbrio peixe-ambiente seja mantido. Por isso estudos mais detalhados sobre as diferentes espécies e em especial o jundiá e as espécies nativas em conjunto com as carpas (policultivo) devem ser realizados para que um balanço nutricional adequado renda maiores frutos ao criador, com menores custos. Estudos mais detalhados com aminoácidos essenciais, minerais, probióticos, vitaminas, lipídeos e proteínas ainda devem ser melhores explorados para que tal espécie mostre todo o seu potencial produtivo quando cultivado, pois em relação a parte nutricional os desafios são grandes e os resultados vão aparecendo aos poucos.

Fontes alternativas de tratamento para diferentes espécies de parasitas também estão sendo testadas, como a utilização de extratos de plantas, testes com espécies vegetais (mamão, arnica, alho, gincko biloba, entre outras) que atuam na cicatrização do tecido, na melhora do

sistema de defesa do organismo ou que tenham alguma atividade sobre a atuação dos organismos infecciosos que parasitam os animais na tentativa de minimizar a atuação dos parasitos nos peixes. Estas fontes alternativas nos dão uma boa base para futuras pesquisas a serem desenvolvidas para tentarmos controlar ou eliminar o parasito do ambiente de cultivo e nos deixam desafios a serem superados nos nossos futuros estudos. Comentar sobre a utilização de plantas na água e na ração.

Muitos trabalhos têm sido realizados em nosso laboratório (Laboratório de Fisiologia de Peixes da UFSM), no Setor de Piscicultura da UFSM, na Universidade de Passo Fundo, Fundação Universidade de Rio Grande e Universidade Federal de Santa Catarina e laboratórios de outras instituições, no intuito de melhorar as condições de qualidade da água nos ambientes de cultivo, aprimoramento das diversas frações da ração oferecida aos peixes, sistemas de criação em policultivo e ictioparasitologia de peixes, porém ainda devem ser realizadas mais pesquisas em todas as áreas mencionadas na tentativa de maximizar o crescimento e desenvolvimento dos peixes. Os testes dos diversos fatores relacionados ao desenvolvimento dos peixes muitas vezes são testados um a um, necessitando-se a realização de novos experimentos com a interação dos fatores já testados e que exercem uma melhora no cultivo das espécies, pois somente com a interação dos vários fatores é que ocorrerá o máximo rendimento dos animais no ambiente de cultivo, gerando assim mais lucro ao criador e uma demanda maior de peixes no mercado.

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