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ÁCIDO HÚMICO E HIPÓXIA MODERADA ALTERAM PARÂMETROS OXIDATIVOS E FISIOLÓGICOS EM DIFERENTES TECIDOS DE JUNDIÁS (*Rhamdia quelen)*

DISSERTAÇÃO DE MESTRADO

Ana Paula Konzen Riffel

Santa Maria, RS, Brasil,
2011

ÁCIDO HÚMICO E HIPÓXIA MODERADA ALTERAM PARÂMETROS OXIDATIVOS E FISIOLÓGICOS EM DIFERENTES TECIDOS DE JUNDIÁS (*Rhamdia quelen*)

Ana Paula Konzen Riffel

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Orientador: Prof. Dr. Bernardo Baldisserotto

Co-orientadora: Profª. Drª. Maria Amália Pavanato

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Aos meus familiares

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“A mente que se abre para uma nova idéia
jamais voltará ao seu tamanho original”.
(Albert Einstein)

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Farmacologia
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ÁCIDO HÚMICO E HIPÓXIA MODERADA ALTERAM PARÂMETROS OXIDATIVOS E FISIOLÓGICOS EM DIFERENTES TECIDOS DE JUNDIÁS (*Rhamdia quelen*)

Autora: Ana Paula Konzen Riffel
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Co-orientadora: Drª. Maria Amália Pavanato

Data e local da defesa: Santa Maria, 15 de dezembro de 2011.

O ambiente aquático apresenta diferentes níveis de oxigênio dissolvido (DO) e de substâncias húmicas (HS), as quais são compostos derivados da biomassa orgânica em decomposição. As HS são fracionadas em ácido húmico (HA), ácido fúlvico e humina. Este estudo investigou o efeito do HA e hipóxia em diferentes parâmetros bioquímicos e fisiológicos em jundiás (*Rhamdia quelen*). Os peixes foram expostos a diferentes níveis de HA, 0, 2,5 e 5 mg/L, por 120 h. Após esse período, cada grupo foi dividido em dois grupos, normóxia e hipóxia. A exposição aos diferentes níveis de DO ocorreu por 96 horas, totalizando 216 h de experimento. Ao final do período experimental, foram coletadas amostras de sangue e os peixes foram eutanasiados para coleta das brânquias e cérebro. Hematócrito, hemoglobina e íons plasmáticos foram dosados. Os parâmetros oxidativos, substâncias que reagem ao ácido tiobarbitúrico (TBARS), hidroperóxidos lipídicos (LOOH), catalase (CAT), superóxido dismutase (SOD), glutationa peroxidase (GPx) glutationa-S-transferase (GST) e grupos tióis não-protéicos também foram determinados. A fim de verificar a capacidade antioxidante, foram dosados os compostos fenólicos totais presentes no HA. Hematócrito, hemoglobina e níveis de íons plasmáticos aumentaram em normóxia. Em hipóxia, o K^+ aumentou enquanto outros parâmetros não foram afetados. No sangue, SOD, GST e grupos tióis aumentaram e a peroxidação lipídica (LPO) diminuiu em normóxia. Em hipóxia, a maioria dos parâmetros diminuiu, exceto a SOD. Nas brânquias, todos os parâmetros diminuíram em normóxia, enquanto a CAT aumentou em hipóxia. No encéfalo, LPO e GPx diminuíram em normóxia, enquanto LPO, SOD e GPx em hipóxia. Todas as alterações descritas ocorreram com o aumento da concentração de HA. A atenuação da LPO e outras modificações observadas nesta investigação podem ser atribuídas a presença de compostos fenólicos no HA e na depressão metabólica ocasionada pela hipóxia.

Palavras chave: Substâncias húmicas; níveis de oxigênio; estresse oxidativo.

// ABSTRACT

Dissertation of Master's Degree
Post-Graduating Program in Pharmacology
Federal University of Santa Maria

HUMIC ACID AND MODERATE HYPOXIA ALTER OXIDATIVE AND PHYSIOLOGICAL PARAMETERS IN DIFFERENT TISSUES OF SILVER CATFISH (*Rhamdia quelen*).

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Co-adviser: Drª. Maria Amália Pavanato

Date and place of defense: December 15, 2011, Santa Maria.

The aquatic environment presents different levels of dissolved oxygen (DO) and humic substances (HS), which are compounds derived from organic biomass in decomposition. The HS are composed of humic acid (HA), fulvic acids and humin. This study investigated the effect of HA and hypoxia on different biochemical and physiological parameters in silver catfish *Rhamdia quelen*. The fish were exposed to a different level of HA, 0, 2.5 and 5 mg/L, for 120 h. After this period, each group was subsequently divided into two groups, normoxia and hypoxia. Exposure to the different levels of dissolved oxygen lasted 96 h, totaling 216 h of experiment. At the end of the experimental period, blood sampling was perfomed and fish were euthanized prior to gills and brain excision. Hematocrit, hemoglobin and plasma ion levels were assessed. The oxidative parameters thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LOOH), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and non protein thiol groups were also determined. In order to verify the antioxidant capacity of HA, total phenolic compounds were measured. Hematocrit, hemoglobin and plasma ion levels increased in normoxia. In hypoxia, K^+ increased while the other parameters were unaffected. In blood, SOD, GST and thiol groups increased and lipid peroxidation (LPO) decreased in normoxia; in hypoxia, most parameters decreased, excepting for SOD. In gills, all parameters decreased in normoxia, while CAT increased in hypoxia. In brain, LPO and GPx decreased in normoxia, while LPO, SOD and GPx decreased in hypoxia. All of the described alterations occurred with increased concentrations of HA. The attenuation of LPO and the other changes observed in this investigation can be mainly attributed to the presence of phenolic compounds in HA and to the hypoxia-induced metabolic depression.

Keywords: humic substances; oxygen levels; oxidative stress.

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LISTA DE ABREVIATURAS E SIGLAS

ANOVA	Análise de variância
ATP	Adenosina trifosfato
CAT	Catalase
CDNB	1-cloro-2,4-dinitrobenzeno
DNA	Ácido desoxirribonucléico
DO	Oxigênio dissolvido
DOC	Carbono orgânico dissolvido
DOM	Matéria orgânica dissolvida
EROD	Etóxiresorufina-O-deetilase
GAE	Equivalentes de ácido gálico
GPx	Glutationa peroxidase
GR	Glutationa redutase
GSH	Glutationa reduzida
GSSG	Glutationa oxidada
GST	Glutationa-S-transferase
H_2O_2	Peróxido de hidrogênio
HA	Ácido húmico
$HClO_4$	Ácido perclórico
HIF-1	Fator induzido por hipóxia
HS	Substâncias húmicas
KCl	Cloreto de potássio
LPO	Peroxidação lipídica
LOOH	Hidroperóxidos lipídicos
MDA	Malondialdeído
NADPH	Nicotinamida adenina dinucleotídeo fosfato reduzido
NaOH	Hidróxido de sódio
NOM	Matéria orgânica natural
O_2	Oxigênio molecular
$O_2^{\bullet -}$	Radical ânion superóxido
$OH^{\bullet}$	Radical hidroxila

PBS	Tampão fosfato salino
PMSF	Fluoreto de fenilmetilsulfonil
ROS	Espécies reativas de oxigênio
SED	Erro padrão da diferença entre as médias
SOD	Superóxido dismutase
TBA	Ácido tiobarbitúrico
TBARS	Substâncias reativas ao ácido tiobarbitúrico
TCA	Ácido tricloroacético

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1 INTRODUÇÃO

O jundiá (*Rhamdia quelen*) é um peixe teleósteo de couro pertencente a família Heptapteridae, cuja distribuição vai do sudeste do México a região central da Argentina. Essa espécie possui hábitos noturnos, preferindo águas calmas (GOMES et al., 2000). Devido a sua distribuição e biologia, os jundiás enfrentam frequentemente na natureza baixos níveis de oxigênio dissolvido (DO). Os níveis de DO, podem influenciar a sobrevivência e o crescimento ds jundiás, uma vez que estudos realizados com esta espécie demonstraram modificações osmorregulatórias (ROSSO et al., 2006), metabólicas (BRAUN et al., 2006) e oxidativas (AZAMBUJA et al., 2011) em baixos níveis de DO.

O oxigênio (O_2), além de desempenhar funções vitais no metabolismo dos seres aeróbicos, também é potencialmente nocivo aos mesmos. Sua toxicidade ocorre através dos intermediários formados na redução parcial do O_2. Esses intermediários são espécies reativas de oxigênio (ROS) como o radical ânion superóxido ($O_2^{\bullet -}$), peróxido de hidrogênio (H_2O_2), e o radical hidroxil ($OH^{\bullet}$) (HALLIWELL e GUTTERIDGE, 2007). Essas ROS atacam os ácidos graxos poliinsaturados, ocasionando a lipoperoxidação (LPO) e prejudicando a integridade celular. No entanto, os organismos dispõem de um amplo sistema de defesa antioxidante, com componentes enzimáticos e não enzimáticos, que atuam interceptando as ROS (HALLIWELL e GUTTERIDGE, 2007). A avaliação de pró-oxidantes e antioxidantes em peixes como biomarcadores tem sido amplamente utilizada para estudos relacionados a respostas adaptativas, manifestações de toxicidade, ou perturbações no estado de óxido-redução (VAN DER OOST et al., 2003).

Os peixes e demais organismos aquáticos enfrentam variações nos níveis de OD diárias e sazonais. Reduções nos níveis de OD ocorrem devido a fenômenos naturais como o aumento da temperatura (BALDISSEROTTO, 2009) e o consumo de OD pelos seres aquáticos, principalmente, no período noturno (ARANA, 2010), no qual não ocorre fotossíntese, ou por ação antropogênica (SAMPAIO et al., 2008).

A hipóxia ambiental é caracterizada quando os níveis de OD são menores que 2,8 mg/L (DIAZ e ROSEMBERG, 1995). Essa situação provoca alterações fisiológicas nos peixes, como diminuição do crescimento, aumento do volume dos eritrócitos, aumento da concentração da hemoglobina e da afinidade desta com o O_2. O aumento das taxas de ventilação branquial, diminuição da LPO, aumento da eficiência energética e por fim a utilização de vias anaeróbicas também podem ser observados em situações hipóxicas. A

maioria dessas modificações está relacionada a alterações na expressão de fatores de transcrição, como o fator induzível a hipóxia (HIF-1), sendo este também influenciado também pelas reações de óxido-redução (NIKINMAA, 2002).

Além dos níveis de OD, outro fator importante para a biota aquática é a concentração de substâncias húmicas (HS). As HS são compostos derivados de biomassa em decomposição e são encontrados no meio aquático, solo e no ar na forma de partículas de poeira. Sua composição química exata depende da fonte de degradação da matéria orgânica, sendo as maiores frações constituídas de carbono, O_2 e nitrogênio (ROCHA et al., 2000). As substâncias húmicas também podem ser separadas em ácidos húmicos (HA), ácidos fúlvicos e humina. No ambiente aquático, a concentração HS é variável, podendo chegar a 100 mg/L (STEINBERG, 2003a). A maior fração de HS é constituída de HA, macromoléculas orgânicas que possuem vários grupamentos funcionais, como grupos carboxílicos, cetonas, quinonas e fenólicos (STEVENSON, 1994; CHENG et al., 1999; FIORENTINO, 2006). Os compostos fenólicos conferem ao HA atividade antioxidante, suprimindo reações que geram ROS e inibindo a LPO (MICHALAK, 2003).

Considerando que não existem estudos relacionados a ação de HA em diferentes níveis de OD, este trabalho buscou investigar o efeito desses fatores no hematócrito, hemoglobina, íons plasmáticos e parâmetros oxidativos em jundiás, a fim de contribuir para o conhecimento das estratégias utilizadas pela espécie frente a esses fatores. Além disso, devido aos efeitos do HA previamente descritos, este trabalho poderá contribuir com informações sobre a presença de HA em viveiros com o intuito de trazer benefícios ao bem-estar dos animais, possibilitando menores perdas aos produtores.

‘

2 REVISÃO BIBLIOGRÁFICA

2.1 *Rhamdia quelen*

O *Rhamdia quelen*, popularmente conhecido como jundiá, é um peixe teleósteo da família Heptapteridae e apresenta distribuição neotropical, do sudeste do México até a região central da Argentina (SILFVERGRIP, 1996; GOMES et al., 2000) (Figura 1). Ele possui hábitos noturnos, preferindo águas calmas com fundo de areia e lama em lagos e poços fundos de rios, escondendo-se durante o dia entre pedras e troncos (GUEDES, 1980; GOMES et al., 2000).

Figura 1 - Exemplares de jundiá, *Rhamdia quelen* (Heptapteridae).
Adaptado de: BALDISSEROTTO, 2004.

O jundiá é um peixe de couro, onívoro, com coloração que varia de marrom-avermelhado à cinza, cuja intensidade pode ser alterada conforme o local que habita. Geralmente, as fêmeas têm maior sobrevida e são maiores que os machos, que apresentam comprimento máximo de 66 cm e 52 cm, respectivamente (GOMES et al., 2000). Além disso, a faixa ideal de pH para o seu crescimento varia entre 8,0 e 8,5 (LOPES et al., 2001) e a temperatura adequada é 24ºC (GARCIA et al., 2008).

Essa é uma espécie amplamente utilizada na piscicultura, onde a manutenção da qualidade da água é de fundamental importância para sobrevivência, crescimento e reprodução. Quando submetido a alterações bruscas nos níveis de OD, temperatura, pH,

matéria orgânica dissolvida, dureza, alcalinidade e resíduos nitrogenados, o jundiá pode ter variações em seu metabolismo, o que constituem desvios da homeostase. Ou seja, situações de estresse podem causar uma demanda energética ainda maior que a sustentável pelo metabolismo aeróbio (OBA et al., 2009).

2.2 Balanço oxidativo

Na atmosfera primitiva, que foi composta basicamente de hidrogênio, metano, dióxido de carbono e amônia, a vida era limitada à utilização de vias anaeróbicas (MARCON, 1997). Posteriormente, os primeiros organismos fotossintetizantes, as cianobactérias, introduziram gradativamente o O_2 na atmosfera, possibilitando o desenvolvimento de organismos aeróbicos com estruturas corpóreas maiores e mais complexas (FRIDOVICH, 1974), como os vertebrados, que se acredita que tenham sido originados primeiramente no ambiente aquático (GRIFFITH, 1987; NIKINMAA, 2002). A divergência dos peixes parece ter ocorrido em torno de 500 milhões de anos atrás, período no qual o ambiente passou por grandes variações nos níveis de O_2, causadas principalmente por modificações na temperatura ambiental. Sendo assim, o O_2 passou a desempenhar um papel fundamental na evolução e sobrevivência dos peixes (NIKINMAA, 2002). Entretanto, o mesmo elemento essencial à sobrevivência apresentou, igualmente, consequências deletérias aos organismos expostos a ele através do aumento da geração das ROS (HALLIWELL e GUTTERIDGE, 2007).

As ROS também podem ser formadas sob condições fisiológicas, principalmente durante os processos de oxidação biológica, como a respiração celular acoplada à fosforilação oxidativa, para formação de ATP na mitocôndria (HALLIWELL e GUTTERIDGE, 2007). A formação dessas espécies ocorre em aproximadamente 5% de todo o processo de redução do O_2 até água. Dentre esses compostos intermediários estão o $O_2^{\bullet -}$, o H_2O_2 e o $OH^{\bullet}$, os quais podem ser espécies radicalares ou não-radicalares. Quando as ROS apresentam pelo menos um elétron desemparelhado são denominadas radicais livres, que são definidos como qualquer espécie capaz de existir de forma independente e que contenha um ou mais elétrons não-pareados no seu orbital mais externo, característica que lhe confere alta reatividade (HALLIWELL e GUTTERIDGE, 2007).

O $O_2^{\bullet-}$ é a primeira ROS a ser formada através da redução monovalente do O_2 a água, e a partir dele serão geradas as demais ROS através de reações sequenciais (PAVANATO e LLESUY, 2008) (Figura 2). O H_2O_2 é o segundo intermediário gerado nesse processo. Ele é uma espécie reativa não-radicalar, citotóxica e pode facilmente se difundir entre as células vivas através das aquaporinas, podendo gerar o $OH^{\bullet}$ através da reação de Haber-Weiss catalisada por íons metálicos, e da reação de Fenton, reação do H_2O_2 com ferro ou cobre (FRIDOVICH, 1974). O $OH^{\bullet}$ é um dos mais potentes oxidantes em sistemas biológicos, com curta meia vida, podendo atravessar membranas e reagir com biomoléculas como lipídios, proteínas e DNA (HALLIWELL e GUTERIDGE, 2007).

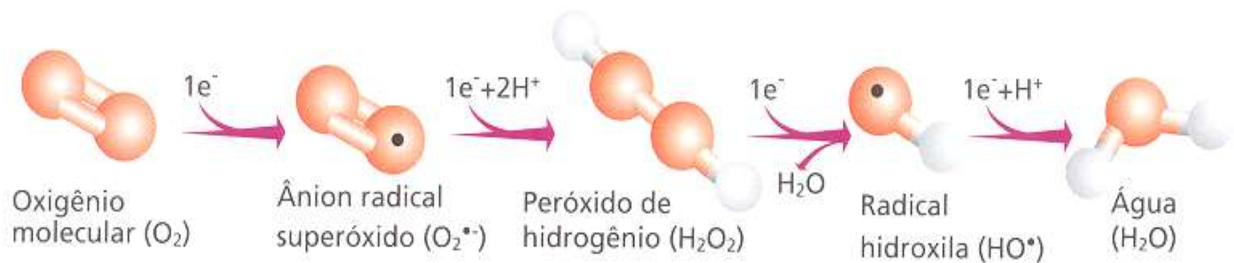

Figura 2 - Formação das ROS a partir da redução parcial do oxigênio.
Fonte: OHARA (2006).

A reação das ROS com os ácidos graxos poliinsaturados da membrana celular leva a LPO, podendo resultar na destruição de membranas celulares, falência dos mecanismos de trocas de metabólitos (canais iônicos) e receptores, e em condições extremas a morte celular (BENZIE, 1996). Os animais aquáticos se caracterizam por apresentar grande quantidade de ácidos graxos poliinsaturados, que podem ser atacados por ROS, levando a perda de função celular, sendo, por isso, as medidas de LPO muito significativas em peixes (STOREY, 1996; LI et al., 2010).

Uma diversidade de defesas antioxidantes foi desenvolvida nos organismos aeróbicos para atenuar as consequências da toxicidade causada pelo O_2 (MARTÍNEZ-ÁLVAREZ et al., 2005). Sendo assim, um antioxidante é considerado como qualquer substância que retarda ou impede significativamente a oxidação do substrato. Os antioxidantes atuam prevenindo a formação das ROS, impedindo ou interrompendo as reações que geram radicais livres, reparando os danos em biomoléculas danificadas e quelando metais que catalisam a formação de ROS (HALLIWELL e GUTTERIDGE, 2007). Os peixes, de modo geral, possuem menores níveis de defesas antioxidantes em relação aos pássaros e mamíferos, além de

apresentarem diferenças de acordo com as características da espécie (WILHELM FILHO, 2007).

O sistema de defesa antioxidante é composto por antioxidantes enzimáticos e não-enzimáticos, que atuam conjuntamente na proteção celular (HALLIWELL e GUTTERIDGE, 2007). O sistema antioxidante enzimático opera prevenindo a cascata de reações de oxidação, interceptando ou inativando intermediários de ROS, fechando o ciclo de LPO e é de fundamental importância no esforço de conter a toxicidade do O_2 quando os níveis de outros componentes antioxidantes estão escassos (MARTÍNEZ-ÁLVAREZ et al., 2005). Dentre as enzimas que compõem esse sistema, a superóxido dismutase (SOD), a catalase (CAT), a glutationa peroxidase (GPx) e a glutationa redutase (GR) recebem destaque, uma vez que constituem a primeira linha de defesa antioxidante, por evitarem o acúmulo do $O_2^{\bullet -}$ e do H_2O_2, atenuando assim a formação das demais ROS.

O sistema antioxidante não-enzimático, por sua vez, atua principalmente pela supressão, eliminação ou desativação das ROS. Ele é constituído por moléculas hidrossolúveis, como a GSH e o ácido ascórbico; e lipossolúveis, dentre os quais estão o α-tocoferol. Níveis elevados de antioxidantes não-enzimáticos têm sido detectados em invertebrados marinhos e peixes (MARTÍNEZ-ÁLVAREZ et al., 2005).

O desequilíbrio entre os antioxidantes e ROS é denominado estresse oxidativo e ocorre devido ao aumento na velocidade de geração de ROS e/ou diminuição na atividade do sistema de defesa antioxidante, resultando em aumento sustentado das concentrações em estado estacionário de ROS (SIES, 1991). Nos peixes existem inúmeras situações que induzem ao estresse oxidativo, dentre elas, repetidos episódios de alterações agudas ou crônicas na concentração de DO (BRAUN et al., 2006). Essas situações, além de gerar estresse oxidativo, podem afetar a produtividade dos viveiros e a aquicultura (ARANA, 2010).

2.3 Níveis de oxigênio

Em ambientes aquáticos, a quantidade de DO depende da pressão parcial de O_2 da atmosfera. Considerando que a pressão parcial do O_2 na água é menor do que a pressão parcial atmosférica, há uma menor disponibilidade de DO na água (BALDISSEROTTO, 2009). Assim, as taxas de difusão do OD na água são cerca de 10 000 vezes menores do que

no ar; consequentemente, qualquer consumo de DO, por processos biológicos ou não, pode rapidamente diminuir as taxas do mesmo (NIKINMAA e REES, 2005).

A diminuição desses níveis de DO são os maiores limitantes da aquicultura (ARANA, 2010) e podem ser ocasionados naturalmente (Figura 3) por altas temperaturas associadas a decomposição da matéria orgânica (SAMPAIO, et al., 2008), pela diminuição das taxas fotossintéticas das algas, ou por fatores antropogênicos, como a adição de nutrientes em corpos aquáticos (DIAZ e ROSEMBERG, 1995; WU, 1999; SAMPAIO, et al., 2008).

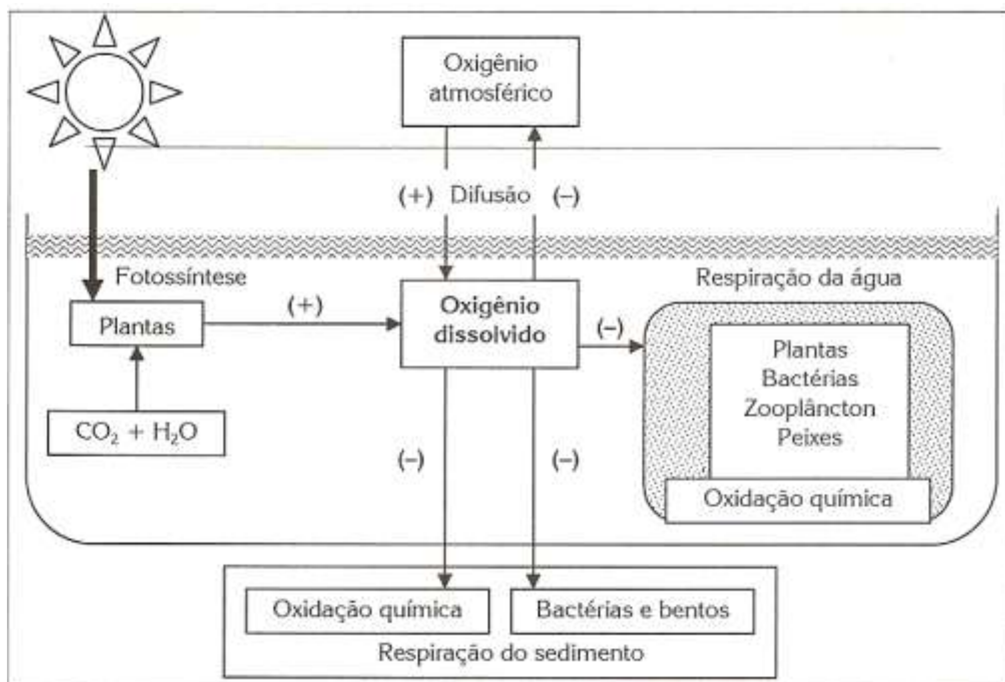

Figura 3 - Principais ganhos e perdas de oxigênio em viveiros.
Fonte: FAST e BODY 1992; ARANA 2010.

Os níveis de DO ideais requeridos pelos peixes aproximam-se de 6 mg/L (BOYD e TUCKER, 1992; BALDISSEROTTO, 2009). Níveis de DO abaixo de 2,8 mg/L caracterizam a hipóxia ambiental (DIAZ e ROSEMBERG, 1995), a qual interfere na biologia dos seres aquáticos. Em situações hipóxicas, os peixes podem aumentar as taxas de ventilação, a fim de aumentar o fluxo de água sobre as brânquias (PERRY, 2011), bem como regular a área funcional das brânquias para obter melhor desempenho. O aumento da concentração de hemoglobina e do número e volume dos eritrócitos também são respostas comuns à hipóxia

(JENSEN e WEBER, 1982; NIKINMAA, 2005), a qual envolve a ativação da eritropoetina (NIKINMAA, 2005).

Estudos realizados com jundiás demonstraram que os mesmos são tolerantes à hipóxia e que a concentração letal de DO para essa espécie é de 0,52 mg/L (BRAUN et al., 2006). BRAUN et al. (2008) também demonstraram que os jundiás em hipóxia (1,96±0,08 mg/L) aumentaram os níveis de LPO e da SOD em fígado e músculo. Por outro lado, jundiás transportados em bolsas plásticas por 7 horas, as quais apresentaram concentração final de 2,29±0,36 mg/L de DO apresentaram uma diminuição da LPO no fígado acompanhada da diminuição da concentração da CAT e da atividade das enzimas GST e SOD. Nas brânquias, houve um aumento da atividade da SOD, enquanto a LPO manteve-se estável. No cérebro, os níveis de LPO aumentaram sem alteração das enzimas antioxidantes, sugerindo que cada tecido responde de forma diferente à hipóxia (AZAMBUJA et al., 2011).

A transferência de jundiás de ambientes normóxicos para ambientes hipóxicos também altera a osmorregulação do animal. A exposição de jundiás a 2,5 mg/L de DO provocaram perda iônica, a qual é estabilizada em relação a Na^+ e Cl^- em 120 horas (ROSSO et al., 2006). Baixos níveis de DO também aumentaram os níveis iônicos em plasma e vesícula biliar de juvenis de *Rhamdia quelen* após 24 horas de exposição (BECKER et al., 2009).

Além disso, a hipóxia também exerce influência nas características ecológicas desses animais aquáticos, podendo levar à mortalidade em massa, principalmente em ambientes marinhos, assim como diminuir a diversidade e riqueza das espécies (WU, 2002). O desenvolvimento dos peixes pode ser afetado por inúmeros fatores, dentre eles os níveis de DO. Considerando que a piscicultura busca alta produtividade aliada ao baixo custo, a utilização de substâncias como a substância húmica (HS), em especial, o ácido húmico (HA), poderia ser uma alternativa aos produtores que buscam alcançar maior rentabilidade do processo produtivo.

2.4 Substâncias Húmicas

As HS compreendem um grupo de compostos orgânicos complexos derivados de biomassa em decomposição (ROCHA et al., 2000). São assim denominadas por serem produtos do processo de humificação, atuando na estabilidade do ciclo global de energia e

sendo encontrados em ambientes aquáticos, no solo e até mesmo no ar na forma de partículas de poeira (STEINBERG et al. 2003b). Na literatura encontram-se vários sinônimos ou termos intrinsecamente ligados ao HS, como: carbono orgânico dissolvido (DOC), matéria orgânica natural (NOM) e matéria orgânica dissolvida (DOM). As HS representam até 80% do carbono orgânico em água doce (STEINBERG et al., 2008).

No ecossistema aquático, as HS são partículas inferiores a 450 nm de diâmetro, cuja composição aproximada é de 50 a 60% carbono, 35 a 40% de O_2, 4 a 5% de hidrogênio, 1 a 2% de nitrogênio, e menos que 1% de enxofre e fósforo (WOOD, 1996). Essa constituição química depende da fonte de degradação dos materiais orgânicos, não sendo possível dessa forma determinar sua estrutura molecular específica (BITTNER, 2006). As substâncias húmicas também podem ser separadas em ácidos húmicos (HA), ácidos fúlvicos e humina (ROBARDS et al., 2004).

Em ambientes de água doce, normalmente a concentração de HS varia de 0,5 a 50 mg/L, podendo em alguns casos chegar a 100 mg/L (STEINBERG, 2003a). De acordo com ROBARDS et al. (2004), a concentração de HS pode alterar a cadeia alimentar, turbidez, pH e concentração de íons da água.

Até pouco tempo, as HS eram consideradas inertes no meio ambiente, mas hoje sabe-se que as mesmas podem atuar diretamente sobre os organismos vivos. O principal interesse em estudar as HS deve-se a sua capacidade de formar complexos alterando a toxicidade de metais e poluentes. Em um estudo em cladóceros *Ceriodaphnia dúbia*, KIM et al. (1999) demonstraram que houve uma diminuição na toxicidade e biodisponibilidade do cobre, conforme o aumento da concentração de DOM. JONES e HUANG (2003) também demonstraram que as HS diminuem a toxicidade do arsênio. Assim, as HS constituem importantes ligantes naturais.

Além dos efeitos químicos como quelação e complexação de elementos, os efeitos biológicos como a absorção das HS passaram também a ser observados. A absorção e interação do HS com as membranas lipídicas foram demonstradas em algas *Ceratophyllum demersum*, no crustáceo *Gammarus pulex* e no anfíbio *Rana arvalis* (STEINBERG, 2003b). Em *Gammarus lacustris* e *G. tigrinus* expostos a NOM não houve diferença nos níveis de LPO em relação ao controle nas primeiras horas de exposição. No entanto, após 6 dias foi observado aumento significativo na LPO, aliado ao aumento das enzimas CAT e GST (TIMOFEYEV et al., 2006).

As HS também demonstraram efeitos contra bactérias e patógenos através da ação bacteriostática (GRYNDLER et al., 2005) ou através da liberação de ROS sobre iluminação.

Um estudo de PAUL et al. (2004) demonstrou que a fotólise de HS aquáticas leva à formação de uma complexa mistura de substâncias reativas, como o $O_2^{\bullet -}$, $OH^{\bullet}$, H_2O_2, que poderiam ser absorvidas pelos organismos aquáticos.

Devido à variedade de grupos funcionais, as HS dissolvidas têm o potencial de interferir em diversas estruturas e vias bioquímicas em organismos aquáticos (STEINBERG et al., 2008). Dentre as frações do HS, o HA é o mais abundante, e, portanto, merece destaque em relação aos demais (STEVENSON, 1994).

2.4.1 Ácidos húmicos

Os HA são macromoléculas orgânicas encontradas no solo, sedimentos e água (AESCHBACHER et al., 2010) e consistem de uma complexa mistura de polímeros com estruturas aromáticas, geralmente possuindo grupos carboxílicos, cetonas, quinonas, terpenos e fenólicos (STEVENSON, 1994; CHENG et al., 1999; FIORENTINO, 2006).

Os compostos fenólicos são produtos secundários do metabolismo de vegetais, tendo como função na planta a proteção contra condições adversas, através de sua atividade antioxidante (NACKZ e SHAHIDI, 2004). Esta atividade antioxidante é atribuída à tendência dos compostos fenólicos em quelar metais, suprimindo reações que geram radicais livres, e também por inibir a LPO através do aprisionamento dos radicais alcoxil que, geralmente, são os iniciadores da LPO. Os compostos fenólicos também atuam estabilizando e protegendo a bicamada lipídica do ataque e difusão dos radicais livres, diminuindo também a fluidez da mesma (MICHALAK, 2003).

Estudos da ação do HA e parâmetros oxidativos em peixes são escassos. Em bagres africanos (*Clarias gariepinus*), ADEKUNLE e AJUWON (2010) observaram que não houve alteração nos níveis de malondialdeído (MDA) nas concentrações de 100 e 250 mg/L de HA, enquanto que em concentrações mais elevadas (500 e 1000 mg/L) houve aumento nos níveis de LPO. De acordo com HALLIWELL et al. (1995), em determinadas circunstâncias um antioxidante pode proteger o organismo, enquanto em outras situações, como frente ao aumento demasiado na concentração, o antioxidante pode, pelo contrário, não exercer efeito protetor e inclusive, causar dano ao organismo.

Em um trabalho realizado com trutas arco-íris (*Oncorhynchus mykiss*) expostas por 30 dias a 7 µg/L de cobre e 7,5 mg/L de HA, houve diminuição da acumulação tecidual e alteração osmorregulatória provocada por este metal, indicando a atuação do HA como quelante e diminuindo a biodisponibilidade de metais (MCGEER et al., 2002).

Outros efeitos relacionados ao HA sobre os organismos estão sumarizados na tabela 1:

Tabela 1 - Efeitos do HA sobre diferentes fatores fisiológicos e bioquímicos.

Fator	Papel do HA
Feromônios	Redução da capacidade do douradinho (*Carassius auratus*) de detectar feromônios na água, diminuindo a comunicação química nessa espécie (HUBBARD et al., 2002).
Hormônio gonadotrófico	Inativação do hormônio em rã comum (*Rana temporária)*, diminuindo a estimulação da maturação do oócito (ZENKEVICS et al., 2005).
Toxicidade	Redução da toxicidade do alumínio no molusco *Lymnaea stagnalis* (DOBRANSKYTE et al., 2006); Além disso, o HA combinado com dióxido de titânio reduz as taxas de absorção de cádmio em zebrafish (*Danio rerio*) (HU et al., 2011).
EROD (Etóxiresorufina-O-deetilase)	Aumento da EROD hepática e branquial no peixe esgana-gata (*Gasterosteus aculeatus*) (ANDERSSON et al., 2011).

De acordo com SACHS e BERNHARD (2011) o uso de HA, preferencialmente purificado ou sintético, contribui para uma avaliação mais confiável das ações e interações desse composto sobre os organismos vivos.

Considerando a relevância do HA e do DO na ecofisiologia aquática e na aquicultura, este trabalho se justifica pela ausência de dados referentes a esses fatores, uma vez que os peixes constituem excelentes biomarcadores da qualidade da água. Devido ao fato do jundiá ser uma espécie amplamente distribuída, com grande potencial de adaptação e importante valor comercial, aumenta a necessidade de conhecer seus mecanismos fisiológicos de

adaptação às condições extremas presentes no habitat. Por outro lado, a ocorrência de situações hipóxicas são comuns nos ambientes, bem a como a presença de HS em diferentes concentrações. Além disso, estudos relativos a ação do HA em diferentes níveis de DO sobre parâmetros oxidativos e fisiológicos são inexistentes.

‘

3 OBJETIVOS

3.1 Objetivo geral

Avaliar os parâmetros fisiólogicos e oxidativos em jundiás frente a distintas concentrações de DO e HA.

3.2 Objetivos específicos

Verificar o efeito do HA e DO sobre o hematócrito e hemoglobina em jundiás;

Avaliar os níveis de íons plasmáticos em jundiás expostos a diferentes concentrações de HA e DO;

Determinar os níveis de LPO através da medida de TBARS e de LOOH em plasma, brânquias e cérebros de jundiás expostos a diferentes concentrações de HA e DO;

Analisar a atividade das enzimas antioxidantes SOD, CAT, GPx e GST nos eritrócitos, brânquias e cérebros dos animais dos diferentes grupos experimentais;

Analisar o conteúdo de grupos tióis não-protéicos em eritrócitos de jundiás submetidos aos diferentes grupos experimentais.

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4 MANUSCRITO

O manuscrito está disposto conforme as especificações requisitadas pela revista *Chemico-Biological Interactions*, ao qual foi submetido para publicação.

Humic acid and moderate hypoxia alter oxidative and physiological parameters in different tissues of silver catfish (*Rhamdia quelen*).

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Footnotes-----------------------------------

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Abstract

This study investigated the effect of humic acid (HA) and hypoxia on different biochemical and physiological parameters in silver catfish *Rhamdia quelen*. The fish were exposed to different level of HA (0, 2.5 and 5 mg L^{-1}) for 120 h. After this period, each group was subsequently divided into two groups, normoxia and hypoxia. Exposure to the different dissolved oxygen levels lasted 96 h, totaling 216 h of experiment. At the end of the experimental period, blood sampling was perfomed and fish were euthanized prior to gills and brain excision. Hematocrit, hemoglobin and plasma ion levels were assessed. The oxidative parameters thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LOOH), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and non-protein thiol groups were also determined. In order to verify the antioxidant capacity of HA, total phenolic compounds were measured. Hematocrit, hemoglobin and plasma ion levels increased in normoxia. In hypoxia, K^+ increased while the other parameters did not show regression relation. In blood, SOD, GST and thiol groups increased and lipid peroxidation (LPO) decreased in normoxia; in hypoxia, most parameters decreased, excepting SOD. In gills, all parameters decreased in normoxia, while CAT increased in hypoxia. In brain, LPO and GPx decreased in normoxia, while LPO, SOD and GPx decreased in hypoxia. All of the described alterations occurred with increased concentrations of HA. The attenuation of LPO and the other changes observed in this investigation can be mainly attributed to the presence of phenolic compounds in HA and to the hypoxia-induced metabolic depression.

Keywords: humic substances; oxygen levels; oxidative stress.

1. Introduction

The humic substances (HS), organic compounds derived from biomass in decomposition, constitute the largest fraction of dissolved organic carbon found in aquatic ecosystems [1]. The HS can be separated into humic acids (HA), fulvic acids and humin [2]. The HA are compounds of high molecular weight, consisting of polymeric structures with many functional groups such as carboxylic and phenolic groups in greater quantity, and smaller contributions of ketones, amines and alkoxyl [3]. The interest in studying the HS and their compounds is due to their ability to modulate the toxicity of pollutants and xenobiotics [4], to act as chelator and reduce the bioavaliability of metals [5] and to alter the food chain and other factors such as turbidity, pH and ionic concentration [2].

Aquatic organisms suffer physiological, biochemical and behavioral changes according to the physicochemical characteristics of their environment. The beneficial or adverse effect of HS on aquatic organisms has been extensively discussed, as well as whether HA is taken up by organisms that come in contact with it [6]. Studies in zebrafish embryos (*Danio rerio*) indicate that HS can exert positive or negative effects according to their concentration, source of origin and physiological conditions of the animal [7]. Other studies indicate that HS can modulate enzymatic activity, such as GST [4], as well as act on the electron transport chain of freshwater plants and cyanobacteria, decreasing photosynthetic rates and consequently the release of oxygen in the aquatic environment [8].

The dissolved oxygen levels are a limiting factor to fish and other aquatic organisms. Besides the decline in photosynthetic rates and the decomposition of organic matter, low oxygen levels can be caused by anthropogenic factors such as addition of nutrients and pollutants [9], or by natural factors such as high temperatures and vertical stratification [10].

Hypoxia in water bodies is defined as dissolved oxygen levels lower than 2.8 mg L^{-1} [11]. Hypoxia induces several physiological alterations in aquatic organisms, as increases in

gill ventilation rate and energetic efficiency in metabolic processes, and reduction in growth rate and food conversion [10]. In addition, variations in dissolved oxygen levels are directly related to reactive oxygen species (ROS), wich are reactive intermediates in partial oxygen reduction such as superoxide anion, hydrogen peroxide and hydroxyl radicals [12]. These subproduts are generated in all aerobic cells by normal breathing and are toxic to these cells at high levels, leading to the oxidation of lipids, proteins and DNA, as well as mutagenesis and cell death [13]. At low levels, they perform important physiological roles such as activation and modulation of signaling pathways and activity of transcription factor sensitive to oxidation-reduction reactions [14].

Aerobic organisms have developed an antioxidant defense system in order to combat the deleterious effects caused by ROS, firstly composed of non-enzymatic mechanisms such as non-protein thiol groups, α-tocopherol and ascorbic acid. Subsequently, it evolved into an antioxidant defense enzyme system comprised of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST). These enzymes are located in different cellular compartments and are widely used as biomarkers of changes in environmental conditions due to their sensivity to various factors [15].

Oxidation-reduction reactions also act as sensors to activation of genes in hypoxia through hypoxia inducible factor 1 (HIF-1), a heterodimeric transcription factor constructed from subunits α and β, the former being related to oxygen concentration [16]. In order to meet the demand of oxygen caused by hypoxia, HIF-1 is responsible for numerous important physiological functions in fish [17], such as gene activation of erythropoietin, glicolitic enzymes and glucose transporters [14].

The silver catfish *Rhamdia quelen* is a species of Neotropical distribution. It occurs from southeastern Mexico to southern Argentina and prefers calm waters along the shores and

vegetation. Lethal concentration of dissolved oxygen levels for this species is 0.52 mg L^{-1} [18], what makes of it an excellent model for studying variations in oxygen levels. Due to its wide distribution, it is also suitable for assessments involving HA. Oxidative and osmorregulation alterations have been verified in the species under hypoxic conditions [19, 20].

There are no studies on the impact of HA and dissolved oxygen levels upon biochemical and physiological parameters in fish. Thus, this study was aimed at evaluating the effects of these factors on certain parameters in various tissues of silver catfish.

2. Material and methods

2.1 Reagents

Humic acid - HA (CAT: H1, 675-2 Aldrich), which corresponds to 44% of dissolved organic carbon[21], was purchased from Sigma Aldrich. Phenylmethylsulfonyl fluoride (PMSF), 1-chloro-2,4-dinitrobenzene (CDNB), L-glutathione reduced (GSH), epinephrine and glycine were acquired from Sigma Chemical Co. (USA). Hydrogen peroxide, trichloroacetic acid (TCA), thiobarbituric acid (TBA) and albumin were obtained from Merck. All other reagents were of analytical grade.

2.2 Determination of total phenolic compounds

The determination of phenolic compounds in HA is an indirect measure of the antioxidant capacity of these compounds. The determination of phenolic compounds was performed through HA extracts. Extractions were performed by using the method described

by Pérez-Jiménez and Saura-Calixto [22]. For chemical extraction, 1 g HA was placed in a test tube and 20 mL of acidic methanol/water (pH 2.0) was added. The tube was thoroughly shaken at room temperature for 1 h. The tube was centrifuged at 2500 xg for 10 min, the supernatant was recovered, 20 mL of acetone/water was added to the residue and shaking and centrifugation were repeated. Both methanolic and acetonic extracts were combined. This procedure was conducted in triplicate.

The total phenolic compounds were determined according to the Folin-Ciocalteau procedure. The reaction medium contained diluted HA extracts, 0.2 N Folin-Ciocalteau reagent and satured sodium carbonate (75 g L^{-1}). Mixtures were kept in the dark at ambient conditions for 2 h to complete the reaction. The absorbance of the resulting blue color was then measured at 765 nm. Galic acid was used as a standard and the results are expressed as galic acid equivalents (mg GAE) g HA^{-1}. The reaction was conducted in triplicate.

2.3 Experimental protocol

The fish (143.90±11.76 g, 25.12±0.62 cm) were acclimated to laboratory conditions for 21 days in 250 L tanks under controlled temperature (23±1.0 ºC) and constant aeration (6.54±0.26 mg L^{-1} O_2). The fish were then divided into three groups and exposed to different concentrations of HA, 0, 2.5 and 5 mg L^{-1}, for 216 h (9 days). At the 120th h, each group was further divided into two groups, normoxia (6.72±0.10 mg L^{-1}), and hypoxia (2.09±0.24 mg L^{-1}), and with three replicates each (n=8). The group exposed to 0 mg.L^{-1} HA and normoxia was considered the control. Decreased aeration and fish oxygen consumption provided reduction in the levels of oxygen, with hypoxic levels being reached in 6 h. The experiment was performed in 250 L tanks.

Dissolved oxygen levels were controlled by increasing or decreasing aeration. Feeding was performed daily with commercial feed, ceasing at the final 96 h of experiment. Siphoning

and water exchange was also performed daily, followed by dissolved oxygen levels verification.

2.4 Water parameters

Dissolved oxygen levels and temperature were measured with an YSI oxygen meter (Model Y5512; YSI Inc., Yellow Springs, OH, USA). The pH was verified with DMPH-2 pH meter (Digimed, São Paulo, SP, Brazil). Nesslerization verified total ammonia nitrogen (TAN) levels according to the method of Eaton et al. [23]. Un-ionized ammonia (NH_3) levels were calculated according to Colt [24]. Water hardness was analyzed by the EDTA titrimetric method. Alkalinity was determined according to Boyd and Tucker [25].

2.5 Tissue collection

At the end of the experimental period, animals were submitted to blood sampling via caudal puncture. The fish were then euthanized by sectioning the spinal cord and gills and brain dissected out and frozen pending determination of oxidative stress parameters.

The blood was aliquoted for measurement of hematocrit and hemoglobin concentration. The remainder was centrifuged in heparinized vials at 1110 xg for 5 min and plasma separated for determination of plasma ion levels, lipid hydroperoxide (LOOH) and thiobarbituric acid reactive substances (TBARS). The fraction containing the red cells were again separated into two aliquots: one for enzymatic dosages, to which 4 mM magnesium sulfate and 1 mM acetic acid were added, and the other for determination of non-protein thiol groups, to which 20% trichloroacetic acid (TCA) was added. Both aliquots were centrifuged at 1110 xg for 5 min, the supernatant separated and then frozen.

The gills and brain were homogenized in a medium containing 120 mM potassium chloride (KCl) and 30 mM buffer phosphate (pH 7.4) with 1 mM phenylmethylsulfonyl fluoride (PMSF). Samples were centrifuged at 1110 xg in a refrigerated centrifuge for 20 min and supernatants separated for oxidative analyses [26].

2.6 Hematocrit and hemoglobin

The determination of hematocrit was performed by the microhematocrit method. Centrifugation was carried out at 10000 xg for 10 min and the percentage of packed red cells was obtained by means of a hematocrit card reader. Hemoglobin concentration was performed using the Drabkin reagent [27], read spectrophotometrically at 540 nm and expressed as g dl^{-1} blood.

2.7 Plasma ion levels

The concentrations of sodium (Na^+) and potassium (K^+) were determined using flame photometer Micronal B262. Chloride (Cl^-) concentrations were obtained according to Zall et al. [28] and read in a plate reader at 480 nm. Standard solutions were made in distilled water, each having five different concentrations of standard curve. Results are expressed in mmol L^{-1}.

2.8 Prooxidants assay

Lipid peroxidation (LPO) was monitored through two methods: determination of LOOH and TBARS. The former was performed with a modified version of the method by Jiang et al. [29]. This technique can detect the primary products of peroxidation, using Fe^{2+}

oxidation by lipid hydroperoxides in acid medium in the presence of xylenol orange dye, forming a complex with Fe^{3+}. Reading was performed in a plate reader at 560 nm. Results are reported as nmol mg $protein^{-1}$. TBARS, in turn, was determined according to Wills [30], measuring the end products of LPO such as malondialdehyde. Aliquots of the supernatant were added to a pyrex tube containing TCA 10% and Thiobarbituric Acid (TBA) 0.67% and incubated at 100 ºC for 45 min. The mixture was allowed to cool on ice for 5 min and then centrifuged at 1000 xg for 5 min, in order to extract the resulting chromogen (Schiff's base). The absorbance of the organic phase was determined at 535 nm in a spectrophotometer. Results are reported as nmol mg $protein^{-1}$.

2.9 Protein assay

Tissue proteins were quantified based on Lowry et al. [31] using bovine albumin as standard. Reading was performed in spectrophotometer at 625 nm.

2.10 Antioxidant defenses assay

Catalase (CAT) activity was evaluated by measuring the decrease in absorption at 240 nm in a reaction medium consisting of 50 mM phosphate buffer (pH 7.4) and 2 mM hydrogen peroxide (H_2O_2), thereby determining the pseudo-first-order reaction constant (k’) of the decrease in H_2O_2 absorption, as outlined in Boveris and Chance [32]. Results are reported as pmol mg $protein^{-1}$.

Total superoxide dismutase (SOD) activity was determined as the inhibition rate of autocatalytic adenochrome generation at 480 nm in a reaction medium containing 1 mM epinephrine and 50 mM glycine/ sodium hydroxide (pH 10.2). Enzyme activity is expressed

as SOD units mg protein^{-1}. One SOD unit was defined as the amount of enzyme needed for 50% inhibition of adenochrome formation, as described by Misra and Fridovich [33].

Glutathione peroxidase (GPx) activity was measured by monitoring NADPH oxidation at 340 nm. The reaction medium consisted of 30 mM PBS, pH 7.0, 0.17 mM GSH, 0.2 U ml $^{-1}$ GR, and 0.5 mM tert-butyl-hydroperoxide. GPx activity is reported as μmol min^{-1} mg protein^{-1} [34].

Glutathione-S-transferase (GST) activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was determined spectrophotometrically at 340 nm according to Habig et al. [35]. The assay was performed at 25 ºC using 100 mM potassium phosphate buffer, pH 6.5, with reduced glutathione (GSH) and CDNB (dissolved in ethanol) at a final concentration of 1 mM each. Activity was calculated from the changes in absorbance at 340 nm. One unit of GST activity was defined as the amount of enzyme catalyzing the conjugation of 1 pmol of CDNB with GSH per min at 25 °C. The enzymatic activity is expressed as pmol min^{-1} mg protein^{-1}.

Non-proteic thiol groups were also measured in red blood cells. They are non-enzymatic antioxidants and represent an indirect measure of GSH. The method is based on GSH reacting with 5,5'-ditio-bis-2-nitrobenzoic (DTNB) acid. The final product formed is the yellow 2-nitro-5-mercapto-benzoic (TNB) acid. The samples were read spectrophotometrically at 412 nm [36]. The content of thiol groups is expressed as μmol mg protein^{-1}.

3. Statistical analysis

Statistical analysis was performed using the software Statistica® 7.0. A Levene test was used to verify whether the data were parametric, and a two-way ANOVA followed by Duncan's test to assess differences between groups. Regression analysis was conducted using

the Sigma Plot® 11.0. The results are expressed as the mean ± standard error (S.E.). The minimum significance level was set at 95% ($p < 0.05$).

4. Results

4.1 Total phenolic compounds

Results demonstrate 160.69 ± 4.47 mg GAE g HA^{-1} of total phenolic compounds.

4.2 Water Parameters

The evaluated water parameters remained stable throughout the experimental period. The pH was maintained at 7.19±0.045, and the water temperature at 21.32±0.26 ºC. Hardness (20.90±1.6 mg L^{-1} $CaCO_3$), alkalinity (24.07±1.9 mg L^{-1} $CaCO_3$) nitrite (0.87±0.07 mg L^{-1}), total ammonia (2.92±0.30 mg L^{-1}) and non-ionized ammonia (0.038±0.007 mg L^{-1}) remained in the desired range.

4.3 Hematocrit and hemoglobin

Hematocrit (Fig. 1A) and hemoglobin levels (Fig. 1B) increased with the increase of HA in normoxia. There was no significant difference between treatments in hypoxia. Likewise, normoxic and hypoxic groups did not show any significant difference (Figure 1).

4.4 Plasma ion levels

Plasma Na^+, K^+ and Cl^- levels increased with increased concentrations of HA in normoxia (Fig. 2A, 2B and 2C, respectively). In hypoxia, however, plasma Na^+ and Cl^- levels were not affected by HA, while plasma K^+ levels increased significantly depending on the

concentration of HA. Plasma K^+ levels were higher in all of the groups subjected to hypoxia compared to normoxia (275.2%, 276.6%, 195.7 in 0, 2.5 and 5mg/L respectively), but this pattern was observed only in the absence of HA for Na^+ levels (43.3%). Plasma Cl^- levels were not affected by dissolved oxygen levels (Figure 2).

4.5 Blood

The levels of LPO measured through TBARS (Fig. 3A) and LOOH (Fig. 3B) in plasma of silver catfish exposed to HA decreased in hypoxia and normoxia compared to controls. In erythrocytes, CAT concentrations remained stable in all groups (Fig. 3C). SOD acitivity increased dependenting on the levels of HA in normoxia and hipoxia (Fig. 3D). A decrease in GPx activity with increased concentrations of HA at both oxygen levels was also observed (Fig. 3E). On the other hand, GST activity increased with increased HA concentrations in normoxia, but GST remained stable in hypoxia (Fig. 3F). Non-protein thiols also raised with increased concentrations of HA in normoxia (Fig. 3G).

In the same HA concentration, SOD and GPx activities decreased in all of the groups exposed to hypoxia compared to normoxia (SOD: 62.38%, 60.71% and 71.35% at 0, 2.5 and 5 mg L^{-1} HA, respectively; GPx: 40.16%, 29.68% and 33.6% at 0, 2.5 and 5 mg L^{-1} HA, respectively). At concentrations of 5 mg L^{-1} HA, the non-protein thiol levels also decreased significantly (38.23%) in the hypoxia group compared to normoxia (Figure 3).

4.6 Gills

There was a significant decrease in the levels of LPO measured by TBARS (Fig. 4A) and LOOH measured by xilenol orange (Fig. 4B) in gills of silver catfish with increased HA concentrations, both in normoxia and hypoxia. Similarly, a decrease in the activities of the antioxidant enzymes CAT (Fig. 4C), SOD (Fig. 4D), GPx (Fig. 4E) and GST (Fig. 4F) were

observed in normoxia with increased concentrations of HA. In hypoxia, CAT increased with increased HA concentrations, and the other enzymes decreased in the same conditions. Thus, all of the tissue oxidative changes occurred in a concentration-dependent manner with respect to HA, and the results of TBARS, LOOH, CAT, SOD and GPx in normoxia were inversely proportional to the concentration of HA (Figure 4).

Moreover, when groups in hypoxia and normoxia in the same HA concentration were compared, increased SOD activity (55.03%) and decreased GST activity (45.77%) were observed in hypoxia in the absence of HA when compared to control. In the presence of HA, CAT activity increased 81.75% at 2.5 mg L^{-1} HA and 152.34% at 5 mg L^{-1} HA; SOD activity increased 23.37% at concentrations of 2.5 mg L^{-1} and 38.57% at 5 mg L^{-1}. Other enzymes showed no significant alterations (Figure 4).

4.7 Brain

As it was observed in gills, levels of LPO by TBARS (Fig. 5A) and LOOH (Fig. 5B) decreased with increased concentrations of HA in brain of fish both in normoxia and in hypoxia. With regard to the antioxidant enzymes, SOD activity remained stable in normoxia. In hypoxia, however, it decreased depending on the concentration of HA (Fig. 5C). GPx activity decreased significantly at both dissolved oxygen levels with increased concentrations of HA (Fig. 5D). The activity of GST was unaffected in fish exposed to hypoxia and normoxia (Fig. 5E).

Levels of LPO measured through TBARS in fish exposed to 0 and 2.5 mg L^{-1} HA increased 84.80% and 95.43%, respectively, in hypoxia compared to normoxia. Activity of GPx was also higher at 2.5 mg L^{-1} (108.33%) and 5 mg L^{-1} (76.05%) in hypoxia compared to the corresponding HA levels in normoxia (Figure 5). Determination of CAT activity was not possible due to the sensitivity of the method.

5. Discussion

The HS are ubiquitous compounds in freshwater, but they can be highly reactive mainly due to the carboxylic and phenolic groups [3]. The growing recognition that HS interact with biological membranes leads to the statement that the substances do not only exert chemical effects, such as chelation or complexation of elements, but also affect biochemical, physiological and molecular mechanisms [8]. This interface between biological membranes and HS probably results from interactions between lipid membranes and hydrophobic domains of HS [6, 1]. In general, organisms exhibit diverse responses to HS, mostly because HS come from different sources, thus presenting differences in constitution and functional groups. In addition, studies in the natural environment can also suffer the effect of the photolysis of HA, a component of HS, releasing ROS into the environment [14]. Some studies indicate that HS may reduce the antioxidant activity of organisms [4]. Nonetheless, reports on the effect of HS and their compounds, such as humic acid, fulvic acids and humin, on oxidative parameters in fish are scarce.

5.1 Total phenolic compounds

Several studies note the presence of phenolic compounds in HS. Phenolic compounds are bioactive substances widely distributed in plants [37], which constitute a potential source of HA when decomposed. Phenolic compounds are generally lipophilic [38] and have antioxidant activity that occurs via the trapping of alkoxyl radicals, which are usually the initiators of lipid peroxidation reactions. The attenuation of LPO caused by phenolic compounds is also due to the membrane stabilization and decreased fluidity, preventing the spread of free radicals [39]. The results of the present study demonstrate that the HA used in the experiments has a considerable amount of total phenolic compounds, which may have

acted as antioxidants, possibly non-enzymatic, reducing the levels of LPO and influencing the behavior of the other analyzed antioxidants.

5.2 Hematocrit and hemoglobin

The increase in hematocrit and hemoglobin in silver catfish kept in normoxia occured depending on the HA levels. *In vitro* studies with human endothelial cells demonstrated that HS increased iron intracellular concentration [40]. Iron is inserted into the heme group of hemoglobin, and participates in the synthesis of hemoglobin in the mitochondria and in other biochemical and physiological processes in the cell [41]. Increase in hemoglobin was also observed in sea bass (*Dicentrarchus labrax*) that received different phenolic compounds via intra-abdominal single dose [38]. In addition, alterations in erythrocytes production and hemoglobin are related to antioxidant protection. Blood thiol groups are involved in the protection of hemoglobin against spontaneous oxidation to metahemoglobin, which could be caused by ROS such as hydrogen peroxide [42]. In the present experiment, thiol groups increased in normoxia. Thus, the presence of thiol groups protecting the cell, the increased iron ion intracellular and the presence of phenolic compounds may have contributed to the increased levels of hematocrit and hemoglobin. Furthermore, hypoxia may have caused an increase in the volume of red blood cells, consequently increasing the hematocrit [43].

No alterations in hematocrit and hemogobin were observed between hypoxia and normoxia, what may have occured because of the moderate, but not severe, hypoxic level employed. There was a metabolic decline, though probably not sharp enough to activate erythropoietin, which is the mediator of erythrocyte production.

5.3 Plasma ion levels

The black waters are ion-poor. Humic substances apparently limit gill permeability and excessive ion loss in these diluted waters by mantaining gill tight junctions [44]. Thus, it is possible that HA reduced ion loss by silver catfish gills, consequently increasing plasma ion levels. Plasma ion levels in silver catfish exposed to hypoxia and no HA were higher than in those kept in normoxia. Such effect of hypoxia exposure was previously demonstrated in this species [20, 45].

5.4 Blood

Previous studies have demonstrated that blood cells have high levels of antioxidants [46], and red blood cells in particular have been studied to investigate specific cellular responses of enzymes involved in cellular protection against ROS, which are produced in large quantities in these cells [39]. The blood of silver catfish showed lower LPO in the plasma of groups exposed to HA in normoxia. On the other hand, the components of the antioxidant system showed a different behavioral pattern in erythrocytes. Catalase activity showed no difference between groups, while the levels of GPx decreased. Both CAT and GPx can catabolyze H_2O_2, but GPx has much higher affinity for H_2O_2 than CAT [47, 48], suggesting that this enzyme may play a more important role *in vivo* at low H_2O_2 concentrations. Moreover, the results obtained for these two enzymes may have been due to maintenance of adequate levels of hydrogen peroxide to be used as regulator of cell signaling [49].

The enzymes GST and SOD increased in the presence of HA, accompanied by an increase in non-protein thiol groups. Non-protein thiols are non-enzymatic antioxidants that act scavenging, inactivating and eliminating free radicals [50]. One of the most abundant intracellular non-protein thiol is glutathione in its reduced form, which acts as a substrate for other enzymes such as GST. Thus, the increase in thiols may be related to the supply of

substrate for the activity of GST in order to detoxify compounds such as HA. In contrast, non-protein thiols and SOD can also act in concert inactivating the superoxide anion. According to Marcon, Winterbourne and Koppenol [46, 51, 52], the superoxide anion acts as a sink for other oxiradicals, which are inhibited when SOD and reduced glutathione are elevated in fish erythrocytes.

In opposition to the results found in the present experiment, a study in African mud catfish (*Clarias gariepinus*) exposed to HA derived from compost waste in concentrations ranging from 100 to 1000 mg L^{-1} found increased LPO and decreased SOD, CAT and thiol groups compared to control. The latter two parameters decreased in a concentration-dependent manner. These results probably reflect the extremely high HA concentrations, as well as their source [53].

In hypoxia, SOD activity also increased with the increase in HA concentration, while CAT and GST were unaffected. The other parameters (LPO, non-protein thiol groups and GPx) decreased compared to control. These results may have been generated by metabolic depression. It is generally established that decreased environmental oxygen concentration or its full absence decrease ROS levels [54].

There was a reduction in LOOH in all of the hypoxic groups compared to normoxia, accompanied by a reduction in SOD and GPx. The levels of GSH decreased significantly only in silver catfish exposed to 5 mg L^{-1} HA in hypoxia when compared to normoxia. These results were probably a consequence of the sum of metabolic depression caused by hypoxia and high levels of HA.

5.5 Gills

The gills are the primary targets of compounds dissolved in water, not only for their large contact surface that facilitates interaction with the external factors, but also because their defense system is not so robust [55].

In gills of silver catfish subjected to normoxia, the decreased LPO levels that occurred with increased HA concentrations were accompanied by reductions in antioxidant enzymes activities. Decreased enzymatic activity was possible also linked to the presence of HA, and the diminution in LPO was probably due to the existence of a non-enzymatic antioxidant defense mechanism contributing to the control of LPO.

The oxidative parameters of silver catfish in hypoxia showed a tendency to decrease with increased concentrations of HA. Catalase activity was the only exception, showing a significant increase with increased concentrations of HA. The teleost gills are an alternative route to excretion of ammonia and hydrogen peroxide [46]. The metabolic decline as a result of hypoxia may have hindered this route, leading to an increase in CAT in order to reduce toxicity in the tissue.

The comparison between hypoxia and normoxia demonstrated that the levels of SOD were higher in all groups in hypoxia and the levels of CAT increased only in the groups exposed to HA in hypoxia. There was an increase in SOD activity and maintance of CAT activity in spot (*Leiostomus xanthurus*) exposed to 2 mg L^{-1} O_2, suggesting an independent action of the two enzymes in this situation [56].

In general, the values obtained for the oxidative parameters in gills of silver catfish in hypoxia were lower than the values found in normoxia, suggesting that these individuals suffered a synergistic effect exerted by HA and the lower metabolism caused by hypoxia, resulting in greater attenuation of the antioxidant defenses.

5.6 Brain

There are no reports regarding the effect of HS on brain oxidative parameters. In silver catfish kept in normoxia, there was a tendency to decreased LPO and GPx with increased concentration of HA, while SOD and GST were unaffected. Attenuation of lipoperoxidation may have occurred via a non-enzymatic mechanism, more specifically through GSH activity, which increased in blood of silver catfish. Reduction in GPx suggests sensitivity of the enzyme to HA. Although the mechanism behind this interaction is unclear, synthesis and transport of GSH, as well as the presence of phenolic compounds in HA, may be involved in tissue protection.

Results obtained in hypoxia were similar to that found in normoxia, excepting for SOD, which also demonstrated decreased activity. This suggests a possible effect of the phenolic compounds found in HA.

When compared to normoxia, all groups in hypoxia displayed an increase in LPO, while the activity of GPx raised only in the groups exposed to HA. A similar pattern was observed in brain of silver catfish transported in plastic bags for 7 h [19].

Increased LPO may be related to the adequate oxygen supply to the brain, since organisms under hypoxic conditions tend to protect primarily the vital organs [57]. A study in goldfish *Carassius auratus* showed increased oxygen supply to the brain due to its redistribution [58]. Cerebral redistribution is regulated by adenosine, a strong vasodilator that acts through many pathways, which may exarcebate the generation of ROS and increase LPO [59]. The activity of GPx also showed a significant increase, indicating that it could be attempting to decrease LPO.

6. Conclusion

Low concentrations of HA attenuated LPO levels in silver catfish, as well as the majority of the enzymatic antioxidants. In hypoxia, the synergistic action of HA and low

oxygen levels led to lower metabolic action and lower metabolic activity of the antioxidant enzymes. The antioxidant activity was probably due to the action of the phenolic compounds in the HA, which decreased LPO. Thus, exposure to these combined factors led to physiological and biochemical imbalances in the individuals. Further studies are needed to elucidate the mechanism of action of HA on this fish species.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure captions

Figure 1. Hematocrit and hemoglobin in blood of *Rhamdia quelen* maintained in hypoxia and normoxia. (A) $y=23.0643+0.7436x$ ($r^2=1$) in normoxia where y is hematocrit (%). (B) $y=5.7602+0.1307x$ ($r^2=0.9861$) in normoxia, where y is hemoglobin (g dL^{-1}). x = humic acid levels (mg L^{-1}) to all figures (Mean±SE).

Figure 2. Plasma ion levels in *Rhamdia quelen* maintained in hypoxia and normoxia. *indicate significant difference from normoxia in the same humic acid level. (A) $y=144.1105+11.1204x$ ($r^2=0.9926$) in normoxia, where y is Na^+ levels (mmol L^{-1}). (B) $y=3.4944+0.2820x$ ($r^2=0.7758$) in normoxia and $y=13.5257+0.3126x$ ($r^2=0.7758$), where y is K^+ levels (mmol L^{-1}). (C) $y=81.3389+0.5435x$ ($r^2=0.7758$) in normoxia, where y is Cl^- levels (mmol L^{-1}). x = humic acid levels (mg L^{-1}) to all figures (Mean±SE).

Figure 3: Relationship between oxidative parameters in blood and humic acid levels in *Rhamdia quelen* maintained in hypoxia and normoxia. *indicate significant difference from normoxia in the same humic acid level. (A) $y=0.0207–0.0014x$ ($r^2=0.8218$) in normoxia and $y=0.0156–0.0008x$ ($r^2= 0.8120$) in hypoxia, where y= thiobarbituric acid reactive substances (TBARS) (nmol mg $protein^{-1}$). (B) $y=0.6110–0.4732x$ ($r^2=0.9749$) in normoxia, and $y=2.5753–0.1698x$ ($r^2=0.9958$) in hypoxia where y = lipid hydroperoxides (LOOH) (nmol mg $protein^{-1}$). (C) catalase (CAT) (pmol mg $protein^{-1}$). (D) $y=0.6475+0.1041x$ ($r^2=0.8860$) in normoxia and $y=0.2379+0.0352x$ ($r^2=0.9934$) in hypoxia, where y = superoxide dismutase (SOD) (uSOD mg protein $^{-1}$). (E) $y=0.0350–0.0020x$ ($r^2=0.9588$) in normoxia and $y=0.0211–0.0007x$ ($r^2=0.9723$) in hypoxia, where y = glutathione peroxidase (GPx) (μmol mg $protein^{-1}$). (F) $y=0.0404+0.0069x$ ($r^2=0.8866$) in normoxia, where y= glutathione-S-transferase (GST) (pmol mg $protein^{-1}$). (G) $y=0.0092–0.0014x$ ($r^2=0.8733$) in normoxia, and $y=0.0320–0.286x$ ($r^2=0.9749$) in hypoxia where y is non protein thiol groups (μmol mg $protein^{-1}$). x = humic acid levels (mg L^{-1}) to all figures (Mean±SE).

Figure 4. Relationships between oxidative parameters in gills of *Rhamdia quelen* maintained in hypoxia and normoxia and humic acid levels. *indicate significant difference from normoxia in the same humic acid level. (A) $y=0.0207-0.0014x$ ($r^2= 0.8218$) in normoxia and $y=0.2093–0.0060x$ ($r^2=0.8936$) in hypoxia, where y= thiobarbituric acid reactive substances (TBARS) (nmol mg $protein^{-1}$). (B) $y=8.7792–0.3179x$ ($r^2=0.9964$) in normoxia

and y=7.2222–0.3543x (r^2=0.9901) in hypoxia where y= lipid hydroperoxides (LOOH) (nmol mg protein^{-1}). (C) y=0.2977–0.0286x, (r^2=0.9278) in normoxia and y=0.2394+0.0393x (r^2=0.9268) in hypoxia, where y= catalase (CAT) (pmol mg protein^{-1}). (D) y=0.9871–0.1139x (r^2=0.9986) in normoxia and y=1.5190–0.1188x (r^2=0.9852) in hypoxia, where y = superoxide dismutase (SOD) (uSOD mg protein $^{-1}$). (E) y=0.0113–0.0011x (r^2=0.9172) in normoxia and y=0.0095–0.0008x (r^2=0.9720) in hypoxia, where y = glutathione peroxidase (GPx) (µmol mg protein^{-1}). (F) y=0.2289–0.0357x (r^2=0.9245) in normoxia and y=0.1419–0.0052x (r^2=0.9999) in hypoxia, where y = glutathione-S-transferase (GST) (pmol mg protein^{-1}). x = humic acid levels (mg L^{-1}) to all figures (Mean±SE).

Figure 5: Relationships between oxidative parameters in brain of *Rhamdia quelen* maintained in hypoxia and normoxia and humic acid levels. *indicate significant difference from normoxia in the same humic acid level. (A) y=0.2535–0.0089x (r^2=0.9979) in normoxia and and y=0.4887–0.0312x (r^2=0.8447) in hypoxia, where y = thiobarbituric acid reactive substances (TBARS) (nmol mg protein^{-1}). (B) y=9.2458-0.3570x (r^2=0.9834) in normoxia and y=0.4887–0.0312x (r^2=0.8447) in hypoxia where y= lipid hydroperoxides (LOOH) (nmol mg protein^{-1}). (C) y=4.7350–0.2550x (r^2=0.9801) in normoxia, where y = superoxide dismutase (SOD) (uSOD mg protein^{-1}). (D) y=0.0362–0.0035x (r^2=0.8136) in normoxia and y=0.0528–0.0029x (r^2=0.9494) in hypoxia, where y= glutathione peroxidase (GPx) (µmol mg protein^{-1}). (E) Glutathione-S-transferase (GST) (pmol mg protein^{-1}). x = humic acid levels (mg L^{-1}) to all figures (Mean±SE).

Figure 1

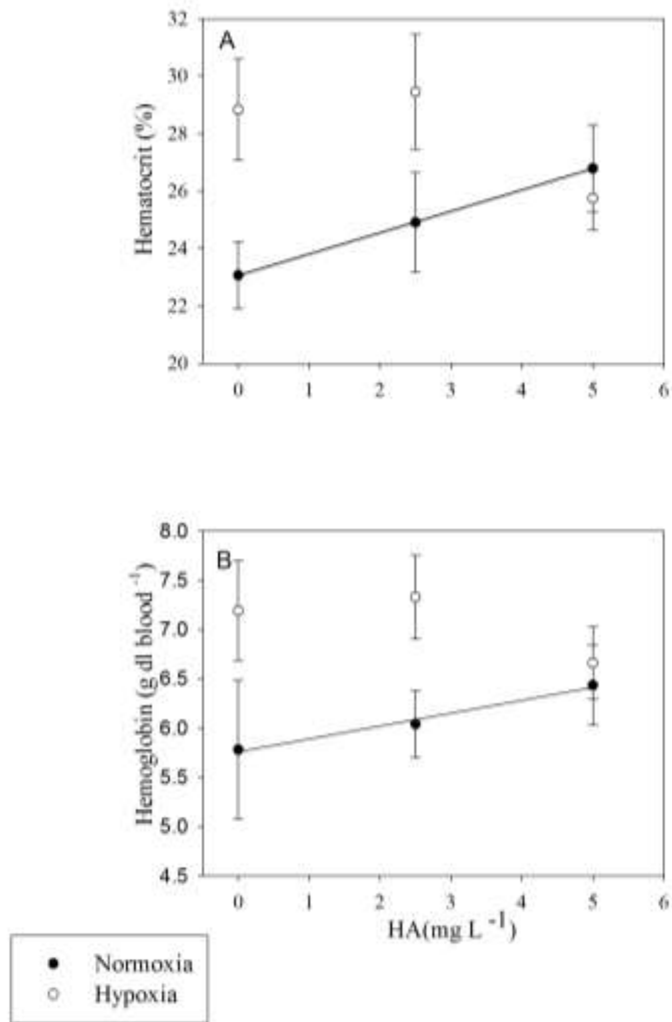


Figure 2.

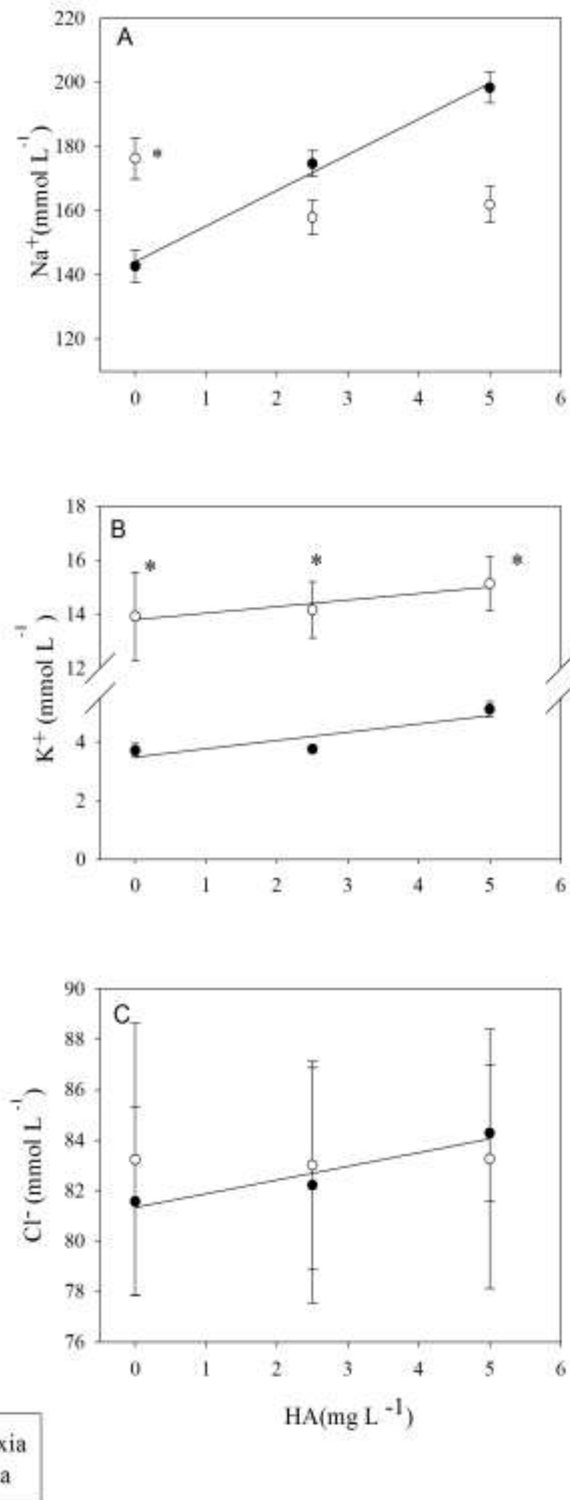


Figure 3.

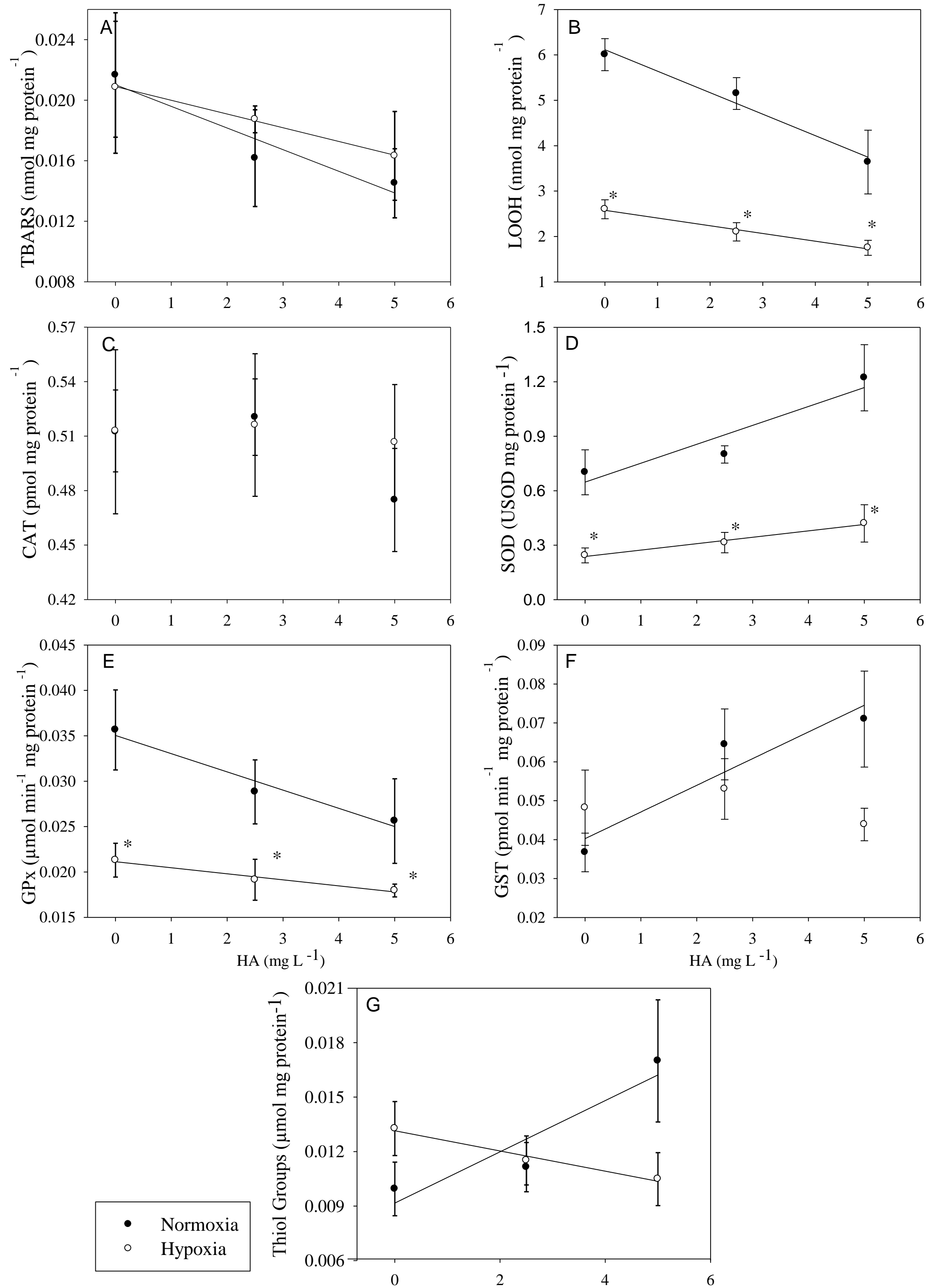


Figure 4.

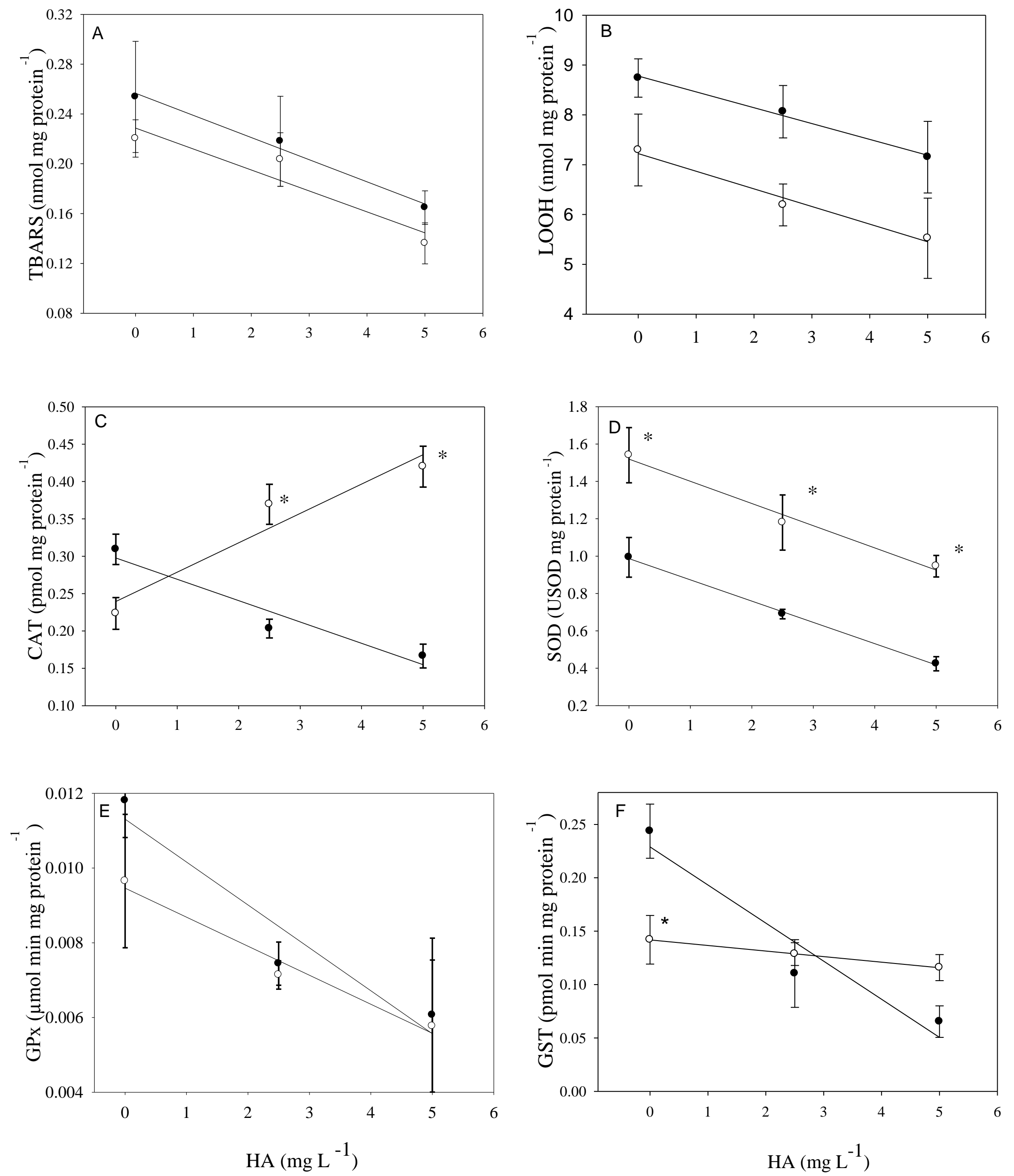
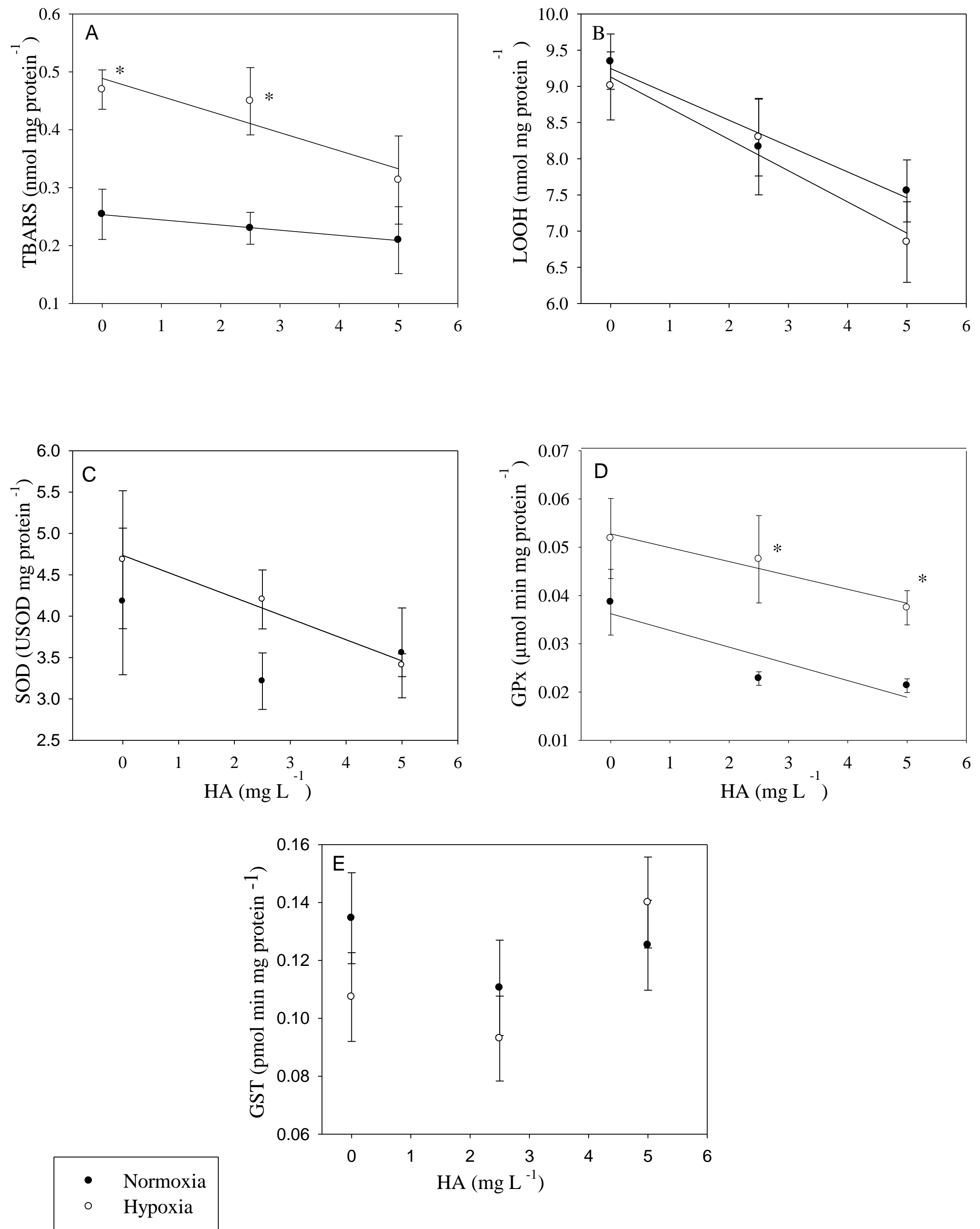


Figure 5



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5 CONCLUSÕES

A exposição aos níveis de HA e OD altera diversos parâmetros em jundiás, a maioria de forma concentração dependente. O aumento do hematócrito e da hemoglobina podem ser atribuídos aos grupos funcionais presentes no HA. Já os íons plasmáticos sofrem alteração devido a baixa concentração iônica da água, a qual induz respostas compensatórias nos peixes como a redução da perda de íons.

O HA em baixas concentrações e o OD também levam a atenuação da LPO de forma concentração dependente em diferentes tecidos de jundiás. Simultaneamente, houve depleção da maioria das enzimas, reforçando a idéia da atuação dos grupos fenólicos.

Esses resultados indicam que os jundiás suportam variações na disponibilidade do O_2 ambiental concomitante a presença de HA em baixas concentrações. Frente a esses fatores, os indivíduos sofrem ajustes finos, que possivelmente incluem a diminuição do metabolismo aeróbico, a ativação do metabolismo anaeróbico e ajustes osmorregulatórios. Como o jundiá demonstra tolerância e adaptações a diferentes variáveis, essa espécie constitui um excelente biomarcador para ambientes naturais bem como um excelente animal para o cultivo.

A presença do HA em pequenas concentrações em tanques pode diminuir a LPO e trazer algumas vantagens já descritas na literatura. Maiores avaliações em diferentes parâmetros precisam ser realizadas para concluir se sua ação traz vantagens concretas para a homeostase e bem-estar do animal e, conseqüentemente, ganhos aos produtores.

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