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**NORMÓXIA, HIPÓXIA E REOXIGENAÇÃO EM JUNDIÁS FRENTES À
EXPOSIÇÃO AO MANGANÊS**

**Santa Maria, RS
2016**

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Farmacologia, Área de Concentração em Farmacologia dos Processos Oxidativos, da Universidade Federal de Santa Maria (UFSM, RS), como requisito para obtenção do grau de **Doutor em Farmacologia**.

Orientadora: Prof^a. Dra. Marilise Escobar Bürger

Co-orientador: Prof. Dr. Bernardo Baldisserotto

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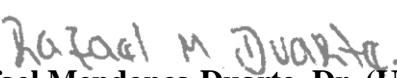
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Dedico esta tese à minha família, pessoas essenciais para a realização desse sonho.

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“ Nunca vá dormir sem um sonho, ou levantar-se sem uma razão. Lembre-se que nenhum dia é igual ao outro e ninguém é como você. Apenas uma pessoa é capaz de te fazer feliz para toda a vida e essa pessoa é você mesmo. Tenham coragem, não tenham medo de sonhar coisas grandes”

(Papa Francisco)

RESUMO

NORMÓXIA, HIPÓXIA E REOXIGENAÇÃO EM JUNDIÁS FRENTE À EXPOSIÇÃO AO MANGANÊS

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CO-ORIENTADOR: BERNARDO BALDISSEROTTO

Nas últimas décadas, o aquecimento global, desencadeado pelo efeito estufa, tem aumentado a incidência dos episódios de hipóxia pelo mundo, alterando as condições de águas doces e oceanográficas. Desse modo, os períodos de hipóxia, antes sazonais, tem ocorrido com maior frequência, principalmente pela interferência do homem no ambiente, gerando preocupações acerca da biota aquática. Como resultado dessas interferências, além da queda nos níveis de oxigênio, pode ser observado também o aumento da poluição das águas, gerada por atividades extrativistas como prospecção de minérios e combustíveis fósseis. Nesse processo, o manganês (Mn) pode sofrer extravasamento para o meio contaminando os afluentes e comprometendo a biodiversidade. Com base no exposto, este estudo avaliou o impacto das variações nos níveis de oxigênio da água (normoxia, hipóxia e reoxigenação) sobre os prejuízos gerados pela exposição ao Mn em diferentes tecidos de jundiás (*Rhamdia quelen*), espécie teleóstea tolerante à hipóxia, de grande relevância econômica. A aclimatação de jundiás à hipóxia (~ 3,09 mg L⁻¹) foi capaz de reduzir o acúmulo de Mn em diferentes tecidos quando peixes foram expostos ao Mn (nas concentrações de 9,8 mg L⁻¹ e 8,1 mg L⁻¹, para o primeiro e demais estudos, respectivamente). Adicionalmente, aclimatação à hipóxia minimizou danos oxidativos a lipídios de membrana e reduziu a carbonilação de proteínas, previnindo alterações induzidas por Mn na atividade da catalase (CAT) e sódio/potássio ATPase (Na⁺/K⁺ ATPase), assim como sobre a expressão da prolactina (PRL) e somatolactina (SL), hormônios hipofisários relacionados ao ciclo reprodutivo, reforçando a hipótese do desenvolvimento de hormesis. Finalmente, quando foi estabelecido o protocolo de reoxigenação (restauração dos níveis de oxigênio para normoxia: ~ 7,0 mg L⁻¹), jundiás aclimatados à hipóxia durante 10 ou 20 dias e, subsequentemente reoxigenados, apresentaram ajustes tanto no perfil hematológico quanto nas respostas antioxidantes, que contribuíram para a redução dos danos oxidativos gerados pela exposição ao Mn sob normoxia, observados pela elevação da geração de espécies reativas (ER), aumento da carbonilação proteica e elevação das transaminases plasmáticas. A aclimatação à hipóxia também produziu alterações morfológicas na superfície branquial, importantes para o processo de adaptação a baixos níveis de oxigênio, que na sua maioria, permaneceram mesmo após o estabelecimento da reoxigenação. Quando o labirinto de ansiedade e memória foi acessado nos testes comportamentais, não foram observadas alterações sobre a memória de jundiás aclimatados à hipóxia ou normoxia, expostos ou não ao Mn. Entretanto, os peixes que acessaram pela terceira vez o labirinto, após o estabelecimento da reoxigenação, mostraram memória referente à interação social. Adicionalmente, jundiás aclimatados à hipóxia mostraram um aumento per se na frequência ventilatória, a qual mostrou-se pouco responsável frente a coespecíficos, enquanto que jundiás sob normoxia responderam agressivamente contra coespecíficos em todo período experimental. Contudo, a exposição ao Mn, sob normoxia ou hipóxia e durante a reoxigenação reduziu a resposta de jundiás frente a coespecíficos. Em relação ao estresse coordenado pelo eixo endócrino, jundiás aclimatados à hipóxia, por 10 e 20 dias, apresentaram expressão aumentada da proopiomelanocortina-A (POMC-A) enquanto a expressão desse hormônio hipofisário foi diminuída durante a exposição ao Mn sob hipóxia. A expressão da proopiomelanocortina-B (POMC-B), PRL ou SL permaneceu constante em todo o período experimental independentemente dos níveis de oxigênio ou da exposição ao Mn. Jundiás aclimatados à hipóxia por 20 dias, assim como nos expostos ao Mn sob hipóxia, apresentaram escurecimento da pele, que persistiu após reoxigenação. Associadamente, todos esses parâmetros permitem concluir que espécies tolerantes à hipóxia como o jundiá desenvolvem durante um curto período de estresse por privação de oxigênio, mecanismos de ajustes fisiológicos e morfológicos que aumentam suas defesas antioxidante e reduzem a seu consumo de energia para, dessa forma, estarem mais aptos a enfrentar situações mais hostis, como excessivas concentrações de Mn ou uma subsequente reoxigenação. Até o momento, os efeitos de uma hipóxia a longo prazo sobre jundiás em seu ambiente natural são desconhecidos, contudo, é possível que episódios sustentados de hipóxia comprometam a relação comportamental de submissão e dominância entre os peixes, modificando as relações hierárquicas como forma de reduzir a agressão e os riscos de injúria, o que pode, por um lado, prolongar a sobrevivência, mas por outro, causar a descaracterização dessa espécie, que apresenta naturalmente um comportamento carnívoro de dominância.

Palavras-chave: *Rhamdia quelen*, estresse oxidativo, hipóxia moderada, alterações histológicas, alterações comportamentais, expressão gênica hipofisária.

ABSTRACT

NORMOXIA, HYPOXIA AND REOXYGENATION ON SILVER CATFISH EXPOSED TO MANGANESE

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In recent decades, the global warming has increased the incidence of the hypoxia episodes in the world, changing the freshwater and oceanographic conditions. Thus, hypoxia periods, before seasonal, have occurred more frequently, mainly by human interference in the environment, raising concerns about the aquatic biota. As a result, it can be also observed by a decrease in oxygen levels and, in addition, increased water pollution generated by extractive activities like prospection of both minerals and fossil fuels. In this process, manganese (Mn) may suffer extravasation into the environment contaminating tributaries and compromising biodiversity. Based on this, this study evaluated the impact of changes in water oxygen levels (normoxia, hypoxia and reoxygenation) on the impairments generated by exposure to Mn in different silver catfish (*Rhamdia quelen*) tissues, a hypoxia-tolerant teleostean species of economic relevance. The acclimation of silver catfish to hypoxia ($\sim 3.09 \text{ mg L}^{-1}$) was able to reduce Mn accumulation in different tissues when fish were exposed to Mn 9.8 mg L^{-1} and 8.1 mg L^{-1} (in the first and other studies, respectively). Additionally, hypoxia acclimation was able to minimize the oxidative damages to the membrane lipids and reduce protein carbonylation, preventing Mn-induced changes on both catalase (CAT) activity and Na^+/K^+ -ATPase, as well as on prolactin (PRL) and somatotropin (SL) gene expression, pituitary hormones related to reproductive cycle, reinforcing the hormesis development hypothesis. Finally, when reoxygenation was established (restoration of the oxygen levels to normoxia: $\sim 7.0 \text{ mg L}^{-1}$), silver catfish acclimated to hypoxia and subsequently reoxygenated, presented adjustments in both hematological profile and antioxidant responses, which contributed to the reduction of the oxidative damages generated by exposure to the Mn under normoxia, observed by increased RS generation, increased protein carbonylation and increased serum transaminases. Hypoxia acclimation also produced morphological changes in the gill surface, important to the adaptive process to low oxygen levels, which, mostly, remained even after the establishment of reoxygenation. When the maze of social interaction memory or anxiety was accessed in the behavioral tests, no changes were observed on the memory of silver catfish acclimated to hypoxia or normoxia, exposed or not to Mn. However, fish accessed by the third time the maze trial, after the establishment of reoxygenation, showing memory related to social interaction. Additionally, silver catfish acclimated to hypoxia exhibited an increase *per se* in ventilatory rate, which was poorly reactive towards conspecifics, while fish under normoxia replied aggressively against conspecifics throughout the trial period. However, exposure to Mn under normoxia or hypoxia, and after reoxygenation, reduced the answer against conspecifics. Regarding stress coordinated by endocrine axis, silver catfish acclimated to hypoxia showed increased proopiomelanocortin-A (POMC-A) expression, while the expression of this pituitary hormone decreased during the exposure to Mn under hypoxia. The proopiomelanocortin-B (POMC-B), PRL and SL expression remained constant throughout the experimental period, regardless of the oxygen levels or Mn exposure. Silver catfish acclimated to hypoxia for 20 days, as well as in those animals exposed to Mn under hypoxia showed skin darkening, which persisted after reoxygenation. Taken together, these parameters allow to conclude that hypoxia-tolerant species as silver catfish develop over a short stress period by oxygen deprivation, both physiological and morphological adjustments mechanisms that increase their antioxidant defenses and reduce their energy consumption in order to face more hostile situations, such as excessive Mn concentration or a subsequent reoxygenation. Until now, the long-term hypoxia effects on silver catfish in its natural environment are unknown. However, it is possible that sustained hypoxia episodes cause behavioral impairments in the submission and dominance relationships among fish, changing the hierarchical relationships and reducing the aggression, injury risks, which may prolong survival but in contrast cause mischaracterization of this species, which naturally presents a carnivorous dominance behavior.

Keywords: *Rhamdia quelen*; oxidative stress; moderate hypoxia, histological changes, behavioral changes, pituitary gene expression.

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LISTA DE ABREVIATURAS E SIGLAS

ARE: *antioxidant response element*

ATSDR: Agência de registro de doenças e substâncias tóxicas (*Agency for Toxic Substances and Disease Registry*)

CAT: catalase

CONAMA: Conselho Nacional do Meio Ambiente

DO₂: oxigênio dissolvido (*dissolved oxygen*)

EO: estresse oxidativo

Epo: eritropoietina (*Erythropoietin - Danio rerio (zebrafish)*)

ERA: elemento de resposta antioxidante

ERO: espécies reativas de oxigênio

ER: espécies reativas

GH: hormônio do crescimento (*growth hormone*)

GPx: glutatona peroxidase

GR: glutatona redutase

GSH: glutatona reduzida

GSSG: glutatona oxidada

GST: Glutatona-S-transferase

H₂O: água

H₂O₂: peróxido de hidrogênio

HIF: fator induzível por hipoxia (*hypoxia inducible factor*)

Mn: manganês

Mn-SOD: manganês superóxido dismutase

Na⁺/K⁺-ATPase: sódio potássio adenina trifosfatase

NADP⁺: fosfato de nicotinamida adenina dinucleotídeo

NADPH: fosfato de nicotinamida adenina dinucleotídeo oxidase

NeuroD: fator de diferenciação neurogênico (*neurogenic differentiation factor*)

NFκβ: fator neurotrófico kappa β (*neurotrophic factor kappa β*)

O₂: oxigênio molecular

O²⁻: ânion superóxido

OH⁻: ânion hidroxila

•O²⁻: radical superóxido

•OH⁻: radical hidroxila

PC: proteína carbonilada (*protein carbonyl*)

pH: potencial hidrogeniônico

PL: peroxidação lipídica

POMC: proopiomelanocortina (*proopiomelanocortin*)

PRL: prolactina (*prolactin*)

SL: somatolactina (*somatolactin*)

SNC: sistema nervoso central

SNA: sistema nervoso autônomo

SOD: superóxido dismutase

TBArS: substâncias reativas ao ácido tiobarbitúrico

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APRESENTAÇÃO

Esta tese está estruturada em seções dispostas da seguinte forma: Capítulo I – REFERENCIAL TEÓRICO e PROPOSIÇÃO, Capítulo II – PRODUÇÃO CIENTÍFICA (Artigo 1, Artigo 2, Artigo 3 e Resumo expandido) e Capítulo III – DISCUSSÃO e CONCLUSÕES.

Os itens Materiais e Métodos, Resultados, Discussão e Referências encontram-se inseridos nos próprios artigos científicos, e inseridos no Capítulo II – **PRODUÇÃO CIENTÍFICA** e representam a produção integral deste estudo.

Por fim, no Capítulo III, encontram-se os itens **DISCUSSÃO e CONCLUSÕES**, nos quais há interpretações e comentários gerais sobre a produção científica contida neste estudo.

CAPÍTULO I

1 REFERENCIAL TEÓRICO

1.1 HIPÓXIA

A hipóxia aquática, definida como diminuição dos níveis de oxigênio dissolvido na água, tem sido intensamente estudada nos últimos anos (NIKINMAA e TERVONEN, 2004; NILSSON e OSTLUND-NILSSON, 2008; De BOECK e cols., 2013; MOYSON e cols., 2015; BOROWIEC e cols., 2015). A hipóxia desenvolve-se naturalmente em lagos cobertos de gelo ou estratificados como uma consequência de situações de eutrofização (DIAZ, 2001; DIAZ e ROSENBERG, 2008). Por outro lado, padrões intermitentes de hipóxia são comuns em correntes marinhas e estuários, principalmente em decorrência dos ciclos diários de respiração e fotossíntese (BREITBURG, 1992; DIAZ, 2001; TYLER e cols., 2009).

Apesar das situações naturais de queda nos níveis de oxigênio da água, nas últimas décadas, tem ocorrido uma série de alterações nas condições oceanográficas mundiais devido as ações antropogênicas no ambiente (FICHEZ e cols., 2005; DEBENAY e FERNANDEZ, 2009; CEARRETA e cols., 2013; SOUSA e cols., 2014). Como um dos resultados a longo prazo dessas interferências cita-se o aquecimento global, o qual afeta negativamente o teor de oxigênio dissolvido nos rios, lagos e oceanos e tem aumentado a incidência de zonas de mínimo de oxigênio oceânico que estão se expandido também para águas mais rasas (KEELING e GARCIA 2002; HELLY e LEVIN, 2004). Além disso, outros fatores contribuem para essa aumentada incidência de hipóxia aquática, tais como mudanças globais no clima, urbanização e a consequente poluição (DIAZ, 2001; FICKE, MYRICK e HANSEN, 2007).

De acordo com relatórios da OECD - “*Organisation for Economic Co-operation and Development*” Organização para desenvolvimento e cooperação econômica, constatou-se que as emissões globais de gases que causam o efeito estufa devem aumentar em 50% até 2050, principalmente em razão da maior demanda de energia e do crescimento econômico nos grandes países emergentes. Conforme esses relatórios, se não forem adotadas novas políticas governamentais de diminuição de emissão de poluentes imediatas, a concentração de CO₂ na atmosfera poderá atingir 685 ppm até 2050 o que acarretaria em um aumento na temperatura média do planeta de 3,7 °C a 6 °C até o final do século. Isso implicaria diretamente no derretimento das geleiras na Antártica, desencadeando uma série de mudanças climáticas no planeta (OECD, 2012), incluindo aumento dos episódios de hipóxia aquática, exigindo

complexos processos de ajustes fisiológicos e comportamentais que determinam a adaptação das espécies.

Os efeitos da hipóxia sobre os ecossistemas tem sido motivo de preocupação (CBD, 2012), uma vez que, em ambientes naturais, a hipóxia aguda produz vários efeitos adversos aos organismos aquáticos. O efeito mais direto é a deficiência brusca de oxigênio somada à liberação de sulfeto de hidrogênio, nutrientes e metais tóxicos a partir dos sedimentos de matéria orgânica e vegetal contidos nas profundezas oceânicas, com sucessiva progressão da hipóxia. O número dessas zonas hipóxicas mundiais passou de 10 em 1960 a 405 casos registrados em 2008. Conforme recente relatório da UNEP “*Sick Water*”, estima-se que 245.000 km² de ecossistemas marinhos estejam afetados por hipóxia, revelando impactos na pesca, nos meios de subsistência e na cadeia alimentar (UNEP, 2012).

Similarmente, em águas doces, durante o verão, pode ocorrer o fenômeno de eutrofização (multiplicação de microorganismos, como algas, que habitam a superfície das águas) em combinação com temperatura elevada que é fortemente influenciada por fontes difusas de nutrientes e falta de zonas ribeirinhas. A deterioração progressiva de espécies em ambientes eutróficos está associada com um aumento em áreas hipóxicas ou anóxicas de lagos, rios e estuários (LANDMAN, VAN DEN HEUVEL e LING, 2005). Como exemplo, cerca de 10% todos os lagos da Nova Zelândia são considerados tanto eutróficos ou hipertróficos (SMITH e cols., 1993). Por outro lado, durante o inverno, no norte da Europa, lagos rasos e lagoas tornam-se muitas vezes anóxicos devido à cobertura de gelo espesso que bloqueia a fotossíntese e difusão do oxigênio a partir do ar (NILSSON e RENSHAW, 2004).

No Brasil, entre as principais causas da ocorrência de hipóxia por ação antropogênica em águas doces, pode se citar a contaminação dos aquíferos urbanos por lixões e aterros, que invadem águas subterrâneas por meio da infiltração nos aterros inadequadamente gerenciados, que culminam com a liberação de chorume para rios e córregos, diminuindo assim a disponibilidade de oxigênio dessas águas (LINS e cols., 2010). As fossas sépticas também são responsáveis pela contaminação de aquíferos superficiais, tendo em vista que são adotadas por cerca de 16% das residências urbanas brasileiras e 9% das residências rurais (ANDREOLI e CARNEIRO, 2005). Adicionalmente, estima-se que o uso de fertilizantes e o despejo de esgotos domésticos têm acelerado o processo de eutrofização em reservatórios brasileiros (INSTITUTO INTERNACIONAL DE ECOLOGIA, 2000), principalmente devido ao excesso de nutrientes gerados, especificamente o nitrogênio e o fósforo, os quais servem de substrato para a proliferação de algas (BARROS, 2008). Essa proliferação excessiva de algas gera um desequilíbrio no balanço do oxigênio dissolvido na água aumentando a demanda por oxigênio

pelos organismos, o que causa a solubilização do fosfato, aumentando a concentração de gás sulfídrico, metano e amônia que são prejudiciais às espécies (SPERLING, 1994).

Também relacionado à eutrofização, em determinados biomas, devido ao clima e a formação vegetal típicos de áreas equatoriais (altas temperaturas e alto índice pluviométrico), com vegetação densa (com árvores de mais de sessenta metros de altura), muitas espécies aquáticas podem experimentar variações nos níveis de oxigênio dissolvido na água, diárias ou sazonais, podendo ocorrer até mesmo períodos de profunda hipóxia (VAL e cols., 1985), como exemplo da região amazônica brasileira (ou regiões equatoriais distribuídas pelo mundo, como nas florestas da Indonésia e Malásia), em decorrência da combinação de três fatores: proliferação de algas macrófitas fotossintéticas, água estagnada e decomposição da matéria orgânica (ALMEIDA-VAL e cols., 2000; CHIPPARI-GOMES e cols., 2005; RICHARDS e cols., 2007). Associadamente esses fatores aumentam o aporte de nutrientes ao meio aquático, aumentando o teor de carbono orgânico total e reduzindo os níveis de oxigênio da água que, conforme estabelecido pelo CONAMA (2005; 2011), devem estar, respectivamente, abaixo de 10 mg L^{-1} e, superior a $4 \text{ mg O}_2 \text{ L}^{-1}$ para águas de classe II (ou seja, águas tratadas que destinam-se ao consumo humano e aquicultura).

Neste sentido, esta diminuída disponibilidade de oxigênio faz com que os peixes tolerantes à águas hipóxicas desenvolvam estratégias adaptativas frente a esses ambientes adversos (VAL e ALMEIDA-VAL, 1995; CHIPPARI-GOMES e cols., 2005) a fim de garantir a sobrevivência de sua espécie. Entre essas estratégias observa-se a depressão nas taxas metabólicas e reorganização do fluxo sanguíneo (NILSSON e RENSHAW, 2004), além de alterações morfológicas, comportamentais e anatômicas frente à hipóxia, como ativação do metabolismo anaeróbico (SOMERO e SUAREZ, 2005), aumento a taxa de ventilação nas brânquias (GRAHAM, 1997), aumento da perfusão branquial (RUSSELL, DOMBKOWSKI e OLSON, 2008; SMITH e cols., 2001), aumento da área de superfície branquial (SOLLID e cols., 2006; SOLLID e NILSSON, 2006), alteração do débito cardíaco (SPEERS-ROESCH e cols., 2010), aumento da concentração de hemoglobina no sangue, assim como a afinidade de ligação Hb-O₂ (WELLS, 2009) e a extração de O₂ pelos tecidos e, finalmente, ativação de defesas antioxidantes (DOLCI e cols., 2013) em resposta ao estresse oxidativo.

Em relação aos processos oxidativos prejudiciais aos seres vivos, sabe-se que tanto a hipóxia severa (níveis iguais ou inferiores a $0,5 \text{ mg L}^{-1}$ de oxigênio dissolvido, saturação de O₂ ~ 10 %) quanto a hiperóxia (elevação no teor de oxigênio da água acima de $8,0 \text{ mg L}^{-1}$ de oxigênio dissolvido, saturação de O₂ ~ 80 %) podem atuar como importantes estressores,

constituindo-se em um fator limitante para o crescimento dos organismos (WILHELM FILHO e cols., 2005). Como exemplo do estresse oxidativo induzido por hipóxia, em um estudo de Braun e cols. (2008) utilizando *Rhamdia quelen*, tanto a hipóxia severa quanto moderada aumentaram a peroxidação lipídica, assim como induziram concomitante ativação da enzima antioxidante superóxido dismutase (SOD). Adicionalmente, como consequência da hiperóxia aos organismos, foi observado por Lushchak e cols. (2005) que a exposição a altos níveis de oxigênio dissolvido na água aumenta a geração de espécies reativas de oxigênio (ERO), ânion superóxido (O_2^-), peróxido de hidrogênio (H_2O_2) assim como do radical hidroxila ('OH), radical livre com potencial extremamente reativo (HALLIWELL and GUTTERIDGE, 1989).

Situações de estresse por hiperóxia podem ser experimentadas em ambientes naturais pelos diferentes organismos aquáticos, como por exemplo, o fitoplâncton, que experimenta hiperóxia transitória durante o verão, como consequência da aumentada produção de oxigênio (fotossíntese) pelas algas em decorrência da maior exposição à luminosidade solar (HALLIWELL e GUTTERIDGE, 1989; PEDERSEN, COLMER e SAND-JENSEN, 2013). Entretanto, a radiação ultravioleta (UVA e UVB), mais intensa durante o verão, pode inibir a produção fotossintética para cerca de 8% (HELBLING, VILLAFANE, HOLM-HANSEN, 1993). Condições hiperóxicas podem ocorrer também em situações de transporte de peixes vivos sob uma atmosfera de oxigênio, uma prática amplamente utilizada na indústria pesqueira. Atenção particular a este tipo ocasional de estresse tem sido relatada por Golombieski e cols. (2003), Azambuja e cols. (2011) e Becker e cols. (2012), que investigaram a ação potencial de óleos essenciais que atuam como anestésicos e, dessa forma, podem minimizar o estresse de transporte, induzido tanto por hipóxia aguda, quanto hiperóxia.

Vários estudos demonstraram que essas transições bruscas entre hipóxia/anoxia e normóxia e/ou entre a normóxia e hiperóxia resultam em estresse oxidativo em peixes (HALLIWELL e GUTTERIDGE, 1989; STOREY, 1996; LUSHCHAK, 2002; HERMES-LIMA, 2004) Concomitantemente, quanto aos danos gerados por eventos de hipóxia severa em peixes, estudos defendem que não a hipóxia, mas sim a reoxigenação constitui o fator mais crítico para estas espécies (BARRY, 1994), uma vez que induz excessiva geração de ERO que são potencialmente prejudiciais aos organismos. Em mamíferos, a hipóxia também pode provocar danos ou até mesmo ser fatal, uma vez que o subsequente processo de reoxigenação de órgãos isquêmicos pode ser o gatilho para o início da formação dessas ERO, que resultam do vazamento de elétrons da cadeia mitocondrial e das reações das oxidases mitocondriais como a xantina oxidase, citocromo P₄₅₀ redutase, glicose oxidase, entre outras (WINSTON e DI GIULIO, 1991; KELLY e cols., 1998). Dessa forma, as ERO atuam como potenciais

desencadeadoras de processos de peroxidação lipídica, oxidação de proteínas e danos diretos ao DNA (LEFER e GRANDER, 2000; WHITE e cols., 2000). Em mamíferos, esses danos decorrem, principalmente, da sua menor capacidade antioxidant frete a situações anóxicas/hipóxicas (HERMES-LIMA e ZENTENO SAVÍN, 2002) em relação a outros vertebrados.

Por exemplo, muitos vertebrados de água doce parecem ajustar-se fisiologicamente para lidar com essas flutuações periódicas nos níveis de oxigênio, como as tartarugas de água doce (STOREY, 1996), que mantêm suas defesas antioxidantes elevadas durante todo o período de transições nos níveis de oxigênio, enquanto outras, como *Carassius auratus* (LUSHCHAK e cols., 2001) e carpa comum (*Cyprinus carpio*) (VIG e NEMCSOK, 1989) usam a estratégia antecipatória de aumentar a sua capacidade antioxidant frete ao estado hipóxico ou anóxico, a fim de se prepararem para enfrentar o estresse oxidativo gerado quando os níveis de oxigênio são novamente restaurados à normoxia (HERMES-LIMA e ZENTENO-SAVIN, 2002; HERMES-LIMA, 2015). Tais ajustes são considerados uma adaptação evolutiva nessas espécies e desempenham um papel de proteção durante as condições de deficiência de oxigênio permitindo que estes possam ajustar-se mais rapidamente frete a uma posterior reoxigenação dos tecidos (HO e BURGGREN, 2012).

1.2 O MANGANÊS: DE MICROELEMENTO ESSENCIAL A POLUENTE AMBIENTAL

A compreensão das alterações morfofisiológicas apresentadas pelos indivíduos durante situações de contaminações ambientais ainda permanece limitado considerando a enorme biodiversidade aquática. Por essa razão, vários modelos experimentais têm sido utilizados na tentativa de mimetizar as diferentes situações adversas experimentadas pelos organismos no seu ambiente natural (BIANCHINI e cols., 2002; BRAZ-MOTA e cols., 2015; RANSBERRY, BLEWETT e McCLELLAND, 2016). Na verdade, espécies distintas de peixes podem reagir de maneira diferente frete a exposição a iguais concentrações de um mesmo contaminante (HOWE, MALCOLM e DOBSON, 1999). Neste sentido, a sobrevivência das espécies é dependente de uma série de fatores que compreendem, entre outros, as condições fisiológicas do animal e a capacidade deste em reagir frete ao agente potencialmente tóxico ao qual ele é exposto.

Conectado com isso, considera-se a importância do Mn, em baixas concentrações, como microelemento essencial aos eucariontes atuando como co-ativador da enzima antioxidante Mn-superóxido dismutase (Mn-SOD), encontrada nas mitocôndrias. De fato, a relevância do Mn para a proteção contra o estresse oxidativo é essencial, como foi demonstrado por [Li e cols. \(1995\)](#), que observaram que a ablação do gene que codifica para SOD2 (Mn-SOD) induz morte perinatal em ratos por estresse oxidativo massivo.

O Mn pode ser encontrado naturalmente em águas doces em baixas concentrações ($1 - 200 \mu\text{g L}^{-1}$) ([BARCELOUX, 1999](#)). Entretanto, a constante emissão de agentes químicos ao ambiente pode produzir contaminações expressivas tanto em reservatórios de águas doces, assim como de águas oceânicas ([OECD, 2012; ATSDR, 2000](#)). Dessa forma, a grande quantidade de Mn liberada ao meio ambiente pode atuar como gerador de ERO devido ao seu alto potencial redutor ([ZHANG e cols., 2003](#)), desencadeando a redução do óxido de manganês (MnO_2) à manganês divalente (Mn^{2+}). Sob condições limitadas de oxigênio essa mobilidade redox do Mn aumenta podendo exacerbar a sua toxicidade sobre a biota aquática ([LIMBURG e cols., 2011; ITAI e cols., 2012](#)). De fato, a mobilização de elementos com sensibilidade redox como ferro, manganês e arsênio em lagos hipóxicos foi reportada em várias regiões ([AURILIO, MASON e HEMOND, 1994; MARTIN e PEDERSEN, 2002](#)) com consequentes prejuízos aos organismos expostos a estes elementos.

Embora a concentração máxima permitida de Mn em águas brasileiras para o consumo humano seja de $0,1 \text{ mg L}^{-1}$ ([CONAMA, 2011](#)), o metal pode atingir altas concentrações no ambiente, devido à poluição causada por atividades antropogênicas como extração de carvão ([CROSSGROVE E ZHENG, 2004](#)) e prospecção de combustíveis fósseis, como ocorre na exploração do petróleo ([HATJE e cols., 2008](#)). De fato, valores de até $6,44 \text{ mg L}^{-1}$ já foram encontrados em águas de formação (geradas pela incorporação de efluentes ao petróleo que emerge do subsolo sob alta pressão) na Reserva de Urucu, Amazonas ([BALDISSEROTTO e cols., 2012; GABRIEL e cols., 2013](#)). Além disso, altos níveis de Mn na água (superior a 1 mg L^{-1}) já foram detectados em áreas próximas à depósitos de resíduos tóxicos nos Estados Unidos ([ATSDR, 2000](#)).

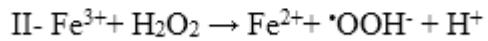
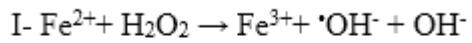
Como outro indício de contaminação por Mn, devido a fatores ligados ao mau gerenciamento de resíduos tóxicos (descarte inadequado de fungicidas e agroquímicos usados em plantações, entre outros), cita-se o episódio ocorrido em 2010, no Rio dos Sinos, RS, o qual foi responsável pela mortalidade de mais de três toneladas de peixes. Neste caso específico, foram encontrados níveis quatorze vezes superiores ao valor habitual de Mn presente nesse rio em amostras enviadas ao laboratório de Zoologia e Botânica do Centro Universitário Metodista

do Sul (IPA), Porto Alegre (RS). Porém, os laudos finais sobre o poluente responsável pela alta mortalidade dos peixes foram inconclusivos, uma vez que o Rio dos Sinos é alvo de poluição constante por resíduos de origem doméstica e industrial, encontrando-se entre o quarto rio mais poluído do Brasil ([FEPAM, 2011](#)).

Quando em excesso, o Mn, exerce toxicidade diretamente devido à ligação inadequada a moléculas biologicamente sensíveis (reação característica dos metais de transição) ou pela formação de ERO potencialmente danosas, principalmente via reação de Fenton (Figura 1), na qual o Mn, por possuir carga divalente, pode deslocar o ferro e substituí-lo na reação rompendo, consequentemente, a homeostase (equilíbrio entre a deficiência de metal e seu excedente) nos organismos. O Mn pode também exercer sua toxicidade indiretamente, por causar disfunção mitocondrial devido a sua propensão para acumular-se preferencialmente na matriz mitocondrial ([LICCIONE e MAINES, 1998; BROWN e TAYLOR, 1999](#)), ocorrendo ainda elevada afinidade para o uniporte de cálcio (Ca^{2+}) ([GAVIN; GUNTER e GUNTER, 1990; 1999](#)). Nesse processo, o Mn^{2+} substitui o Fe^{2+} nos citocromos da cadeia respiratória de elétrons (com estrutura semelhante à hemoglobina) ([MISSY e cols., 2000](#)) causando a redução incompleta do oxigênio molecular (O_2) e assim a formação de radicais livres como $\cdot\text{O}_2^-$ e compostos oxigenados tais como H_2O_2 .

Em peixes, a exposição ao Mn pode produzir toxicidade por duas vias de aquisição, seja através da dieta (por influência dos níveis de Mn presentes na ração) ou devido a direta absorção do metal através da água. De acordo com [Howe, Malcolm e Dobson \(1999\)](#), no ambiente aquático, devido à variação entre as superfícies corporais disponíveis para absorção e trocas gasosas entre as espécies, não é permitido estabelecer uma concentração definida de Mn que cause toxicidade a todos os organismos, havendo apenas uma concentração máxima aceitável do metal que diminua riscos às espécies em geral.

Figura 1 – Reação de Fenton



Fonte: Adaptado de [KREMER, 2003](#), p. 1734.

Em jundiás (*Rhamdia quelen*) mantidos em laboratório, a exposição ao Mn sob normoxia, em concentrações de 4,2 a 16,2 mg L⁻¹, é responsável pela redução da viabilidade das mitocôndrias de brânquias e de fígado (DOLCI e cols., 2013). Possivelmente, exacerbação desse efeito tóxico pode ser encontrado em ecossistemas naturais, uma vez que o fluxo iônico de Mn estaria aumentado frente a quantidades reduzidas de oxigênio dissolvido (pela deposição da matéria orgânica), migrando do sedimento às diferentes camadas de água, podendo atingir concentrações até mil vezes superiores aquelas naturalmente observadas em águas marinhas (22 mg L⁻¹) (ALLER, 1994). Adicionalmente, Dolci e cols. (2014) demonstraram efeitos prejudiciais do Mn (9,8 mg L⁻¹) como indutor de danos oxidativos em diferentes tecidos de jundiás, bem como alterações na expressão de genes hormonais hipofisários dessa espécie.

Estudos de Campos e Beaugé (1988) demonstraram que o manganês pode substituir o magnésio (coativador essencial para a atividade da enzima) (JORGENSEN, HAKANSSON e KARLISH, 2003) na estrutura da Na⁺/K⁺-ATPase, ligando-se fortemente e com alta afinidade à Na⁺/K⁺-ATPase durante a fosforilação promovida por ATP. Essa alta afinidade de ligação do Mn com a Na⁺/K⁺-ATPase está relacionada com os desequilíbrios induzidos por Mn na atividade dessa enzima, uma vez que ela é responsável pela manutenção dos gradientes de Na⁺ e K⁺ através das membranas celulares (MAGISTRETTI e PELLERIN, 1999).

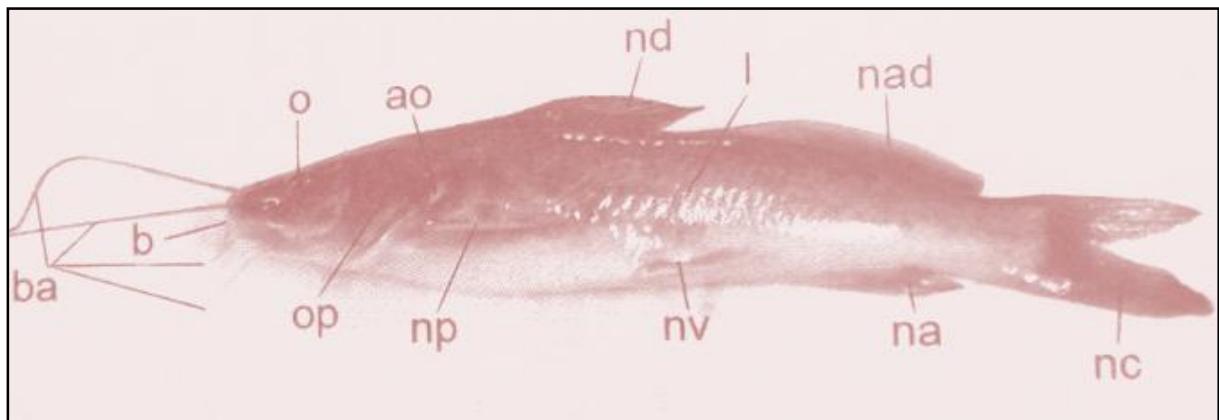
1.3 PEIXES COMO MODELO EXPERIMENTAL

1.3.1 Jundiá

O jundiá (Figura 2) é um peixe onívoro, com tendência piscívora, de ampla distribuição geográfica, tendo sua ocorrência registrada desde a região central da Argentina até o sul do México, possuindo distribuição neotropical (SILFVERGRIP, 1996; GOMES e cols., 2000). De acordo com Silfvergrip (1996), após ampla revisão taxonômica do gênero, baseada em características da morfologia interna, concluiu que o gênero *Rhamdia* é formado de apenas onze espécies dentre as cem anteriormente descritas. Segundo o mesmo autor, *Rhamdia quelen* pertence à seguinte divisão taxonômica: Classe: Osteichthyes, Série: Teleostei, Ordem: Siluriformes, Família: Heptapteridae, Gênero: Rhamdia, Espécie: *Rhamdia quelen*.

Com a finalidade de estudar os efeitos da aclimatação a hipóxia e da potencial toxicidade do manganês (Mn) em peixes, se encontrou no jundiá um modelo promissor, uma vez que se trata de uma espécie nativa brasileira ([BALDISSEROTTO \(2009\)](#) bem adaptada a diferentes ambientes e amplamente utilizada em viveiros de piscicultura ([GOMES e cols., 2000](#)).

Figura 2 – Jundiá (*Rhamdia quelen*).



Fonte: Adaptado de Biologia e Fisiologia de Peixes Neotropicais de Água Doce, [BALDISSEROTTO, CYRINO e URBINATI, 2014](#), p. 18.

ao: abertura opercular; b: boca; ba: barbillões; l: linha lateral; na: nadadeira anal; nad: nadadeira adiposa; nc: nadadeira caudal; nd: nadadeira dorsal; np: nadadeira peitoral; nv: nadadeira ventral; o: olho; op: opérculo.

O jundiá pode atingir cinquenta centímetros de comprimento e três quilos de peso corporal. Possui hábito noturno sendo encontrado em locais calmos e profundos dos rios. Os alevinos suportam transferência de água de 0 %, a 10,0 g % (água do mar) e 9,0 g L⁻¹ de sal comum (NaCl) por 96h, indicando ser uma espécie estenoialina, de modo que o uso de sal comum para tratamento de doenças pode ser aplicado nesta espécie sem prejuízos ([MARCHIORO, 1997](#)). Experimentos de [Marchioro \(1997\)](#) demonstraram que os alevinos também suportam uma variação de pH na faixa de 4,0 a 8,5 (dureza de 30,0 mg L⁻¹ CaCO₃), porém essa exposição a águas ácidas ou alcalinas provoca uma redução dos níveis corporais de Na⁺ e K⁺, como já relatado para outros teleósteos de água doce.

O jundiá pode ser considerado uma espécie euritolerante, uma vez que alevinos aclimatados a 31° C suportam temperaturas de 15 à 34° C. A aclimatação a temperaturas mais baixas produz uma maior tolerância à redução de temperatura, mas o limite superior de tolerância praticamente não se altera ([CHIPPARI-GOMES, GOMES e BALDISSEROTTO,](#)

1999). De acordo com Gomes e cols. (2000) o jundiá é uma espécie ovulípara e com desova em locais de águas limpas, calmas e de fundo pedregoso.

Essa espécie tem despertado grande interesse nos piscicultores da Região Sul do Brasil (BARCELOS, FAGUNDES e FERRREIRA, 2013), pela sua resistência ao manejo, docilidade e crescimento acelerado, inclusive no inverno (CARNEIRO e cols., 2002; FRACALOSSI, ZANIBONI FILHO e MEURER, 2002). Adicionalmente possui boa taxa de conversão alimentar e, sobretudo, apresenta carne saborosa, sem espinhos intramusculares.

Os estudos com o jundiá tem se intensificado no Brasil. Contudo, ainda existem alguns entraves para sua produção, principalmente pela susceptibilidade dos alevinos ao protozoário conhecido como íctio (*Ichthyophthirius multifiliis*) assim como o crescimento heterogêneo da espécie (FRACALOSSI e cols., 2007).

1.3.2 As brânquias e a circulação sanguínea

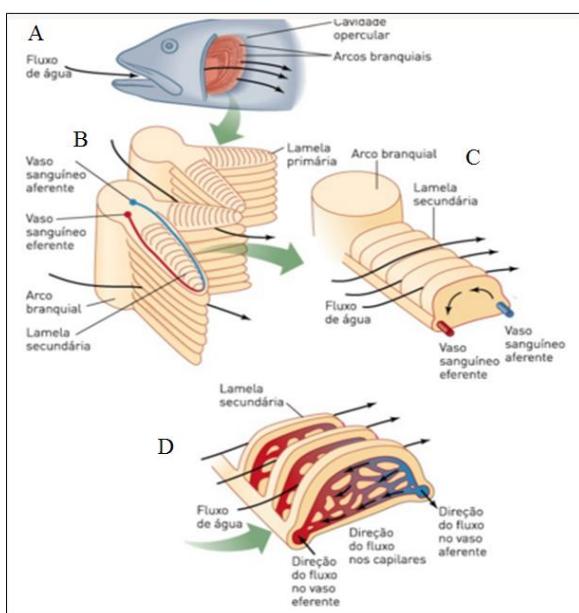
As brânquias são estruturas responsáveis pela respiração em peixes adultos (exceto naqueles de respiração aérea), e são constituídas por quatro arcos branquiais de cada lado da faringe, que por sua vez, são formados por duas fileiras de filamentos branquiais. Nos filamentos encontram-se as lamelas (de 20 a 100 µm, dependendo da espécie e nível de atividade da espécie). As lamelas são compostas por células pilares e cobertas por uma membrana basal e o epitélio, o qual é contínuo com o filamento e constituído por uma camada interna de células não diferenciadas e uma externa de células pavimentosas. A espessura do epitélio pode variar de 3 µm em peixes de natação rápida, a 10-14 µm em peixes de natação lenta (BALDISSEROTTO, 2013).

As brânquias representam 90% da superfície total do corpo e estão em contínuo contato com a água através de uma superfície fina e extensa, assumindo três funções principais: trocas gasosas, regulação iônica e excreção de produtos do metabolismo (EVANS, PIERMARINI e CHOE, 2005; DOLCI e cols., 2014). Devido ao íntimo contato com o ambiente externo, as brânquias constituem o primeiro alvo de poluentes por via aquática (PERRY e LAURENT 1993), sendo suscetíveis a danos causados por metais.

A circulação de sangue dentro da lamela ocorre em um sentido, enquanto a circulação de água para fora da lamela ocorre no sentido oposto, formando um sistema contracorrente (Figura 3 C, D). A água que sai das brânquias tem pouco oxigênio, cruzando com o sangue que entra nas brânquias, com menos oxigênio ainda (pressão parcial de oxigênio menor). Porém, a

medida que o sangue circula através das lamelas, vai recebendo mais oxigênio por difusão da água que está passando entre as lamelas, que sempre tem maior pressão parcial de oxigênio. Este arranjo permite que 80-85 % do oxigênio da água se difunda para o sangue que passa pelas brânquias (BALDISSEROTTO, 2013).

Figura 3 – Representação esquemática da respiração branquial de peixes teleósteos, demonstrando o fluxo de água e sangue através das brânquias.



Fonte: CHACON e LUCHIARI, 2011, Site: <<http://www.geefaa.com>> Acesso em novembro de 2015.

Adicionalmente, nos filamentos branquiais podem ser encontradas fibras nervosas, células mucosas (produtoras de muco), células neuroepiteliais (produzem serotonina e são quimiorreceptoras, respondendo a hipóxia), células de cloreto (regulam trocas iônicas), células não diferenciadas e células pavimentosas (BALDISSEROTTO, 2013).

Em alguns peixes tolerantes à hipóxia, como *Ictalurus punctatus*, a diminuição da pressão parcial de oxigênio na água e no sangue (hipóxia) é detectada por quimiorreceptores localizados nos três primeiros arcos branquiais, desencadeando o fechamento dos canais de K⁺ da membrana plasmática, estimulando assim o quimiorreceptor. Na tentativa de manter a pressão de oxigênio constante, este peixe aumenta a ventilação branquial através da elevação da frequência respiratória, demonstrando a aclimatação à hipóxia dessa espécie (BURLESON

CARLTON e SILVA, 2002). Esse aumento da ventilação aumenta o volume de água que passa através das brânquias aumentando a disponibilidade de oxigênio para as trocas gasosas. Assim, a exposição de *Ictarus punctatus* (respiração aquática exclusiva) à hipóxia pode resultar em aumento da sua área branquial na tentativa de aumentar a eficiência de captação de oxigênio (**BALDISSEROTTO, 2013**).

1.3.3 O fígado

O fígado de teleósteos é uma glândula anexa, derivada embrionariamente do intestino, situando-se dentro da cavidade abdominal e separado da cavidade pericárdica por um septo transversal. Possui formas diversas, de coloração escura, tendo como anexo a vesícula biliar (com funções biliar, glicogênica e adipogênica), também de formatos diversos (**MOREIRA, 2001**). É uma estrutura bem desenvolvida com abundante secreção de bile e, histologicamente, quando comparado ao fígado de mamíferos, não apresenta lóbulos (**GUILLAUME e CHOUBERT, 2001**). O fígado secreta emulsificadores que são carregados pela bile até o intestino, auxiliando a digestão de lipídios (**WEATHERLEY e GILL, 1987**).

O fígado de peixes é, sobretudo, um órgão dotado de uma numerosa quantidade de enzimas antioxidantas (**DAS, SARKAR e BHATTACHAARYA, 1998**) e detoxificantes (**GLUSCZAK e cols., 2000**), sendo o principal local de produção e exportação de glutationa reduzida (GSH) (**WU e cols., 2004**). É importante salientar que a GSH atua como um sequestrante poderoso de radicais livres e espécies reativas de oxigênio (ROS), através de reações enzimáticas em que a GSH é oxidada para gerar glutationa oxidada (GSSG), que é subsequentemente reduzida para GSH pela atividade de NADPH dependente de glutationa reduzida (GR) (**WU e cols., 2004**). Além disso, a GSH é cofator essencial para enzimas desintoxicantes que atuam contra o estresse oxidativo, tais como glutationa peroxidase (GPx) e glutationa transferase (GST) (**RAHMAN, 2007; VALKO e cols., 2006; JAESCHKE, 2010**).

Adicionalmente a GSH desempenha um papel importante na regulação da ligação das proteínas dissulfeto, assim como na remoção de eletrófilos e oxidantes, devido à presença de cisteína na sua estrutura (**MARI e cols., 2009**). Além da GSH e as enzimas envolvidas na sua regeneração, a catalase (CAT) constitui-se em outra enzima com alta atividade no fígado (**YOUNG e WOODSIDE, 2001**) e possui função de catalisar a conversão do peróxido de

hidrogênio (H_2O_2) à moléculas mais estáveis como água e oxigênio molecular, reduzindo dessa forma os danos oxidativos.

1.3.4 O rim – células cromafins e inter-renais

Os rim e ureteres, órgãos secretor e excretor, respectivamente, regulam o conteúdo de água no corpo e mantem o equilíbrio osmótico com o meio. Apresentam-se como duas massas sanguíneas paralelas e dispostas longitudinalmente junto à coluna vertebral. Geralmente os ductos excretores (ao sair dos rim) unem-se, formando apenas um, o qual desemboca em uma bexiga urinária ou diretamente no orifício urogenital, ou na cloaca, dependendo da espécie. Os peixes de água doce possuem rim maior, com maior número de glomérulos que peixes de água salgada, pois vivem em um meio externo menos concentrado e assim tem maior entrada de água por osmose (EVANS, 2010).

Em teleósteos a glândula inter-renal não apresenta uma região cortical e outra medular como nos demais vertebrados, pois as células estão dispostas agrupadamente ao longo do rim. Contudo, possuem as células cromafins, análogas às existentes na medula adrenal, e as inter-renais, equivalentes às do córtex de outros vertebrados (BALDISSEROTTO, 2013). As células cromafins podem ser estimuladas (a partir da liberação de serotonina no sistema nervoso central ou, acetilcolina nas fibras pré-ganglionares do sistema nervoso simpático) a liberar catecolaminas como resposta a estressores (hipóxia ou Mn). Consequentemente, uma das ações da liberação de catecolaminas é a ativação dos sistemas respiratório e circulatório para aumentar a distribuição de oxigênio, de modo que a demanda energética corporal possa ser atendida. Essa ativação do sistema circulatório faz com que a pressão sanguínea se eleve, havendo dilatação dos vasos sanguíneos das brânquias, com consequente aumento de perfusão nas lamelas e captação de oxigênio. Isso causa uma maior permeabilidade branquial e maior perda de íons por difusão e entrada de água por osmose em peixes de água doce (BALDISSEROTTO, 2013).

As células inter-renais produzem cortisol, hormônio com funções catabólicas, e que, de modo geral, estimula o desenvolvimento e proliferação das células de cloreto nas brânquias e aumenta a atividade da bomba Na^+/K^+ -ATPase a qual, em peixes de água doce, desenvolve ação conjunta com prolactina (PRL) (BALDISSEROTTO, 2013). Quando teleósteos são expostos a estressores (tais como hipóxia ou exposição a metais, por exemplo) há uma liberação de serotonina pelo sistema nervoso central, que por sua vez, estimula o hipotálamo a liberar o

fator liberador de corticotrofina (CRH) que faz aumentar a liberação de ACTH pela adenohipófise. A urotensina I (UI), com funções hipotensoras, nesse caso atua como neurotransmissor, sendo também liberada pelo hipotálamo e atuando na adenohipófise. O ACTH atua nas células inter-renais estimulando a secreção de cortisol, cujos níveis aumentam até que, por retroalimentação negativa, inibem a liberação de CRH e UI pelo hipotálamo, reduzindo a liberação do ACTH e do próprio cortisol (MOMMSEN, VIJAYAN e MOON, 1999), havendo portanto a autorregulação dos níveis de cortisol frente à situações estressoras, mantendo o equilíbrio entre as funções orgânicas anabólicas e catabólicas.

1.3.5 O encéfalo

Os peixes teleósteos apresentam um sistema nervoso composto pelo sistema cérebro-espinal (encéfalo, medula espinal, gânglios, nervos craniais e raquidianos) e sistema nervoso autônomo (gânglios, nervos simpáticos e parassimpáticos). O sistema cérebro-espinal possui as mesmas divisões que para os vertebrados superiores, embora apresente um desenvolvimento relativamente diferente. É portanto formado pelo: i) telencéfalo, que se reduz a lóbulos olfatórios anteriores e posteriores; ii) diencéfalo, constituído pelo epitélamo, tálamo, hipotálamo e, em sua parte anterior, pelo quiasma ótico (impulsos olfativos e visuais e associados a outras glândulas); iii) mesencéfalo, constituído pelos lóbulos óticos; iv) metencéfalo, o qual origina o cérebro que é o centro de coordenação muscular (peixes de movimentos rápidos possuem esse centro bem desenvolvido) e, por fim, v) miencéfalo, que forma o bulbo (análogo à medula espinal de vertebrados), sendo o centro de atividades vitais como respiração, ação cardíaca, metabolismo, equilíbrio da linha lateral e ouvido interno (MOREIRA, 2001).

O sistema nervoso autônomo (SNA) é o responsável pelo controle das funções orgânicas nos peixes, como abertura da íris, pressão sanguínea, fluxo sanguíneo através das brânquias, distribuição de sangue para os tecidos, funções cardíacas, motilidade gástrica, entre outras. O SNA também obedece a divisão em i) SNA simpático, com fibras pré-ganglionares colinérgicas (liberam acetilcolina) e pós-ganglionares adrenérgicas (liberam noradrenalina e adrenalina) e, ii) SNA parassimpático, com fibras pré e pós-ganglionares colinérgicas (MOREIRA, 2001).

Adicionalmente, o encéfalo agrega o hipotálamo, localizado na porção inferior do cérebro, o qual funciona como uma interface entre os sistemas nervoso e endócrino. Conectado ao hipotálamo encontra-se a hipófise, dividida em neurohipófise e adenohipófise. Assim,

algumas células neurosecretoras possuem o corpo celular (onde o hormônio é produzido) no hipotálamo, mas o final do axônio (onde é liberado o hormônio) dentro da neurohipófise. Os teleósteos não possuem o sistema vascular porta-hipotalâmico-hipofisário, que conduz o sangue que passa no hipotálamo diretamente até a adenohipófise, como observado nos demais vertebrados. Entretanto, as células neurosecretoras hipotalâmicas liberam hormônios que influenciam a produção e liberação dos hormônios pela adenohipófise (BALDISSEROTTO, 2013).

Entre os hormônios liberados pelo hipotálamo e armazenados na neurohipófise encontra-se o hormônio concentrador de melanóforos (MCH), no qual o subtipo MCH-R1 estimula a agregação de pigmentos nos melanóforos (células que contêm pigmentos escuros), xantóforos (pigmentos amarelos) e eritróforos (pigmentos vermelhos) na pele, responsáveis pela agregação dos grânulos de pigmentos (promovem clareamento da pele) de teleósteos (KAWASHI e cols., 1983), sendo bastante conservado entre as espécies. A liberação de MCH inibe a liberação de ACTH e α-MSH (BALDISSEROTTO, 2013).

Por outro lado, o hormônio liberador de corticotrofina (CRH) (hormônio liberado pelo hipotálamo, que altera a produção e/ou liberação de hormônios da adenohipófise), estimula a liberação de ACTH e α-MSH pela adenohipófise. O α-MSH é um dos hormônios pigmentares mais relevantes do ponto de vista fisiológico, estimulando a translocação de grânulos de pigmento e síntese de melanina, assim como proliferação celular (TAKAHASHI e KAWAUCHI, 2006) e, em teleósteos, induz a dispersão pigmentar (promovem escurecimento da pele) (FUGII, 2000; TAKAHASHI e KAWAUCHI, 2006). Além do CRH, outros hormônios são liberados pelo hipotálamo e desempenham ações na adenohipófise como o fator liberador do hormônio do crescimento (GHRH), que estimula a liberação do hormônio do crescimento (GH) e o fator liberador da prolactina (PrRP) que estimula a liberação de prolactina (BALDISSEROTTO, 2013).

Observada a complexidade do encéfalo em teleósteos, numerosos contaminantes já foram descritos como causadores de prejuízos oxidativos sobre esse órgão, assim como desencadeantes de interrupção hormonal em peixes (LINS e cols., 2010; DOLCI e cols. 2013, 2014; BRAZ-MOTA e cols., 2015). Portanto, o encéfalo pode representar um alvo promissor para o estudo da toxicidade de poluentes (BAGNYUKOVA e cols., 2005b, MODESTO e MARTINEZ, 2010b; BRAZ-MOTA e cols., 2015) assim como para estudar os efeitos das mudanças nos níveis de oxigênio (normoxia/hipoxia/hiperoxia) da água (LUSHCHAK e cols., 2005; PARENTE e cols., 2013).

1.4 PARÂMETROS COMPORTAMENTAIS

O estudo do comportamento em peixes é uma ciência que comprehende uma rede complexa de fatores que guiam os padrões motores, suas motivações e influências evolutivas genéticas, responsáveis pela conservação das espécies. Segundo conceito proposto por Timo-Iaria (1985) o comportamento pode ser dividido em dois componentes característicos: motor ou clássico, representado pela atividade locomotora, movimentos de virar, saltar, assim como de imobilidade; e o componente que está relacionado aos ajustes fisiológicos que ocorrem nessas situações. Tais ajustes ocorrem para dar suporte ao funcionamento das células nervosas e musculares, promovendo ajustes característicos para um dado comportamento (VOLPATO, 2007b).

Para a compreensão adequada do comportamento animal é necessário que se considere o organismo como um todo inserido em seu ambiente natural e, esse local, inserido num ambiente mais complexo, originário de uma história evolutiva. Para que um comportamento seja efetivado em uma resposta motora é necessário que seja analisado associadamente ao sistema nervoso central (SNC), que é considerado um sistema de controle. Além do SNC o sistema endócrino também atua na modulação do comportamento tornando os animais mais ou menos sensível a estímulos externos, como no caso de uma interrupção do eixo endócrino por exposição a substâncias que possuem essa característica (MATTHIESSEN e LOGAN, 1984; TANNER e KNUTH, 1996). Nesse caso, o componente motor e o neurovegetativo (SNA) são ordenados de forma independente, mas que culminam em um resposta coerente.

Neste sentido, o comportamento animal pode ser entendido a partir de alguns referenciais, existindo categorias distintas de comportamento. Alguns comportamentos estão bem documentados para o jundiá como i) fototaxia, caracterizada pela resposta do peixe frente a uma fonte luminosa (CECCARELLI, SENHORINI e VOLPATO, 2000; HUNTINGFORD e TURNER, 1987) e que, no caso do jundiá, naturalmente observa-se fototaxia negativa; ii) tigmotaxia, ou seja, resposta do peixe ao contato com elementos do ambiente, uma vez que os jundiás apresentam tendência a permanecerem nos cantos de um aquário; e iii) geotaxia, que diz respeito ao comportamento em resposta à gravidade, que pode ser influenciado pelo hábito de vida (superfície, meia-água, fundo) e que para os jundiás, caracteriza-se por geotaxia positiva uma vez que essa espécie tende a ficar mais no substrato.

A aprendizagem é um processo que envolve, necessariamente, a participação de processos de memória. Considera-se, portanto, que a aprendizagem ocorre quando, após algumas experiências, o animal muda o seu comportamento, sem que essa mudança seja

atribuída à alteração na motivação, maturação, injúria ou idade. Nesse sentido, a habituação é o tipo mais simples de aprendizagem e trata-se na redução da emissão de um comportamento, em função de seu estímulo desencadeador repetir-se com uma frequência determinada (VOLPATO, 2014) e, neste estudo, foi acessada pelo teste do labirinto de interação social e/ou ansiedade (SOVRANO e cols., 2002; GRASSIE e cols. 2013).

Estudos mais sofisticados têm mostrado que peixes possuem habilidades cognitivas complexas e, portanto, podem ser seres sencientes (ou seja, dotados de capacidade de perceber e distinguir estados internos de prazer e desprazer, calor e frio, conforto e dor), podendo ser não apenas condicionados (MOREIRA e VOLPATO, 2004; MOREIRA, PULMAN e POTTINGER, 2004), mas também formar mapas cognitivos de seus ambientes, assim como avaliar noções de tempo em estudos de condicionamento. Nessa linha de estudo Portavella e cols. (2002) demonstraram que os peixes possuem estruturas cerebrais homólogas à amígdala e hipocampo dos mamíferos e que alterações nessas áreas prejudicam o aprendizado de tarefas mais complexas (SNEDDON, 2006).

Neste sentido, a avaliação da resposta do peixe frente a coespecíficos (substância de alarme) (CHIVERS e SMITH, 1998; WEBSTER e WEISSBURG, 2009) pode fornecer informações sobre alterações que a espécie exibe frente a fatores estressantes. Este teste avalia o comportamento do peixe frente a um predador, pois quando o predador ataca um peixe e causa danos substanciais à pele da presa, células especializadas na epiderme liberam uma substância química que funciona como um sinal de alarme, detectado através do olfato por outros peixes no aquário que geram um padrão de nado estereotipado (de um lado a outro do aquário) na tentativa de evitar o predador, reduzindo dessa forma o risco de predação (SCOTT e cols., 2003). Este teste pode fornecer também informações adicionais como alterações na frequência ventilatória dos animais, e hábito alimentar (SANCHES e cols., 2015) frente à fatores estressantes.

1.5 PARÂMETROS HEMATOLÓGICOS

O estudo dos parâmetros hematológicos constitui ferramenta sensível para o monitoramento de alterações fisiológicas em organismos expostos a estressores ambientais (ZHANG e cols., 2007). Alterações nos níveis de oxigênio da água, como por exemplo a

hipóxia, podem conduzir a ajustes no débito cardíaco ([SPEERS-ROESCH e cols., 2010](#)), alterar os níveis de hematócrito (por percentagem da contagem de eritrócitos) e a afinidade de hemoglobina pelo oxigênio (Hb-O₂), com o objetivo de aumentar a captação do O₂ da água pelos tecidos durante hipóxia ([WELL e cols., 2009](#)).

Entre os mecanismos que contribuem para o aumento da afinidade de oxigênio pela hemoglobina, cita-se a diminuição dos níveis de GTP e ATP intraeritrocitários devido à inibição da produção de ATP pela fosforilação oxidativa, de modo que organismos intolerantes à hipóxia não conseguem manter sua produção de energia sob tal condição. Adicionalmente, durante hipóxia, ocorre hiperventilação, redução do CO₂ do sangue, provocando uma alcalose respiratória devido à elevação do pH sanguíneo e, dependendo do grau de hipóxia, pode ocorrer concomitante aumento de catecolaminas, contribuindo para um aumento adicional na ligação O₂-Hb ([BALDISSEROTTO, 2013](#)).

Por outro lado, a análise de leucócitos permite obter informações como alterações na imunidade desses animais, uma vez que os leucócitos estão envolvidos em defesas celulares não específicas (imunidade inata), como fagocitose e apoptose, através de mecanismos oxidativos por produção de ERO intermediárias com potentes atividades citotóxicas ([NEUMANN e cols., 2001](#)) e também por mecanismos não oxidativos como citocinas (interleucinas IL-1, IL-8), fator de necrose tumoral (TNF) entre outros ([SECOMBES, 1997; ALVAREZ-PELLITERO, 2008](#)).

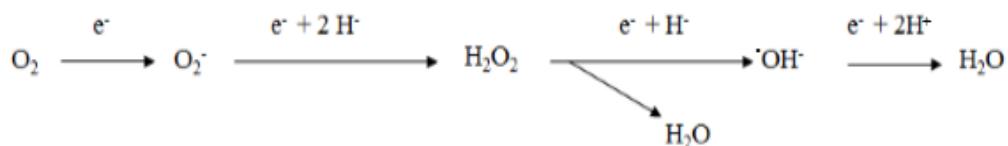
Os diferentes tipos de leucócitos que são reconhecidos em mamíferos também ocorrem em peixes teleósteos, com morfologia e funcionalidade equivalente à mamíferos, incluindo macrófagos, neutrófilos, monócitos, trombócitos, células B e T, células *natural killer* e eosinófilos ([MANNING e NAKANISHI, 1996; SECOMBES, 1997; WHYTE, 2007](#)). Contudo, teleósteos não possuem medula óssea ou nódulos linfáticos. Portanto, a mielopoiese (formação de células precursoras eritrocitárias) geralmente ocorre no rim ou baço, ao passo que o timo, rim e baço são os principais órgãos linfóides ([ZAPATA e cols., 2006](#)).

1.6 MARCADORES DE ESTRESSE OXIDATIVO

Os marcadores de estresse oxidativo (EO) possuem aplicações largamente difundidas em relação aos mecanismos de toxicidade apresentados pelos organismos aquáticos em decorrência da exposição a poluentes químicos ([BIANCHINI e cols., 2002; De BOECK e cols.,](#)

2004; EYCKMANS e cols., 2011). Por isso, espécies territorialistas (OWESON, BADEN, HERNROTH, 2006) assim como algumas espécies de peixes não territorialistas, têm sido amplamente utilizados como bioindicadores sensíveis a poluentes aquáticos associados às espécies reativas (ER) (BIANCHINI e cols., 2002; De BOECK e cols., 2004). O estresse oxidativo se instaura quando a geração de ER (Figura 4) prevalece sobre a capacidade antioxidant. Entre as RS que podem causar danos a proteínas celulares, lipídios e ácidos nucleicos, podendo levar a lesão cumulativa dos órgãos ou até morte celular, estão incluídos o ânion superóxido (O_2^-), peróxido de hidrogênio (H_2O_2), radical hidroxila ($\cdot OH$) gerado via fosforilação oxidativa, reação de Fenton, entre outros (HALLIWELL e GUTTERIDGE, 2007).

Figura 4 – Reação da redução da molécula de O_2 e intermediários reativos.



Fonte: MENEGHINI, 1987 pag. 57-62.

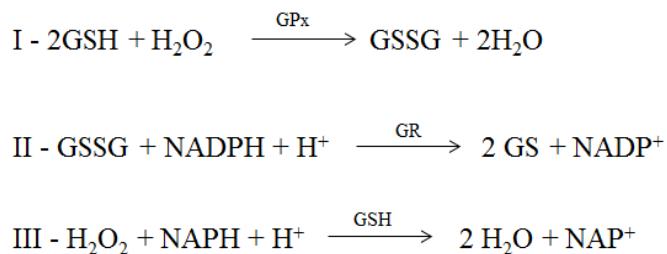
Esses danos podem ser modulados, ou minimizados, por sistemas antioxidantes especializados, que são considerados importantes defesas biológicas contra o estresse oxidativo induzido por íons metálicos (LOPES e cols., 2001). O equilíbrio entre pró-oxidantes (endógenos e exógenos, como os poluentes ambientais) e as defesas antioxidantes (enzimáticas e não-enzimáticas) podem ser úteis para avaliar os efeitos que condições ambientais estressoras, tais como danos oxidativos aos sistemas biológicos induzidos por diferentes classes de poluentes químicos (BIANCHINI e cols., 2002; EYCKMANS e cols., 2011), assim como estudar os danos potenciais induzidos por redução (ou elevação) nos níveis de oxigenação da água (BRAUN e cols., 2008).

O sistema antioxidante visa à manutenção da integridade celular sendo constituído de substratos tais como a glutationa reduzida (GSH) (de baixo pelo molecular), além das enzimas glutationa peroxidase (GPx) e glutationa redutase (GR). Outra importante enzima responsável pela detoxificação de xenobióticos, a nível hepático, constitui-se na glutationa-s-transferase

(GST). Concomitantemente, outras enzimas antioxidantes contribuem para a manutenção da integridade celular nos organismos, como a superóxido dismutase (SOD) e catalase (CAT), além de compostos antioxidantes não-enzimáticos como ácido ascórbico, ácido úrico e tocoferóis (HALLIWELL e GUTTERIDGE, 2007; HERMES-LIMA, 2004). Associadamente, essas enzimas e peptídeos endógenos representam um potente mecanismo de defesa antioxidante e atuam em conjunto para reduzir a toxicidade do Mn. Adicionalmente, a nível nuclear, encontra-se o fator eritroide de transcrição nuclear (Nrf2), um fator transcrecional com sensibilidade redox que desempenha um papel central no elemento de resposta antioxidante – “*antioxidant response element*” – ARE, induzindo a transcrição de enzimas antioxidantes, ou da fase II (GSH, glutationa oxidada – GSSG, GST e glucuronosiltransferases – UGTs) de detoxificação (van DER OOST e cols., 2003; LI e cols., 2008), sendo um elemento central de resposta a danos, estresse e inflamação (HAYDEN e GHOSH, 2008).

Na inativação de um agente pró-oxidante ocorre redução de glutationa oxidada (GSSG) à GSH por meio da GR na presença de NADPH, formando o ciclo redox (LU, 2000) (Figura 5). Em situações em que o sistema de óxido-redução está íntegro, a GSH pode ser recuperada. Entretanto, em condições de excesso de agentes oxidantes e/ou deficiência do sistema protetor, ocorre o desequilíbrio entre o consumo de GSH e a produção de GSSG, causando depleção dos níveis de GSH contribuindo para o estresse oxidativo (CNUBBEN e cols., 2001; LEIER e cols., 1996). Esse processo pode sofrer variações nas respostas biológicas, dependendo do período de permanência do estressor, para os indivíduos de uma mesma espécies.

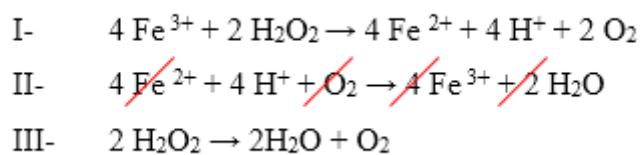
Figura 5 – Ciclo da glutationa reduzida (GSH). A GSH reduz o peróxido de hidrogênio (H_2O_2) à água (H_2O) em presença de glutationa peroxidase (GPx), formando uma ponte dissulfeto e, em seguida, a GSH é regenerada (Equações I a III).



Fonte: Adaptado de BARREIROS e DAVID, 2006, p.117

A catalase (CAT) está presente nos peroxissomas de eucariontes, sendo a principal enzima envolvida na neutralização do H₂O₂ citosólico (decorrente do metabolismo celular dos organismos aeróbicos, além de ser uma componente essencial de células do sistema imunitário por atuar como agente antibacteriano) prevenindo dessa forma o seu acúmulo dentro das células (Figura 6). A espécie reativa de oxigênio (H₂O₂) participa do processo de formação do radical hidroxil (·OH-), extremamente reativo e responsável por gerar lesão em sistemas orgânicos (NORDBERG e ARNER, 2001).

Figura 6 – Reação de dismutação, ou seja, o substrato atua tanto como redutor quanto oxidante. Esta reação também pode ocorrer na ausência de catalase (CAT), embora mais lentamente, devido à presença de metais traços no meio (gerados pela reação de Fenton).

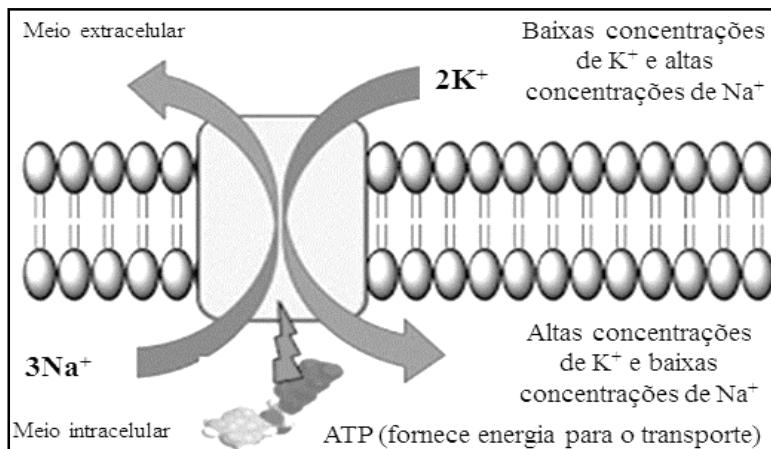


Fonte: Adaptado de KEILIN E HARTREE, 1945.

1.7 A Na⁺/K⁺ ATPASE

A enzima Na⁺/K⁺ ATPase (Figura 7) é uma proteína integral de membrana e está diretamente relacionada com o transporte de íons através da célula, sendo bastante sensível a agentes oxidantes (CHTOUROU e cols., 2011). A Na⁺/K⁺-ATPase possui importante papel na osmorregulação, sendo as brânquias de teleósteos uma fonte excelente e facilmente acessível dessa enzima (GIBBS e SOMERO, 1990; ALAM e FRANKEL, 2006; BAN, ANDO e URANO, 2007).

Figura 7 – Representação esquemática da bomba Na^+/K^+ .



Fonte: Adaptado de MENDES, 2015, Site: http://maxaug.blogspot.com.br/2013_04_01_archive.html

Em *Oncorhynchus mykiss* já foram descritas cinco isoformas de α -subunidades ($\alpha 1a$, $\alpha 1b$, $\alpha 1c$, $\alpha 2$, $\alpha 3$) de bombas Na^+/K^+ (RICHARDS e cols., 2003). Este estudo analisou a atividade da Na^+/K^+ -ATPase uma vez que ela desempenha um papel fundamental na reabsorção ativa e fluida de Na^+ , sendo importante para o transporte ativo de Na^+ e K^+ através da membrana celular sem afetar os movimentos transepiteliais de cátions nas brânquias (BIANCHINI e CARVALHO DE CASTILHO, 1999).

O efeito inibitório do Mn sobre a Na/K ATPase e sobre vários sistemas de captação de sinaptossomas já foi anteriormente descrito (LAI e cols., 1978, 1980, LAI, LIRA e DAVISON, 1981b; LAI, LIM e DAVISON, 1982c; WONG e cols., 1980). Similmente, estudos em roedores demonstraram que a atividade da Na^+/K^+ -ATPase pode ser inibida frente a altas concentrações de Mn (cerca de 6 mg Kg⁻¹) (SHUKLA, MALHOTRA e CHANDRA, 1983).

Em relação a atividade da Na^+/K^+ -ATPase frente à hipóxia, a redução da oxigenação induz a inibição dessa enzima em várias células e tecidos. Quando não revertida, a diminuição da atividade da bomba Na^+/K^+ -ATPase conduz a um acúmulo de Na^+ , inchaço celular progressivo, levando à morte. Entretanto, se essa inibição estiver acompanhada da supressão de vias dissipativas de cátions (por exemplo, inibição da fosforilação oxidativa) ocorrerá a redução do consumo de ATP, de forma a garantir a sobrevivência dos organismos à períodos prolongados de hipóxia. Tal efeito apresenta uma estratégia adaptativa benéfica restrita a certas espécies tolerantes à hipóxia frente a baixos níveis de oxigênio (BOGDANOVA e cols., 2005).

1.8 TRANSAMINASES COMO MARCADORES DE DANO HEPÁTICO

As transaminases plasmáticas, mais especificamente a transaminase glutâmica oxalacética (TGO) e transaminase glutâmica pirúvica (TGP), podem ser encontradas em diferentes tecidos, como eritrócitos, células cardíacas, tecido muscular, pâncreas e rim, embora seu local de principal concentração seja no fígado. Dessa forma, o monitoramento dos níveis de transaminases plasmáticas, associadamente à gama-glutamil transferase (GGT), pode ser útil ao diagnóstico de insultos hepáticos, uma vez que a quantidade de TGO e TGP presentes no sangue está diretamente relacionada com a extensão do dano tecidual (HUANG, e cols. 2006).

A enzima TGO é responsável por catalisar, de forma específica, a transferência do grupo amina da alanina para o α -cetoglutarato, com formação de glutamato e piruvato, sendo que este último é reduzido à lactato por ação da lactato desidrogenase (LDH), enquanto que a coenzima NADH é oxidada a NAD⁺ (LEHNINGER, 2002; GALANTE e ARAÚJO, 2012). Já a enzima TGP catalisa especificamente a transferência do grupo amina do ácido aspártico para o α -cetoglutarato com formação de glutamato e oxalacetato, o qual é reduzido a malato por ação da malato desidrogenase (MDH), enquanto a coenzima NADH é oxidada à NAD⁺ (LEHNINGER, 2002; GALANTE e ARAÚJO, 2012). Essas transferências de grupamentos amina para o α -cetoglutarato efetuados pelas transaminases permitem a metabolização de aminoácidos no fígado, além de eliminar azoto (nitrogênio) do organismo que é tóxico (LEHNINGER, 2002).

Contudo, quando o tecido ou órgão está danificado, essas reações são bloqueadas e ocorre extravasamento dessas transaminases para a corrente sanguínea, elevando os seus níveis para valores muito acima dos de referência. Por exemplo, uma lesão grave tecidual pode fazer com que ocorra um aumento nos níveis de TGO de dez a vinte vezes maiores que os de referência, enquanto os níveis de TGP podem atingir níveis até cinquenta vezes maiores que os de referência. As concentrações plasmáticas normais das transaminases podem variar de valores compreendidos entre 5 a 40 U L⁻¹ para TGO e, 5 a 35 U L⁻¹ para TGP, sendo influenciados por fatores como idade e sexo, sendo que a razão entre TGO/TGP pode representar um marcador confiável de dano hepatocelular (KITAMURA e cols., 1992; HUANG e cols., 2006). Quando essa razão é igual ou superior a dois, existe uma forte correlação com dano hepático mitocondrial, uma vez que a TGO é exclusivamente mitocondrial.

Outra enzima utilizada especificamente para o diagnóstico de dano hepático constitui-se na gama glutamil transferase (GGT). Sua detecção no soro é originada, principalmente, a

partir do sistema hepatobiliar, e sua atividade está elevada no dano hepático. (THRALL e cols., 2007b). A GGT catalisa a transferência de resíduos gama-glutamil para L-aminoácidos ou para outros peptídeos produzindo aminoácidos gama-glutamil e cisteinilglicina, sendo a glutationa (GSH) o seu principal substrato. A clivagem da GSH eleva os níveis extracelulares de cisteína, o precursor limitante para a síntese de GSH no meio intracelular. Como consequência, a GGT participa da manutenção da homeostase redox de células e processos dependentes de reatividade a condições adversas intra e extracelulares (MERONI e cols., 1997; MARES e cols., 2005). A síntese ou atividade da GGT é regulada por diversos fatores (GARCION e cols., 1999), desempenhando funções importantes na metabolização de drogas e outras toxinas. Valores elevados de GGT são encontrados em casos de obstrução biliar intra ou pós-hepática (QUARESMA e cols., 2007) e variam conforme sexo e idade. Em roedores valores séricos elevados de GGT já foram observados após intoxicações por chumbo (SHALAN e cols., 2005).

1.9 MARCADORES DE INTERRUPÇÃO HORMONAL

Um grande número de contaminantes ambientais pode interferir com a regulação hormonal em vertebrados (PAIT e NELSON, 2002). Estes agentes exógenos que interferem na síntese, secreção, transporte, recepção, ação, ou eliminação dos hormônios naturais do corpo são denominados ruptores endócrinos (EDCs) (USEPA, 1998). Estes desreguladores endócrinos podem prejudicar a homeostase, uma vez que a sinalização endócrina controla muitos processos fisiológicos essenciais para os organismos, como o crescimento e desenvolvimento, a resposta ao estresse, e, finalmente, a reprodução e o desenvolvimento da população (SCHOLZ e MAYER, 2008).

Entre os hormônios hipofisários, mais precisamente, os da adenohipófise, estão o hormônio do crescimento (GH), a prolactina (PRL) e a somatolactina (SL). O GH é responsável pelo estímulo do crescimento somático em peixes por estimular a secreção de fatores do crescimento (IGF-I e IGF-II “*insuline like growth factors*”) no fígado, além de atuar também na adaptação de teleósteos de água doce à água do mar e vice-versa (SAKAMOTO e McCORMICK, 2006), assim como estar envolvido na adaptação de espécies eurialinas à água do mar, incluindo salmonídeos e tilápias (*Oreochromis mossambicus*) (McCORMICK, 1996; SAKAMOTO e cols., 2006). A prolactina (PRL) é o principal hormônio responsável pela regulação do equilíbrio hidromineral em peixes de água doce (WENDELAAR BONGA,

LOWIK, VAN DER MELT, 1983). Portanto, possui importância bem estabelecida na adaptação de teleósteos eurialinos em água doce (**CLARKE e BERN, 1980; BROWN e BROWN, 1987**). Estudos demonstram que a expressão gênica da PRL encontra-se aumentada frente à exposição ao Mn (**KIM e cols., 2009; DOLCI e cols., 2014**).

Em relação à somatolactina (SL), hormônio secretado exclusivamente por peixes, ainda existem funções a serem definidas, embora já tenham sido observados efeitos sobre a função imunológica em teleósteos (**CALDUCH-GINER e cols., 1998**), equilíbrio ácido-base (**KAKIZAWA, KANEKO, HIRANO, 1996**), mobilização de energia (**RAND-WEAVER e SUMPTER, 1993; RAND-WEAVER, POTTINGER, SUMPTER, 1995**), biossíntese de esteroides gonadais (**PLANAS e cols., 1992**), regulação do fosfato (**LU e cols., 1995; ZHU e THOMAS, 1995**) e metabolismo do cálcio (**KAKIZAWA e cols., 1993**). Adicionalmente, especula-se que desempenhe um papel na maturação sexual (**BENEDET e cols., 2008**).

Outro hormônio, não exclusivamente hipofisário, constitui-se na proopiomelanocortina (POMC), que é precursora do hormônio adrenocorticotrófico (ACTH), hormônio estimulador de melanócitos (α -MSH) e endorfinas (END) e portanto, expressa em outras estruturas como hipotálamo e queratinócitos da pele (**TAKAHASHI e KAWAUCHI, 2006; MOUNTJOY e cols., 2003**). De fato, a POMC é um precursor de vários hormônios peptídicos importantes envolvidos em funções que vão desde a resposta ao estresse à homeostase energética (**MOMMSEN, VIJAYAN e MOON, 1999; FUGII, 2000; TAKAHASHI e KAWAUCHI, 2006**) e, em mamíferos e peixes, o α -MSH, um dos precursores da POMC está envolvido na supressão do apetite através da interação com receptores de melanocortina-4 (α -MSH-4) (**LEDER e SILVERSTEIN, 2006**).

Os detalhes sobre a homeostase energética nos peixes estão sendo elucidados e muitos dos genes envolvidos em vias de sinalização neuroendócrina em mamíferos estão sendo descobertos também em peixes. Há evidências de que o neuropeptídeo Y, peptídeo relacionado a agouti (MSH, CART – transcrição regulada por cocaína e anfetamina, leptina e grelina) desempenhe função na regulação do apetite em peixes (**VOLKOFF e cols., 2005**). Para a POMC, duas cópias desse gene foram identificadas, sendo provável que tenham resultado a partir da duplicação do genoma de salmonídeos, a POMC-A e POMC-B, adicionando um outro grau de complexidade na determinação da função e homologia com outros vertebrados (**SALBERT e cols., 1992**).

2 PROPOSIÇÃO

Este estudo se propõe a investigar mecanismos responsáveis pela tolerância de jundiás a condições adversas como hipóxia e posterior reoxigenação, uma vez que tais condições tem ocorrido com aumentada frequência no ambiente aquático gerando uma série de alterações nas condições de águas doces mundiais. Associadamente utilizou-se o manganês, por tratar-se de um poluente comumente liberado ao ambiente durante atividades de prospecção de petróleo e extrativismo mineral, possuindo potencial para causar efeitos prejudiciais às espécies aquáticas. O jundiá foi escolhido como modelo por apresentar uma considerável resistência à hipóxia, representando um avanço evolutivo frente a tais situações.

2.1 OBJETIVO GERAL

Investigar se a aclimatação de jundiás à hipóxia moderada modifica parâmetros fisiológicos, morfológicos, bioquímicos, moleculares e comportamentais relacionados ao processo de adaptação. Adicionalmente, investigar a influência do manganês e subsequente estabelecimento da reoxigenação (restauração dos níveis de oxigênio para normóxia) sobre os mesmos parâmetros.

2.1.1 Objetivos específicos

2.1.1.1 Artigo I

- ✚ Aclimatar jundiás à hipóxia ou normóxia, expondo-os posteriormente ao Mn;
- ✚ Avaliar o acúmulo de manganês nos diferentes tecidos (plasma, encéfalo, brânquias, fígado e rim) de jundiás aclimatados à hipóxia ou normóxia expostos ou não ao Mn;
- ✚ Avaliar hematócrito e hemoglobina em jundiás aclimatados à hipóxia ou normóxia expostos ou não ao Mn;
- ✚ Avaliar o status oxidativo através da determinação da geração de espécies reativas (ER), peroxidação lipídica (PL), níveis de proteínas carboniladas (PC) e atividade enzimática da catalase (CAT) em encéfalo, brânquias, fígado e rim de jundiás aclimatados à hipóxia ou normóxia expostos ou não ao Mn;

- ⊕ Avaliar a atividade da enzima Na^+/K^+ -ATPase em encéfalo, brânquias, fígado e rim de jundiás aclimatados à hipóxia ou normóxia expostos ou não ao Mn;
- ⊕ Avaliar possíveis alterações na expressão gênica de hormônios hipofisários de jundiás aclimatados à hipóxia ou normóxia expostos ou não ao Mn, tais como hormônio do crescimento (GH), somatolactina (SL), prolactina (PRL).

2.1.1.2 Artigo 2

- ⊕ Aclimatar jundiás a normóxia ou hipóxia, expondo-os posteriormente ao Mn e, subsequentemente, restabelecer a reoxigenação nesses peixes;
- ⊕ Avaliar o acúmulo de manganês em brânquias de jundiás expostos ao Mn sob normóxia, hipóxia e reoxigenação;
- ⊕ Avaliar o status oxidativo através da determinação do monitoramento da geração de ER, níveis de PC, viabilidade mitocondrial (redução do MTT), assim como avaliar a atividade enzimática da CAT e da Na^+/K^+ -ATPase em brânquias de jundiás aclimatados à hipóxia ou normóxia expostos ou não ao Mn e, subsequentemente reoxigenados;
- ⊕ Avaliar possíveis alterações morfológicas induzidas por Mn através de análises histológicas em brânquias de jundiás sob normóxia, hipóxia e reoxigenação.

2.1.1.3 Artigo 3

- ⊕ Aclimatar jundiás à normóxia ou hipóxia, expondo-os posteriormente ao Mn e, subsequentemente, restabelecer a reoxigenação nesses peixes;
- ⊕ Avaliar o acúmulo de manganês em plasma, fígado e rim de jundiás expostos ao Mn e aclimatados a normóxia e hipóxia e, subsequentemente reoxigenados;
- ⊕ Avaliar o perfil hematológico de jundiás aclimatados à hipóxia ou normóxia expostos ou não ao Mn e, subsequentemente reoxigenados;
- ⊕ Avaliar o status oxidativo através da determinação da geração de ER e níveis de PC em jundiás aclimatados à hipóxia ou normóxia expostos ou não ao Mn e, subsequentemente reoxigenados;

- Avaliar as transaminases plasmáticas (TGO, TGP, GGT e razão TGO/TGP) como marcadores de dano hepático em jundiás aclimatados à hipóxia ou normóxia expostos ou não ao Mn e, subsequentemente reoxigenados.
- Avaliar as defesas antioxidantes como os níveis de glutationa reduzida (GSH) e atividade CAT, além da atividade da Na^+/K^+ -ATPase em fígado e rim de jundiás aclimatados à hipóxia ou normóxia expostos ou não ao Mn e, subsequentemente reoxigenados;

2.1.1.4 Resumo expandido

- Aclimatar jundiás à normóxia ou hipóxia, expondo-os posteriormente ao Mn e, subsequentemente, restabelecer a reoxigenação nesses peixes;
- Avaliar o acúmulo de manganês em encéfalo de jundiás expostos ao Mn aclimatados à normóxia e hipóxia e, subsequentemente reoxigenados;
- Avaliar alterações comportamentais com testes específicos de memória de interação social (labirinto) ou ansiedade, assim como avaliar a responsividade de jundiás aclimatados à hipóxia ou normóxia expostos ou não ao Mn e, subsequentemente reoxigenados, frente à coespecíficos (substância de alarme);
- Avaliar o status oxidativo de jundiás aclimatados à hipóxia ou normóxia expostos ou não ao Mn e, subsequentemente reoxigenados, através da determinação da geração de ER e níveis de PC, assim como análise da atividade das enzimas CAT e Na^+/K^+ -ATPase em plasma de jundiás;
- Avaliar possíveis alterações na expressão gênica de hormônios hipofisários de jundiás, aclimatados à hipóxia ou normóxia expostos ou não ao Mn e, subsequentemente reoxigenados, como proopiomelanocortina A e B (POMC-A, POMC-B), prolactina (PRL) e somatolactina (SL);
- Avaliar possíveis alterações na cor da pele de jundiás aclimatados à hipóxia ou normóxia expostos ou não ao Mn e, subsequentemente reoxigenados.

CAPÍTULO II

3 PRODUÇÃO CIENTÍFICA

Os resultados obtidos nessa tese apresentam-se sob a forma de artigo 1 (publicado), artigo 2 (submetido), artigo 3 (redigido) e resumo expandido (4). Os itens Materiais e Métodos, Resultados, Discussão e Referências, encontram-se no próprios artigos e resumo expandido, os quais estão dispostos da mesma forma que foram publicado (artigo 1), submetido (artigo 2), redigido (artigo 3) e em fase final de redação (resumo expandido).

3.1 ARTIGO 1

Publicado no periódico *Aquatic Toxicology* 157: 175-185, 2014

ACLIMATAÇÃO A HIPÓXIA PROTEGE CONTRA DANOS OXIDATIVOS E MODIFICA A EXPRESSÃO DE PROLACTINA E SOMATOLACTINA EM JUNDIÁS (*Rhamdia quelen*) EXPOSTOS AO MANGANÊS

G.S. Dolci, L.T. Vey, A.J. Schuster, Kr. Roversi, K. Roversi, V.T. Dias, C.S. Pase, R.C.S. Barcelos, C.T.D. Antoniazzi, J.I. Golombieski, W.G. Glanzner, P.A. Anezi Junior, P.B.D. Gonçalves, M.A.G. Nunes, V.L. Dressler, B. Baldisserotto, M.E. Burger

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Hypoxia acclimation protects against oxidative damage and changes in prolactin and somatolactin expression in silver catfish (*Rhamdia quelen*) exposed to manganese



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ABSTRACT

The aim of this study was to assess the Mn toxicity to silver catfish considering Mn accumulation and oxidative status in different tissues, as well as pituitary hormone expression after acclimation to hypoxia. Silver catfish acclimated to hypoxia for 10 days and successively exposed to Mn (9.8 mg L⁻¹) for an additional 10 days exhibited lower Mn accumulation in plasma, liver, kidneys and brain and prevented the hematocrit decrease observed in the normoxia group. Hypoxia acclimation also modified Mn-induced oxidative damage, which was observed by lower reactive species (RS) generation in gills and kidneys, decreased lipid peroxidation (LP) levels in gills, liver and kidneys and decreased protein carbonyl (PC) levels in liver, kidneys and brain. Manganese accumulation showed positive correlations with LP levels in gills and kidneys, as well as with PC levels in gills, liver and brain. In addition, hypoxia acclimation and Mn exposure increased catalase (CAT) activity in gills and kidneys and Na⁺/K⁺-ATPase activity in gills, liver and brain. Silver catfish that were acclimated under normoxia and exposed to Mn displayed increased pituitary prolactin (PRL) and decreased somatolactin (SL) expression. Interestingly, hypoxia acclimation prevented hormonal fluctuation of PRL and SL in fish exposed to Mn. These findings indicate that while the exposure of silver catfish to Mn under normoxia was related to metal accumulation and oxidative damage in tissues together with endocrine axis disruption, as represented by PRL and SL, hypoxia acclimation reduced waterborne Mn uptake, thereby minimizing oxidative damage and changes in hormonal profile. We hypothesized that moderate hypoxia is able to generate adaptive responses, which may be related to hormesis, thereby ameliorating Mn toxicity to silver catfish.

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1. Introduction

Aquatic environmental pollution is estimated with stationary species, which are considered important bioindicators (Oweson et al., 2006). However, some non-stationary fish species may also reflect the environmental contamination by pollutants. According to Chapman (1996), as aquatic contaminants can be incorporated into the biological methods providing information about

anthropogenic activities, bioaccumulation and biomagnification of toxic substances may be involved in the acute or chronic toxicity of aquatic species, thus affecting its genotoxicity or mutagenicity. The current study was designed considering that fish are good biological approaches to water quality assessment, allowing chemical analysis of biota. Linked to this characteristic, silver catfish (*Rhamdia quelen*) is a Brazilian native species that has high potential for commercialization due to its texture, fillet quality and high carcass yield (Gomes et al., 2000). However, silver catfish may be subject to potential life hazards, mainly due to inadequate sewage treatment and release of industrial waste into waters with evidence of metal contamination in the effluents (ATSDR, 2000; Garcia de Oliveira et al., 2008).

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In this context, manganese (Mn) is a metal naturally present in freshwater in the 1–200 mg L⁻¹ range (Barceloux, 1999), reaching up to 264 mg L⁻¹ in the formation water obtained from oil and gas extraction in the Brazilian Amazon Basin (Baldisserotto et al., 2012). The maximum permissible concentrations (MPC) of Mn for Brazilian class I freshwaters have been fixed at 0.1 mg L⁻¹, with an increase to 0.5 mg L⁻¹ for class III waters (CONAMA, 2005).

Despite Mn being important for a variety of physiological functions (Underwood, 1971; Leach and Lilburn, 1978; Simkiss and Taylor, 1989), it may display toxicity at high concentrations, acting as a reactive species (RS) generator through Fenton's reaction. In Fenton's reaction, ferrous iron(II) is oxidized by hydrogen peroxide to ferric iron(III), releasing a hydroxyl radical and a hydroxyl anion (Valko et al., 2005). In fact, for this known reaction, Mn(II) can take place of the iron, with consequent RS release.

Reactive species (RS) include superoxide anion (O²⁻), hydrogen peroxide (H₂O₂) and hydroxyl radical (OH⁻), among others (Halliwell and Gutteridge, 1999), which can be neutralized by antioxidant enzymes such as glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) (Hermes-Lima, 2004), including other systems, such as Na⁺/K⁺ ATPase, an enzyme implicated in neuronal excitability and metabolic energy production (Dogānli et al., 2013).

Manganese becomes bioavailable to generate RS when Mn(III) in water is reduced to Mn(II) by hypoxic/anoxic conditions, especially during the degradation of sedimented organic matter (Dehairs et al., 1989). These hypoxia episodes are well-known in some Brazilian regions, such as the Amazon Basin, where drastic daily fluctuations occur due to the combination of photosynthetic macrophytes, stagnant water, and decaying organic matter (Scott et al., 2004). An excessive load of dissolved organic matter contributes to decrease dissolved oxygen (DO₂) in water streams, with impairments for most species (Terova et al., 2008). On the other hand, some studies have reported a protective effect for a moderate hypoxia level (DO₂: 3.88 mg L⁻¹) that is able to increase hematocrit values in silver catfish (Dolci et al., 2013), resulting in erythrocyte swelling and the formation of new red blood cells in response to hypoxia (Nikinmaa and Tervonen, 2004).

Fish pituitary hormones, namely growth hormone (GH), prolactin (PRL) and somatotropin (SL), regulate the synthesis and secretion of many hormones that control other endocrine glands (Arulkwe, 2001). Different studies have demonstrated the involvement of pituitary hormones in physiological processes such as reproduction, growth, metabolism, and immune responses (Björnsson, 1997; Bole-Feysot et al., 1998; Manzon, 2002; McCormick, 2001; Kaneko, 1996; Benedet et al., 2008). According to Tipsmark et al. (2009), these hormones constitute a reproducible model to analyze the effect of pollutants (Harvey, 1993; Nishioka et al., 1988). Pituitary hormones are also involved in stress responses, as PRL is able to prevent loss of ions in fish gills when confronted by stressors (Chakraborti and Mukherjee, 1995; Manzon, 2002). In addition, Mn is able to stimulate dopamine autoxidation in dopaminergic neurons, indirectly modulating PRL secretion, thus making this hormone a Mn contamination marker (Marreilha dos Santos et al., 2011).

A recent study by our group revealed that the simultaneous presence of Mn and moderate hypoxia was related to decreased Mn accumulation together with decreased oxidative damage in different silver catfish tissues (Dolci et al., 2013), indicating that low oxygen levels may exert some beneficial influence for this species. For a better understanding of Mn and its interaction with oxygen levels, this study aimed to assess the influence of previous hypoxia acclimation with subsequent Mn exposure on metal accumulation and oxidative status in different tissues in addition to the expression of pituitary hormones in silver catfish.

2. Materials and methods

2.1. Fish

One hundred thirty-two (132) silver catfish (128.9±4.4 g; 16.5±0.7 cm) (11 fish/tank, 3 tanks per experimental group in a total of four groups) obtained from fishponds at Universidade Federal de Santa Maria, Southern Brazil, were acclimated in continuously aerated 250 l tanks with controlled temperature (20 °C) for at least one week prior to experiments. During this period, they were fed once a day (42% crude protein) at 12 p.m.

2.2. Reagents

Manganese sulfate hexahydrate [MnSO₄·6H₂O] (Vetec®, Rio de Janeiro, RJ, Brazil) was used to maintain the waterborne Mn level. All chemicals and solvents used were of HPLC grade and purchased from Sigma Aldrich® (Brazil).

2.3. Exposure protocol

The manganese level was determined according to situations of environmental pollution established by literature reports (Baldisserotto et al., 2012; Dolci et al., 2013). After acclimation, the silver catfish were randomly transferred to 250 l tanks (11 fish per tank, three tanks/group), in a semi-static system, with 50% daily water exchange with dissolved oxygen levels previously adjusted through bubbling air (normoxia) and/or nitrogen (hypoxia) for 10 days.

On day 11, the fish were exposed for an additional 10 days to nominal waterborne Mn levels between 0 (control without Mn addition) and 10 mg L⁻¹, yielding the following groups: normoxia (DO₂ [dissolved oxygen]: 7.16±0.08 mg L⁻¹ [92.27% oxygen saturation]; pH: 6.93±0.06), hypoxia (DO₂: 2.83±0.18 mg L⁻¹ [36.47% oxygen saturation]; pH: 6.82±0.04), normoxia plus Mn (DO₂: 7.51±0.07 mg L⁻¹ [96.78% oxygen saturation]; pH: 6.93±0.06) and hypoxia plus Mn (DO₂: 2.70±0.17 mg L⁻¹ [34.79% oxygen saturation]; pH: 6.86±0.04) (Fig. 1). Manganese values were posteriorly detected by inductively coupled plasma optical emission spectrometry (described in Section 2.6.2) and fixed in 0.005 and 9.8 mg L⁻¹, respectively, in four treatments in triplicate. The initial hypoxic environment was created from the oxygen consumption due to the normal fish metabolism, and different DO₂ levels were maintained through aeration with air pumps (air pump AC 2000; 0.0014 MPa pressure) or nitrogen gas when appropriate. Water reservoir tanks containing waterborne manganese at the appropriate

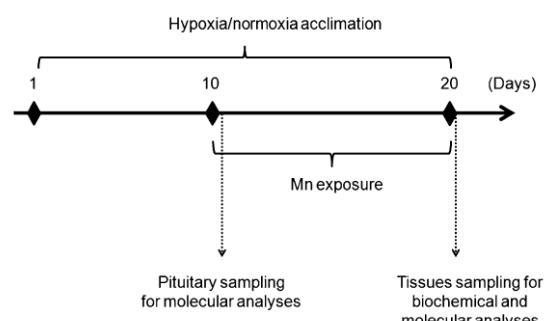


Fig. 1. Experimental design. Fish were acclimated for 10 days to normoxia/hypoxia and at 10 days were exposed or not to waterborne Mn [9.8 mg L⁻¹], for over 10 days, totaling 20 days.

concentration kept under normoxia (with air pumping) or under hypoxic conditions (with nitrogen gas bubbling) were used.

This protocol was approved by the Animal Use Ethics Committee (CEUA), at Universidade Federal de Santa Maria.

2.4. Tissues collection

For molecular analyses of the pituitary hormones, on experimental days 10 and 20, the fish (one per tank, totaling 3 per group) were anesthetized by immersion in buffered tricaine methane sulfonate (MS222) (300 mg L^{-1}) (pH: 7.00), euthanized by decapitation, and their pituitary was collected. For the analysis of Mn accumulation, the same fish used for molecular assays on experimental day 20 were sampled (one per tank, totaling 3 per group). Blood samples from 8 fish from each replicate ($n=3$) were collected from fish anesthetized with MS222 by puncture of caudal vein on experimental day 20. These fish were then euthanized by decapitation and brain, kidneys, liver and gills were removed.

2.5. Water parameters

Dissolved oxygen and temperature (YSI5512 oxygen meter; SI Inc., Yellow Springs, OH, USA), pH (DMPH-2 pH meter Digimed, São Paulo, SP, Brazil) and total ammonia nitrogen (TAN) levels (Eaton et al., 2005) were measured twice a day. Un-ionized ammonia (NH_3) levels were calculated according to Colt (2002). Water hardness (EDTA titrimetric method) and alkalinity (Boyd and Tucker, 1992) were determined once a day. Tanks were cleaned late afternoon daily by siphoning and replaced with pre-adjusted Mn levels ($\text{MnSO}_4 \cdot 6\text{H}_2\text{O}$). At the end of each exposure, Mn wastes were chemically treated and properly disposed.

2.6. Analyses

2.6.1. Plasma and tissues samples

Plasma (1 mL) was obtained after blood centrifugation ($1310 \times g$ for 15 min). The digestion of gills, liver, kidneys, brain and plasma was performed using a conventional heating block (Velp Scientifica, Model DK, Italy) with open glass vessels. The procedure was performed with concentrated nitric acid (14 mol L^{-1}) (Merck, Darmstadt, Germany). H_2O_2 was added, and the digests were heated up to 80°C for 1 h. After cooling, the digests were diluted with purified water (Milli-Q system, Millipore Corp., Bedford, USA) for further analyses.

2.6.2. Sample Mn determination

The waterborne Mn level and Mn accumulation (in plasma and tissues) were determined using an inductively coupled plasma optical emission spectrometer (ICP OES, Model Spectro Ciros CCD, Spectro Analytical Instruments, Kleve, Germany), which was equipped with an axial view configuration and a cross-flow nebulizer coupled to a Scott-type double pass nebulization spray chamber. The wavelength for Mn determination was 257.611 nm , and the radiofrequency power was 1400 W. The flow rates for plasma generation, auxiliary and nebulization gas were 20.1 and 0.9 L min^{-1} , respectively. Argon (99.996%, White Martins–Praxair, São Paulo, SP, Brazil) was used for plasma generation for nebulization and as an auxiliary gas. For accuracy assessment, certified reference material (CRM) of dogfish muscle tissue (DORM-2) from the National Research Council, Canada, was used.

2.6.3. Hematological parameters

The hemoglobin concentration in whole blood (g dL^{-1}) was determined according to Collier (1944). This method is based on iron atom oxidation(II) of hemoglobin molecule by potassium ferricyanide in weakly alkaline pH, forming methemoglobin, which

is converted to cyanmethemoglobin after reacting with potassium cyanide. The reddish color proportional to the concentration of hemoglobin present in the sample (after correction for standard hemoglobin samples) can be measured in a spectrophotometer at a wavelength of 540 nm . The hematocrit was determined by the microhematocrit method as described by Goldenfarb et al. (1971).

2.6.4. Oxidative status determination

2.6.4.1. Sample preparation. Brain, gills, kidneys and liver were homogenized (1:5 w/v) in buffer Tris–HCl (10 mM, pH: 7.4) and centrifuged ($3640 \times g$ for 15 min). The supernatants were used for oxidative stress parameter determination.

2.6.4.2. Reactive species (RS) determination. RS levels were measured using the oxidant-sensing fluorescent probe 2'-7'-dichlorofluorescein diacetate (DCHF-DA) (Hempel et al., 1999). The oxidation (DCHF-DA) to fluorescent dichlorofluorescein (DCF) was determined at 488 nm for excitation and 525 nm for emission. After homogenization and centrifugation of tissues, 3 mL of buffer (10 mM Tris–HCl, pH 7.4) was added. After 10 s, $10\text{ }\mu\text{M}$ (DCHF-DA) (prepared in ethanol) was added to the mixture, and the fluorescence intensity of DCF was measured for 300 s and expressed as a percentage of the untreated control group. The protein content was normalized by quantification according to Lowry et al. (1951).

2.6.4.3. Lipid peroxidation (LP) estimation. Lipid peroxidation (LP) was estimated using thiobarbituric acid reactive substances (TBARS), as described by Ohkawa et al. (1979). This measure is based on a reaction with MDA (malondialdehyde) resulting from oxidative damage in lipid membranes with thiobarbituric acid, in which pink chromogen is generated and can be spectrophotometrically measured at 532 nm . The TBARS levels were expressed as $\text{nmol of MDA g tissue}^{-1}$.

2.6.4.4. Protein carbonyl determination. Protein carbonyl (PC) was measured according to Yan et al. (1995), with some modifications. Soluble protein was mixed with 2,4-dinitro-phenylhydrazine (DNPH; 10 mM in 2 M HCl) or HCl (2 M) and incubated at room temperature for 1 h. Denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, with 3% sodium dodecyl sulfate), ethanol (99.8%) and hexane (99.5%) were added, mixed by shaking, and centrifuged. The protein isolated from the interface was washed twice with ethyl acetate/ethanol 1:1 (v/v) and suspended in denaturing buffer. Each DNPH sample was read at 370 nm in a spectrophotometer against the corresponding HCl sample (blank). The results were expressed as the $\text{nmol carbonyl/g tissue}^{-1}$.

2.6.4.5. Catalase activity assay. Catalase (CAT) activity was spectrophotometrically quantified by the method of Aebi (1984), which monitors the disappearance of H_2O_2 in the presence of tissue at 240 nm . The enzymatic activity was expressed as $\mu\text{mol H}_2\text{O}_2 \text{ g tissue}^{-1} \text{ min}^{-1}$ (1 U decomposes $1\text{ }\mu\text{mol H}_2\text{O}_2/\text{min}$ at pH 7 at 25°C).

2.6.4.6. Na^+/K^+ -ATPase activity assay. The Na^+/K^+ -ATPase activity was determined in gills, liver, kidneys and brain according to Muszbek et al. (1977), with some modifications. Briefly, the aliquots of tissue (20 μL) were added to a reaction medium containing NaCl, MgCl_2 , KCl and Tris–HCl buffer (pH 7.4), with or without the Na^+/K^+ -ATPase enzyme inhibitor ouabain. The method for ATPase activity measurement was based on the determination of the inorganic phosphate (Pi) released to the reaction medium by the hydrolysis of ATP, according to the method proposed by Atkinson et al. (1973). The reaction was initiated with the addition of the substrate ATP to the reaction medium and finished by the addition of the color

Table 1

Primers design for amplification of β -actin, PRL, SL and GH genes based on the sequences of these genes described by Baldisserotto et al. (2014).

Gene	Sequences
β -Actin	
Forward	5'-CGA ATG CCA GGG TAC ATG GT-3'
Reverse	5'-CCA CCT TCA ACT CCA TCA TTGA A-3'
PRL	
Forward	5'-ACC AGA GAC AGG AGC TCG TTC T-3'
Reverse	5'-AGC TCA TGA GAC CGT CCA TGT-3'
SL	
Forward	5'-CGA GCC CAG GAC TTT GTT TG-3'
Reverse	5'-GAC GCG CAC AAG GTT TGA T-3'
GH	
Forward	5'-TTG ACA GTC TTG GTG CTG CTT T-3'
Reverse	5'-GAG CGA CTG CGT TGT TGA AG-3'

reagent (1 mL), containing ammonium molybdate (2%), Triton X-100 and H_2SO_4 (10%) after 15 min of incubation at 37 °C. The formed molybdate–Pi complexes were measured spectrophotometrically at 405 nm. Values were calculated in relation to a standard curve constructed with Pi at known concentrations, and also corrected by the protein content. Protein was measured according to Lowry et al. (1951).

2.6.5. Pituitary hormones expression

For pituitary hormones expression, the total RNA was extracted from samples using Trizol reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions. Total RNA quantity and purity were assessed using a Nanodrop machine (Thermo Scientific). Regarding RNA purity, samples reaching values lower than 1.7 were discarded from the study. Total RNA (1 µg) was treated with DNase (Invitrogen) at 37 °C for 5 min to digest any contaminating DNA. The reverse transcription reaction was performed with an iScript cDNA Synthesis Kit (Bio-Rad) in a final volume of 20 µL. The mRNA expression of the studied hormones was analyzed through qRT-PCR, using the StepOnePlus™ RT-PCR system (Applied Biosystems) with a Power SYBR Green PCR Master Mix (Applied Biosystems). The sequences for growth hormone (GH), prolactin (PRL) and somatotropin (SL) used to design all the primers were prepared according to Baldisserotto et al. (2014) (Table 1). The results were normalized to the expression of the constitutive gene beta-actin. The calculation of relative expression was performed as recommended by Pfaffl (2001).

2.7. Statistical analysis

Statistics were performed using Statistica (Statsoft Inc., Tulsa, USA, version 7). Data homogeneity and normal distribution were initially tested by Levene's test. Data were analyzed by two-way ANOVA ($Mn \times$ dissolved oxygen levels), followed by Tukey's multiple range test, when appropriate. Pearson correlation was applied for the data. Data were expressed as the mean \pm SEM, and $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Water parameters

All of the assessed parameters (pH; DO; hardness, alkalinity; total ammonia; NH_3 ; nitrite) were presented within the expected values, with no variations throughout the experiment (data not shown).

Table 2

Mn accumulation in different tissues of silver catfish submitted to normoxia or hypoxia for 10 days and exposed or not to waterborne manganese for over 10 days.

Tissue	Oxygen level	Mn accumulation ($\mu g g^{-1}$ tissue $^{-1}$)	
		Control	Mn
Plasma ($\mu g L^{-1}$)	Normoxia	0.09 \pm 0.00	3.77 \pm 0.14*
	Hypoxia	0.09 \pm 0.00	2.05 \pm 0.30**
Gill ($\mu g g^{-1}$)	Normoxia	0.36 \pm 0.03	6.86 \pm 0.40*
	Hypoxia	0.40 \pm 0.02	6.64 \pm 0.35*
Liver ($\mu g g^{-1}$)	Normoxia	2.04 \pm 0.02	25.27 \pm 4.10*
	Hypoxia	1.40 \pm 0.26	10.16 \pm 3.22*
Kidney ($\mu g g^{-1}$)	Normoxia	0.97 \pm 0.12	7.53 \pm 0.05*
	Hypoxia	0.75 \pm 0.07	5.64 \pm 0.61**
Brain ($\mu g g^{-1}$)	Normoxia	0.22 \pm 0.03	1.45 \pm 0.04*
	Hypoxia	0.25 \pm 0.02	0.89 \pm 0.00**

Control: [0.005 mg L $^{-1}$]; Mn: waterborne manganese [9.8 mg L $^{-1}$].

* Indicates significant differences between oxygen level in the same Mn concentration.

** Indicates significant difference between Mn and control in the same oxygen level ($P < 0.05$).

3.2. Mn accumulation in different tissues

Two-way ANOVA of Mn accumulation revealed a significant main effect of oxygen level in blood and liver [$F(1,32) = 26.09$, $P < 0.001$; 9.07, $P < 0.05$], respectively. A significant main effect of the metal was also revealed in blood, gills, liver, kidneys and brain [$F(1,32) = 282.73$, $P < 0.001$; 553.69, $P < 0.001$; 37.44, $P < 0.001$; 326.4, $P < 0.001$; 19.20, $P < 0.05$], respectively, and a significant oxygen level \times metal interaction in blood and liver [$F(1,32) = 26.09$, $P < 0.001$; 7.66, $P < 0.05$], respectively.

Silver catfish acclimated to both normoxia and hypoxia and exposed to Mn displayed increased accumulation of the metal in their plasma, gills, liver, kidneys and brain in comparison with their respective controls. Comparing the Mn exposed groups, Mn accumulation in the plasma, liver, kidneys and brain was 2.48-, 1.84-, 1.33- and 1.62-fold greater in normoxia than in hypoxia, respectively (Table 2).

3.3. Hematological parameters

Two-way ANOVA of hematocrit revealed a significant main effect of Mn and a significant oxygen \times Mn interaction [$F(1,8) = 26.89$, $P = 0.0008$; 35.56, $P = 0.0003$, respectively]. For hemoglobin, it revealed a significant main effect of oxygen levels and Mn [$F(1,8) = 16.91$; 16.79, $P = 0.003$, respectively].

There was a reduction of hematocrit of the silver catfish normoxia acclimated and posteriorly exposed to Mn, whereas, under hypoxia, hematocrit was not changed by Mn. In fact, after Mn exposure, hypoxia acclimated group showed higher hematocrit than normoxia group. While hypoxia acclimation increased hemoglobin per se, Mn exposure decreased hemoglobin in hypoxia-acclimated silver catfish. In fact, after Mn exposure, both hypoxia and normoxia groups showed similar hemoglobin values (Table 3).

3.4. Oxidative parameters

3.4.1. Gills

Two-way ANOVA of RS generation revealed a significant main effect of oxygen level and a significant oxygen \times Mn interaction [$F(1,8) = 66.85$, $P = 0.00003$; 10.65, $P = 0.01$, respectively] in gills. For LP generation, it revealed a significant main effect of oxygen level, Mn and a significant oxygen \times Mn interaction [$F(1,8) = 34.88$, $P = 0.0003$; 267.71, $P = 0.0000$; 7.33, $P = 0.02$, respectively] in gills, and for PC generation it revealed a significant main effect of Mn [$F(1,8) = 22.63$, $P = 0.001$].

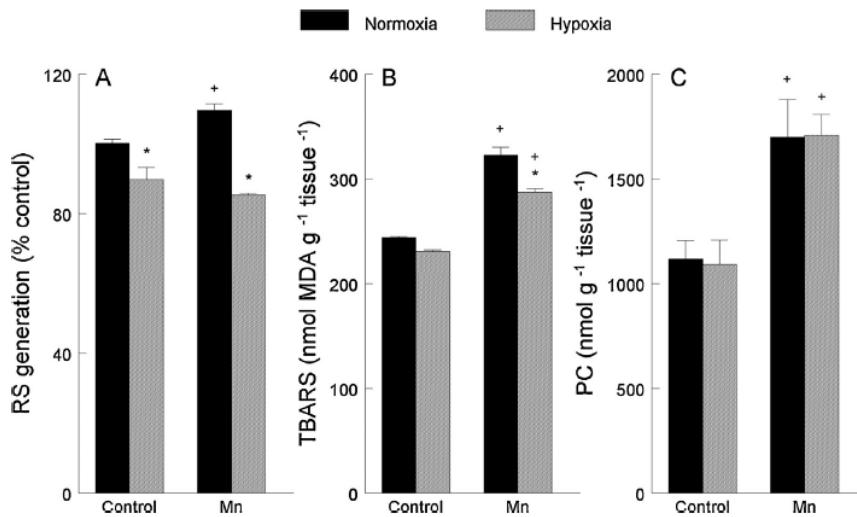


Fig. 2. Reactive species generation (RS) (A), lipid peroxidation (LP) (B) and protein carbonyl (PC) (C) in the gills of silver catfish normoxia or hypoxia acclimated for 10 days and exposed or not to waterborne manganese (Mn) for over 10 days. Control: [0.005 mg L⁻¹]; waterborne Mn [9.8 mg L⁻¹]; *Indicates significant differences between oxygen levels in the same Mn concentration; +Indicates significant differences between Mn and control groups at the same oxygen level ($P < 0.05$). The values are expressed as the mean \pm SEM.

There was a significant decrease of RS generation in the gills of silver catfish acclimated to hypoxia in comparison with normoxia. In normoxia-acclimated fish, Mn exposure increased RS generation (Fig. 2A), LP (Fig. 2B) and PC (Fig. 2C) levels, whereas under hypoxia, only PC levels increased. In fact, Mn exposure in fish acclimated to hypoxia induced lower RS generation and LP levels compared with normoxia-acclimated fish. Interestingly, both LP ($r^2 = 0.97$, $P = 0.0000$) and PC ($r^2 = 0.69$, $P = 0.0008$) levels displayed a significant positive correlation with Mn accumulation in the gills of silver catfish.

3.4.2. Liver

Two-way ANOVA of LP generation revealed a significant main effect of oxygen level and a significant main effect of Mn [$F(1,8) = 119.54$, $P = 0.0000$; 52.10, $P = 0.0000$, respectively] in liver. For PC generation it revealed a significant main effect of oxygen level, Mn and a significant oxygen \times Mn interaction [$F(1,8) = 127.86$; 113.96, $P = 0.0000$; 10.72, $P = 0.01$ respectively].

Dissolved oxygen levels did not affect RS generation in the liver of silver catfish, regardless the Mn exposure (Fig. 3A). On the other hand, LP was significantly lower in hypoxia-acclimated

silver catfish compared with normoxia-acclimated silver catfish (Fig. 3B). The Mn exposure increased LP and PC levels (Fig. 3B and C) in both experimental groups (normoxia and hypoxia). In fact, silver catfish acclimated to hypoxia and subsequently exposed to Mn displayed lower LP and PC levels than those exposed to Mn at normoxia. A significant positive correlation between the PC level and Mn accumulation ($r^2 = 0.75$, $P = 0.0002$) was observed in the liver of silver catfish.

3.4.3. Kidneys

Two-way ANOVA of RS generation revealed a significant main effect of oxygen level, Mn and a significant oxygen \times Mn interaction [$F(1,8) = 50.16$, $P = 0.0001$; 7.89, $P = 0.02$ and 60.68, $P = 0.0000$ respectively]. For LP generation, it revealed a significant main effect of oxygen level, Mn and a significant oxygen \times Mn interaction [$F(1,8) = 450.61$; 1075.07 and 388.44, $P < 0.0000$, respectively], and for PC generation the test revealed a significant main effect

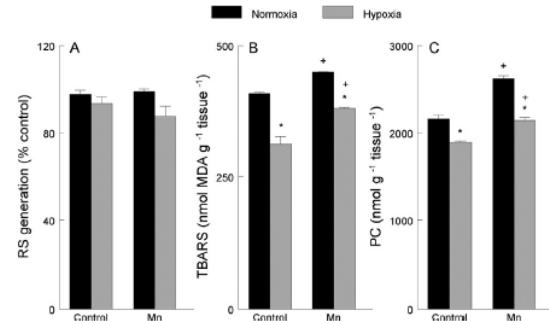


Fig. 3. Reactive species generation (RS) (A), lipid peroxidation (LP) (B) and protein carbonyl (PC) (C) in liver of silver catfish normoxia or hypoxia acclimated for 10 days and exposed or not to waterborne manganese (Mn) for over 10 days. Control: [0.005 mg L⁻¹]; waterborne Mn [9.8 mg L⁻¹]; *Indicates significant differences between oxygen levels in the same Mn concentration; +Indicates significant differences between Mn and control in the same oxygen level ($P < 0.05$). Values are expressed as the mean \pm SEM.

Table 3

Blood parameters. Hematocrit (% cell volume) and hemoglobin (g dL⁻¹) of silver catfish submitted to normoxia or hypoxia for 10 days and exposed or not to waterborne manganese for over 10 days.

Hematological parameters	Oxygen levels	Groups	
		Control	Mn
Hematocrit	Normoxia	29.38 \pm 0.20	24.61 \pm 0.65*
	Hypoxia	27.61 \pm 0.38	27.94 \pm 0.33*
Hemoglobin	Normoxia	6.75 \pm 0.19	6.45 \pm 0.10
	Hypoxia	7.49 \pm 0.10*	6.75 \pm 0.04*

Values are expressed as mean \pm SEM.

Control: [0.005 mg L⁻¹]; Mn: waterborne manganese [9.8 mg L⁻¹].

* indicates significant differences between oxygen levels in the same Mn concentration.

* Indicates significant differences between Mn and control in the same oxygen levels ($P < 0.05$).

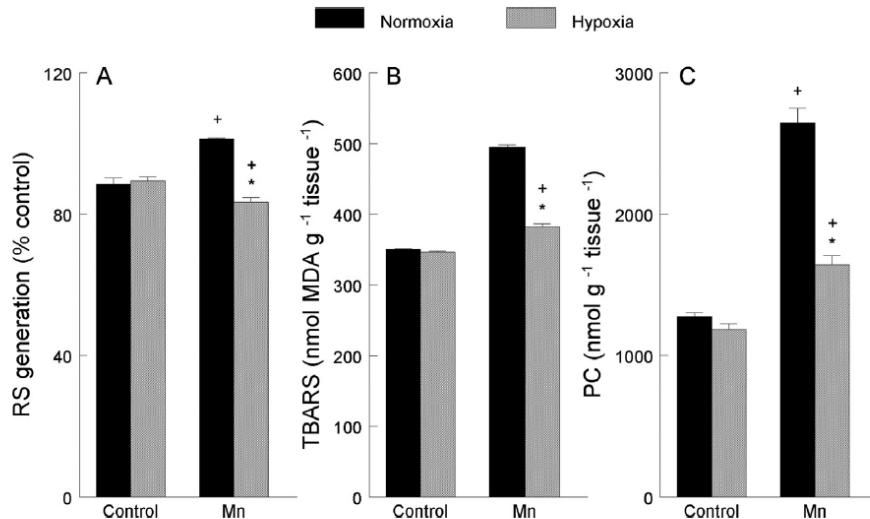


Fig. 4. Oxidative parameters. Reactive species generation (RS) (A), lipid peroxidation (LP) (B) and protein carbonyl (PC) (C) in kidneys of silver catfish normoxia or hypoxia acclimated for 10 days and exposed or not to waterborne manganese (Mn) for over 10 days. Control: [0.005 mg L⁻¹]; waterborne Mn [9.8 mg L⁻¹]; *Indicates significant differences between oxygen levels in the same Mn concentration; +Indicates significant differences between Mn and control in the same oxygen level ($P < 0.05$). Values are expressed as the mean \pm SEM.

of oxygen level, Mn and a significant oxygen \times Mn interaction [$F(1,8)=70.68$; 196.46, $P=0.0000$ and 48.80, $P=0.0001$, respectively].

Dissolved oxygen levels did not alter RS generation (Fig. 4A), LP (Fig. 4B) and PC (Fig. 4C) levels in the kidneys of silver catfish not exposed to Mn. On the other hand, Mn exposure increased RS generation (Fig. 4A) and LP levels (Fig. 4B) in silver catfish of the normoxia group and PC levels of those acclimated to both normoxia and hypoxia (Fig. 4C). Silver catfish acclimated to hypoxia and exposed to Mn presented lower RS generation, LP and PC levels compared with normoxia-acclimated fish exposed to Mn. A

significant positive correlation between LP level and Mn accumulation ($r^2 = 0.82$, $P=0.0006$) was observed in kidneys of silver catfish.

3.4.4. Brain

Two-way ANOVA of RS generation revealed a significant main effect of oxygen level, Mn and a significant oxygen \times Mn interaction [$F(1,8)=17.75$, $P=0.002$; 5.56, $P=0.04$ and 22.70, $P=0.001$, respectively]. For PC levels it revealed a significant main effect of the oxygen level and Mn [$F(1,8)=30.93$, $P=0.0005$; 104.86, $P=0.0000$, respectively].

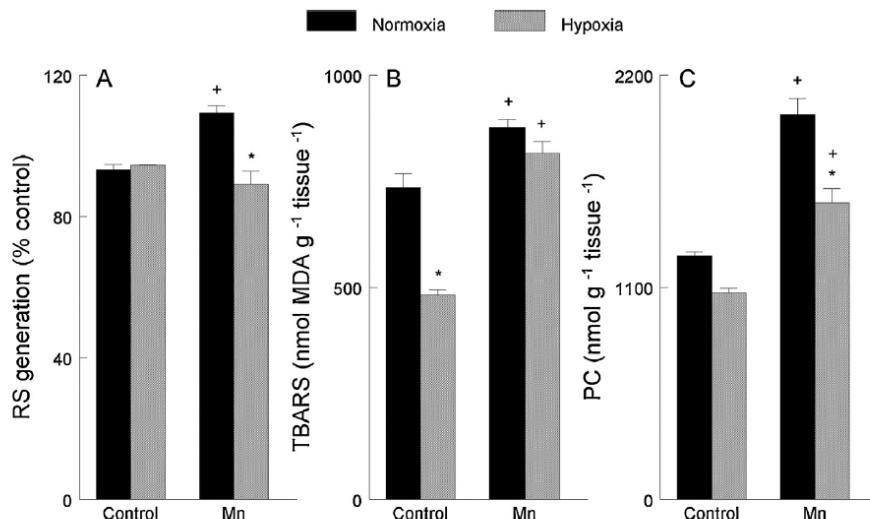


Fig. 5. Oxidative parameters. Reactive species generation (RS) (A), lipid peroxidation (LP) (B) and protein carbonyl (PC) (C) in brain of silver catfish normoxia or hypoxia acclimated for 10 days and exposed or not to waterborne manganese (Mn) for over 10 days. Control: [0.005 mg L⁻¹]; waterborne Mn [9.8 mg L⁻¹]; *Indicates significant differences between oxygen levels in the same Mn concentration; +Indicates significant differences between Mn and control in the same oxygen level ($P < 0.05$). Values are expressed as the mean \pm SEM.

Table 4

Enzymatic activity of silver catfish submitted for 10 days to normoxia or hypoxia and exposed or not to the waterborne manganese for over 10 days.

Activity	Tissues			
	Gill	Liver	Kidney	Brain
<i>CAT (pmol H₂O₂ g tissue⁻¹ min⁻¹)</i>				
Control				
Normoxia	890.3 ± 5.45	848.81 ± 2.53	2493.6 ± 10.13	460.69 ± 5.45
Hypoxia	956.5 ± 2.99*	811.87 ± 3.19*	2454.1 ± 22.99	380.97 ± 2.99*
Mn				
Normoxia	893.8 ± 11.10	834.60 ± 1.77*	1543.2 ± 13.90*	755.54 ± 11.10*
Hypoxia	1106.0 ± 0.38**	814.04 ± 2.61*	2357.0 ± 11.61**	615.09 ± 0.38**
<i>Na⁺/K⁺-ATPase (nmol Pi mg protein⁻¹ min⁻¹)</i>				
Control				
Normoxia	75.1 ± 0.70	67.7 ± 2.94	37.8 ± 3.40	51.6 ± 2.10
Hypoxia	57.7 ± 1.10*	106.2 ± 5.44*	105.8 ± 5.31*	45.2 ± 2.14
Mn				
Normoxia	33.7 ± 0.66*	50.0 ± 1.93*	40.2 ± 2.96	23.6 ± 4.43*
Hypoxia	64.5 ± 0.69**	95.6 ± 2.39*	66.9 ± 0.64**	52.6 ± 0.84*

Values are expressed as mean ± SEM.

Control: [0.005 mg L⁻¹]; Mn: waterborne manganese [9.8 mg L⁻¹].

* indicates significant differences between oxygen levels in the same Mn concentration.

** Indicates significant differences between different Mn concentrations in the same oxygen levels ($P < 0.05$).

In silver catfish not exposed to Mn, the hypoxia acclimation did not affect RS generation and PC levels (Fig. 5A and C), but it decreased LP levels (Fig. 5B) in the brain. The Mn exposure increased PC levels of both normoxia and hypoxia-acclimated fish (Fig. 5C), but this Mn effect was lower in the hypoxia-acclimated group. In fact, while Mn increased RS generation (Fig. 5A) and LP levels (Fig. 5B) of normoxia group, it only increased LP levels in hypoxia-acclimated silver catfish. A significant positive correlation between PC level and Mn accumulation ($r^2 = 0.88$, $P = 0.0000$) was observed in the brain of the silver catfish.

3.5. Enzymatic activities

3.5.1. Gills

Two-way ANOVA of catalase (CAT) activity revealed a significant main effect of oxygen level, Mn and a significant oxygen × Mn interaction [$F(1,8) = 477.79$; 144.18; 131.20, $P = 0.0000$, respectively] in gills. For Na⁺K⁺-ATPase activity, it revealed a significant main effect of the oxygen level, Mn and a significant oxygen × Mn interaction [$F(1,8) = 64.47$; 453.05; 890.91, $P = 0.0000$, respectively].

Silver catfish acclimation to hypoxia increased CAT activity *per se*, decreasing Na⁺K⁺-ATPase activity in gills. However, Mn exposure significantly increased the activity of both enzymes in hypoxia but not in the normoxia-acclimated group (Table 4).

3.5.2. Liver

Two-way ANOVA of catalase (CAT) activity revealed a significant main effect of oxygen level, Mn and a significant oxygen × Mn interaction [$F(1,8) = 124.2$, $P = 0.0000$; 5.5, $P = 0.04$ and 10.1, $P = 0.01$, respectively] in liver. For Na⁺K⁺-ATPase activity, it revealed a significant main effect of oxygen level [$F(1,8) = 148.26$, $P = 0.0000$].

Hypoxia acclimation decreased CAT activity compared to normoxia group. When silver catfish of both levels of oxygen were exposed to Mn, CAT activity decreased in relation to groups not exposed to Mn. In fact, after hypoxia acclimation, Mn exposure reduced CAT activity in relation to normoxia group. On the other hand, hypoxia acclimation increased Na⁺K⁺-ATPase activity in relation to the normoxia group, and this effect remained after Mn exposure. In fact, groups acclimated to hypoxia, exposed to Mn or not, displayed higher activity of Na⁺K⁺-ATPase in relation to the normoxia groups (Table 4).

3.5.3. Kidneys

Two-way ANOVA of CAT activity revealed a significant main effect of oxygen level, Mn and a significant oxygen × Mn interaction [$F(1,8) = 645.41$; 1.181 and 783.80, $P < 0.0000$, respectively] in kidneys. For Na⁺K⁺-ATPase activity, the test revealed a significant main effect of oxygen level, Mn and a significant oxygen × Mn interaction [$F(1,8) = 183.39$, $P = 0.0000$; 27.29, $P = 0.0007$ and 34.92, $P < 0.0003$, respectively].

Though hypoxia acclimation produced no effects *per se* on CAT activity in silver catfish kidneys, Mn exposure decreased the activity of this enzyme in both normoxia and hypoxia-acclimated fish. In fact, after Mn exposure, normoxia-acclimated silver catfish displayed decreased CAT activity compared with the hypoxia group. Hypoxia increased Na⁺K⁺-ATPase activity in relation to normoxia, but Mn exposure reduced this activity. In fact, after Mn exposure, hypoxia-acclimated fish presented higher Na⁺K⁺-ATPase activity than normoxia-acclimated group (Table 4).

3.5.4. Brain

Two-way ANOVA of CAT activity revealed a significant main effect of oxygen level, Mn and a significant oxygen × Mn interaction [$F(1,8) = 141.69$; 817.89, $P = 0.0000$ and 10.78, $P = 0.001$, respectively] in brain. For Na⁺K⁺-ATPase activity, it revealed a significant oxygen level, Mn and a significant oxygen × Mn interaction [$F(1,8) = 17.50$, $P = 0.003$; 14.49, $P = 0.005$ and 42.38, $P = 0.0001$, respectively].

Acclimation to hypoxia decreased catalase (CAT) activity in the brain of silver catfish, and no difference *per se* was found in Na⁺K⁺-ATPase activity. However, Mn exposure increased CAT activity in both normoxia and hypoxia, being the higher value observed in normoxia group. Mn exposure also reduced the Na⁺K⁺-ATPase activity of the normoxia, but not the hypoxia group, whose activity was higher than that observed in the normoxia group (Table 4).

3.6. Pituitary hormone expression

No differences in the expression of pituitary hormones were observed after 10 days of normoxia/hypoxia acclimation. Two-way ANOVA of prolactin (PRL) expression revealed a significant main effect of Mn [$F(1,8) = 10.10$, $P < 0.05$] in pituitary. For somatotropin (SL), the test revealed a significant oxygen level × Mn interaction [$F(1,8) = 4.29$, $P < 0.05$].

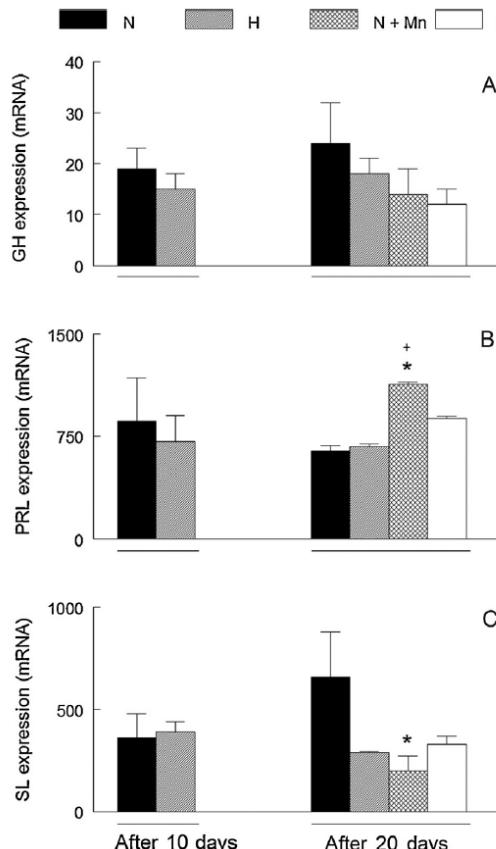


Fig. 6. Growth hormone (GH) (A), Prolactin (PRL) (B) or Somatotropin (SL) (C) expression in the pituitary of silver catfish normoxia or hypoxia acclimated for 10 days and exposed or not to waterborne manganese (Mn) for over 10 days. For better visualization of results, the data were multiplied by 1000. Control: [0.005 mg L⁻¹ Mn]; Mn: [9.8 mg L⁻¹ Mn]; N: [normoxia]; H: [hypoxia]; N + Mn: [normoxia plus manganese]; H + Mn: [hypoxia plus manganese]. *Indicates significant differences between normoxia (control) and Mn plus normoxia; *Indicates significant differences between hypoxia (control) and Mn plus hypoxia; ($P < 0.05$). Values are expressed as the mean \pm SEM.

Neither acclimation of silver catfish to different oxygen levels nor Mn exposure caused changes in GH expression (Fig. 6A). Otherwise, Mn exposure increased PRL (Fig. 6B) and reduced SL (Fig. 6C) expression of normoxia-acclimated silver catfish, with the effects being prevented by hypoxia acclimation.

4. Discussion

It is known that fish exposed to high metal concentrations may take up substantial quantities of these metals (Sultana and Rao, 1998), which are not biodegradable (Hodson, 1988; Carpené et al., 1990; Wicklund-Glynn, 1991). Recently, we demonstrated that silver catfish co-exposed to Mn and normoxia showed higher Mn accumulation in liver, kidneys and gills than those co-exposed to the metal and hypoxia, which is most likely related to cellular adaptation and/or hormesis development (Dolci et al., 2013). In fact, the adaptation process has been related to the increased hypoxia-inducible factor (HIF) expression (Owes et al., 2010), as observed in different organisms during the course of evolution (Wu, 2002; Nikinmaa and Rees, 2005).

These interesting hypotheses were a stimulus to investigate whether a previous acclimation of silver catfish to hypoxia could protect the fish from the deleterious effects of Mn exposure, once this species is considered tolerant to hypoxia (Braun et al., 2006). The expectations were confirmed because silver catfish previously acclimated to hypoxia and sequentially exposed to Mn displayed lower metal accumulation in all tissues, except for the gills, where the Mn level was similar to the normoxia-acclimated group.

This finding is indeed interesting, as that gills comprise a fine and extended surface (90% of the total body area), which maintains primary contact with the water, making this tissue a target of waterborne pollutants (Perry and Laurent, 1993). In fact, due to its constant interaction with the external environment, gills are responsible for functions such as gas exchange, ionic regulation and excretion of metabolic products (Evans et al., 2005), making them susceptible to interference from metals, with accumulation being dependent on the rates of absorption and elimination (Jezierska and Witeska, 2006). Similar to the observed in a study performed with cadmium (Hattink et al., 2005), hypoxia exerted no influence on Mn accumulation in the gills, as it is a transitional tissue for accumulation (Baden et al., 1995), enabling the delivery of the metal to other vital tissues such as plasma, liver, kidneys and brain, what was also observed in the current study.

Moreover, even though the gills of fish acclimated to both normoxia and hypoxia displayed similar Mn levels, our findings indicated a protective influence of hypoxia in this tissue, which was observed through the reduced RS generation and LP levels. Interestingly, this protective influence of hypoxia was not directly reflected for PC, but CAT activity increased in the hypoxia group, confirming the better antioxidant condition of the fish acclimated to this oxygen level (Lushchak et al., 2005; Baker et al., 2007; Tripathi et al., 2013).

Regardless the oxygen level, there was the highest Mn accumulation in livers of silver catfish in relation to all other tissues, thus reinforcing the importance of this vital organ, which is closely involved in xenobiotic detoxification (Heath, 1987; Glusczak et al., 2000). However, our study revealed that none of the factors, the oxygen level and/or Mn exposure, changed the hepatic RS generation, possibly because of the high detoxification potential of this organ. Nevertheless, the beneficial influence of hypoxia acclimation on the liver was notably observed through the lower Mn accumulation, LP and PC levels than those of the normoxia group. This protective action observed in the liver of animals acclimated to hypoxia appears not to be related to CAT, which displayed lower activity in this tissue.

In addition to the gills and liver, the kidneys are one of the first vital organs damaged by water pollutants (Thophon et al., 2003), including tubule degeneration, dilatation of glomerulus capillaries and changes of Bowman's space (Takashima and Hibiya, 1995), and this severe damage may compromise the structural integrity and function of the kidneys. Hypoxia acclimation decreased Mn accumulation in kidneys of silver catfish, and this protective effect was concomitant with a lower RS generation together with lower LP and PC levels. These protective influences from hypoxia acclimation may be associated with antioxidants such as CAT, as CAT activity was maintained in the kidneys of silver catfish exposed to Mn, whereas it was decreased in the normoxia group exposed to Mn. These results indicate that this enzyme has an important detoxifying role in the renal system. Indeed, CAT eliminates hydrogen peroxide, whose accumulation is related to damage in the biological membranes of different tissues (Vutukuru et al., 2006).

The lowest Mn accumulation occurred in the brain of silver catfish in relation to all other tissues, regardless the oxygen level, giving support to fish brain-barrier relative impermeability. In fact, this barrier is structurally similar to the glial perineurium, which was identified in freshwater crayfish, ensuring protection of brain

by maintaining the ions input selectivity (Butt et al., 1990). Indeed, hypoxia-acclimated silver catfish were less affected by Mn than normoxia-acclimated fish, which showed higher RS generation and increased PC levels in the brain.

Considering the Mn exposure, as reported in other studies (Simkiss and Taylor, 1989; Aschner and Aschner, 1991), Mn accumulation in the brain appears to be closely dependent on the oxidation of Mn(II) to Mn(III) by Fenton's reaction, as only Mn(III) may be carried through the brain barrier via transferrin, together with the endocytosis process. Transferrin is a protein containing a Fe-cluster, which is critical for absorption, transport, storage and excretion of metals, being thus able to transpose the relative impermeability of the brain barrier of fish. Silver catfish acclimated to hypoxia displayed reduced Mn accumulation in the brain, possibly due to lower metal oxidation, which may have acted as a limiting agent for the brain barrier permeability. Exposure to Mn of both normoxia- and hypoxia-acclimated fish resulted in increased levels of PC, but the low oxygen level confirmed its protective influence due to the lower PC level in the brain of hypoxia-acclimated fish. In fact, brain protein damage was particularly reduced after hypoxia acclimation, and this was not due to CAT because its activity was lower in both normoxia and hypoxia group. It might be possible that the brain could negatively regulate the consumption of energy through the peripheral nervous system and/or by hormonal routes, as observed in the anoxia-tolerant goldfish (*Carassius auratus*) (Van Ginneken et al., 1996), thus limiting the xenobiotic entrance in the brain.

The positive correlations between Mn accumulation and oxidative parameters (in proteins, by PC levels and lipids, by LP levels) observed in gills, liver, kidneys and brain indicate the close relationship between Mn exposure and oxidative damage development in these vital tissues of silver catfish. Considering that Mn accumulation and the consequent oxidative status in silver catfish were modified by hypoxia acclimation, it is possible that these physiological changes are related to adaptation and/or hormesis. Hormesis was previously described (Bengtsson, 1979; Laughlin et al., 1981) and involves the presence of a small stimulus that may disrupt homeostasis, generating compensatory processes (Calabrese and Baldwin, 2002; Lushchak, 2014).

Xenobiotics may unfavorably affect the Na^+/K^+ -ATPase, whose activity is dependent on the structural integrity of the membrane, and therefore, the enzyme active site would be affected by such exposure, compromising its physiological role (Sancho et al., 2003). Mn exposure decreased the Na^+/K^+ -ATPase activity in the gills and brain of silver catfish acclimated to normoxia. In contrast, besides preventing this Mn-induced effect, hypoxia acclimation increased Na^+/K^+ -ATPase activity in the gills and brain of silver catfish exposed to Mn in comparison to normoxia. Similar to this finding, another study revealed the protective effects of hypoxia on Na^+/K^+ -ATPase activity in gills of *Piaractus mesopotamicus* exposed to copper (Sampaio et al., 2008).

Furthermore, our findings revealed that even though hypoxia increased the Na^+/K^+ -ATPase activity *per se*, after Mn exposure, the activity of this enzyme decreased in the kidneys of hypoxia-acclimated fish. Such finding suggests that Na^+/K^+ -ATPase activity may be more sensitive to Mn-induced damage in this tissue. In fact, previous studies demonstrated that the activity of this functional transmembrane enzyme may be greatly reduced in the kidneys during severe hypoxia (Richards et al., 2007; Wood et al., 2007), also indicating its greater sensibility.

Moreover, as Na^+/K^+ -ATPase acts as a key-enzyme for neurological function in fish (Pandey et al., 2005; Verma et al., 1983), its decreased activity induced by Mn exposure, as observed in the brain of normoxia-acclimated silver catfish, may cause increased neurotransmitter release due to ionic imbalance and consequent depolarization of nerve cells (Kimellberg and Papahad, 1974). Once that hypoxia acclimation prevented a reduction in the

Na^+/K^+ -ATPase activity of silver catfish exposed to Mn, it is possible that moderate hypoxia preserved the ionic properties of the nerve cells, avoiding the acidosis development related to the loss of osmolarity in the brain.

Considering that hematological variations constitute an index of health status in a number of fish species (Blaxhall, 1972; Aubin et al., 2001), such changes have been observed following stress conditions as pollutants exposure, toxic metals, diseases and hypoxia (Duthle and Tort, 1985; Barnhoorn and van Vuuren, 2001). In this sense, our findings revealed that hypoxia acclimation increased the hemoglobin concentration of silver catfish, however, this effect was not maintained after Mn exposure. In addition, Mn exposure reduced the hematocrit of the normoxia-acclimated group, while this metal exerted no effects on the hematocrit of the hypoxia group. In the same way, endorsing this finding, Oweson et al. (2006) showed Mn-induced apoptosis in hematopoietic cells, which might contribute to the decrease of red blood cells (RBC). However, in the current study, the influence of this exposure was prevented by hypoxia.

Literature data indicate that during chronic hypoxia, the hematocrit may increase from hormonal stimulation in the kidneys, which are related to erythropoietin (EPO) secretion. On the other hand, the increased hematocrit in response to erythropoietin may also be modulated by a genetic control, which is related to hypoxia inducible factor (HIF) (Semenza, 2004). Thus far, no studies about HIF in silver catfish have been performed, but the involvement of this factor in the preventive influence of hypoxia on the Mn-induced hematocrit reduction is possible. Thus, this issue requires additional studies.

In addition to these oxidative and damaging effects of Mn on the vital tissues of silver catfish, which were, in general, prevented by hypoxia acclimation, there is also interference of this metal in the expression of pituitary hormones, constituting a promising target for direct analysis of xenobiotics contamination (Tipsmark et al., 2009). Controversial data from literature indicates the influence of xenobiotics on GH expression (Elango et al., 2006), but Mn exposure did not change the expression of this hormone in silver catfish regardless the oxygen levels. However, this study demonstrated for the first time that hypoxia acclimation prevents the changes in PRL and SL expression in a teleost fish provoked by Mn exposure. Indeed, in addition to Mn (Ellingsen et al., 2003; Smargiassi and Mutti, 1999; Takser et al., 2004), PRL levels can be increased by lead (Govoni et al., 1987; Lucchini et al., 2000) and organic mercury exposure (Carta et al., 2003). In rodents, Mn affects the dopaminergic system integrity and also produces an increase of PRL levels (Marreilha dos Santos et al., 2011). Consequently, it is possible that the increased pituitary PRL expression of normoxia-acclimated silver catfish exposed to Mn (and the protective effect of hypoxia acclimation) may be due to a direct effect in the pituitary and/or dopaminergic system.

Moreover, SL, an exclusive fish hormone, is up- or down-regulated during different physiological conditions (Fukada et al., 2005), but its plasma levels and pituitary expression appear to be more related to sexual maturation than responses to environmental factors (Rand-Weaver and Swanson, 1993; Bhandari et al., 2003; Onuma et al., 2003). However, in silver catfish at normoxia, Mn exposure decreased SL expression, possibly by acting in the endocrine axis disruption responsible for the sexual maturation. Thus far, it is not known whether this hormonal change induced by Mn may result in reproductive dysfunction in silver catfish, requiring additional studies. On the contrary, hypoxia acclimation was able to prevent this hormonal change, reinforcing our initial hypothesis about hormesis development, which may increase the defense conditions against external contaminants.

Several studies have demonstrated that moderate hypoxia may be beneficial to a number of aquatic organisms (Hamdoun and

Epel, 2007; Nikinmaa and Rees, 2005; Dolci et al., 2013) because the continuous presence of a small stimulus such as low oxygen levels induces compensatory responses, thus activating physiological changes. All these concomitant alterations may contribute to preventing contaminant-induced hormonal disruptions, enabling aquatic organisms to react against damages caused by environmental contaminants (Calabrese and Baldwin, 2002) such as Mn, thus reflecting the hormesis concept. Taken together, Mn uptake, oxidative status, Na^+/K^+ -ATPase and pituitary hormones expression in silver catfish demonstrated the beneficial influence of hypoxia acclimation, and its action may be related to adaptation mechanisms and/or hormesis development, as already proposed.

Finally, the current study demonstrated that hypoxia is able to ameliorate the oxidative status induced by Mn in silver catfish that are hypoxia-acclimated, and the action mode may be partially linked to CAT activity and possibly to hormesis-induced physiological adaptations. Furthermore, Na^+/K^+ -ATPase activity can be considered a biomarker for Mn toxicity, as it was modified by this metal in gills and brains of silver catfish. In addition, it was demonstrated for the first time that previous hypoxia acclimation is able to prevent Mn-induced physiological changes in the pituitary axis of silver catfish.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2014.10.015>.

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3.2 ARTIGO 2

Submetido ao periódico *Aquatic Toxicology*

ACLIMATAÇÃO A HIPÓXIA PODERIA CAUSAR ALTERAÇÕES MORFOLÓGICAS EM BRÂNQUIAS E PROTEGER CONTRA DANOS OXIDATIVOS INDUZIDOS PELO MANGANÊS MESMO APÓS REOXIGENAÇÃO?

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Could hypoxia acclimation cause gill morphological changes and protect against Mn-induced oxidative injuries in silver catfish (*Rhamdia quelen*) even after reoxygenation?

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Abstract

Aquatic organisms face multiple stressors in natural ecosystems, which have been related to the development of adaptive mechanisms that are poorly understood. Among these, exposure to hypoxia has shown beneficial influences on different species, including silver catfish (*Rhamdia quelen*), especially in situations of aquatic contamination with pollutants such as manganese. Considering that hypoxia is seasonal in the natural aquatic environment, we decided to assess whether these beneficial adaptive mechanisms, initially observed in silver catfish, could be maintained when reoxygenation is established. Silver catfish previously acclimated to moderate hypoxia (~3 mg L⁻¹, 41 % O₂ saturation) for 10 days and subsequently exposed to Mn (~8.1 mg L⁻¹) for additional 10 days displayed lower (47%) Mn accumulation in the gills, and it was maintained (62.6%) after reoxygenation, in comparison to normoxia. Biochemical assays involving oxidative status in the gills allowed us to observe increased reactive species (RS) generation and protein carbonyl (PC) level together with decreased mitochondrial viability induced by Mn. On the other hand, while hypoxia *per se* was beneficial on RS generation and PC level, this acclimation was able to minimize Mn toxicity, as observed by the minor increase of RS generation and the minor reduction of mitochondrial viability, together with decreased PC level. Interestingly, after reoxygenation, part of the protective influences observed during hypoxia acclimation against Mn toxicity were maintained, as observed through a lower level of PC and higher mitochondrial viability in relation to the group exposed to Mn under normoxia. Also, only groups exposed to Mn under hypoxia showed increased activity of both catalase (CAT) and Na⁺/K⁺-ATPase in the gills, however, while CAT activity remained increased after reoxygenation, Na⁺/K⁺-ATPase activity was decreased by Mn, regardless of the oxygen level. Taken together, these findings indicate that exposure of silver catfish to Mn under normoxia was related to higher metal accumulation and increased oxidative damage in the gills, while Mn exposure under hypoxia reduced waterborne Mn uptake, and minimized oxidative damage, causing small but significant morphological changes in the gills. Based on these outcomes, it is possible to propose that events of moderate hypoxia are able to generate rearrangements in the gills of silver catfish exposed to Mn contamination, whose beneficial influence persists in part after establishment of water reoxygenation. These beneficial responses may be related to the development of hormesis, thereby reducing Mn toxicity to silver catfish.

Keywords: *Rhamdia quelen*; Moderate hypoxia; Na⁺/K⁺-ATPase; Oxidative stress; Hormesis; Histological analyses.

1. Introduction

In the fie

In the field of aquatic pollution, global concerns have been raised regarding the release of contaminants into natural waters (Yan et al., 2016), generally related to oil and fuel spills with immediate consequences to ecosystems and economic activities (Rocha et al., 2016). However, data on a possible toxicity together with hypoxia on aquatic species are still lacking. In recent years, the prevalence and intensity of episodes of aquatic hypoxia have increased worldwide (Diaz and Rosenberg, 2008). Different factors have contributed to this, including water pollution by substances rich in nitrogen and phosphorus used in agriculture, pollution generated by environmental accidents, or even natural processes such as water eutrophication. All these factors can consume large amounts of oxygen leading to aquatic hypoxia and organic pollution, and this problem has been worsened by global warming (Sappal et al., 2015).

Constant episodes of hypoxia (waterborne low oxygen level) can lead to morphological and physiological changes in aquatic organisms (Richards, 2009). In fact, these organisms experience daily changes in oxygen levels, which represent adaptive processes related to natural evolution of vertebrates, particularly in aquatic ecosystems, allowing the development of tolerance to hypoxia (Ho and Burggren, 2012). According to Ho and Burggren (2012), two weeks of exposure to low levels of oxygen are sufficient to maintain equilibrium in zebrafish offspring, which were subsequently exposed to severe hypoxia for longer periods. Moreover, it appears that early exposure to hypoxia may lead aquatic animals to cope better with hypoxic environments when they face it in adulthood.

Similarly, silver catfish (*Rhamdia quelen*), a Brazilian native species with high potential for commercialization (Gomes et al., 2000), may experience hypoxia episodes in farm ponds with low water renewal, which can be temporary or permanent depending on environmental conditions (Baldisserotto and Silva, 2004). Moreover, our group have reported the protective effects of hypoxia acclimation against manganese-induced oxidative damage, together with the beneficial influence of hypoxia on the reproductive hormones expression in this species (Dolci et al., 2014).

Otherwise, acute severe hypoxia can also affect thousands of km² of seawater in the world, creating death zones in oceans, and decreasing fish production in these areas (Wu, 2002, Keeling and Garcia, 2002; Helly and Levin, 2004). In addition, aquatic ecosystem contamination by toxic substances is another frequent problem, mainly due to anthropogenic

sources of pollution such as inadequate sewage treatment and release of industrial waste into waters, what lead to the increase of hypoxia (ATSDR, 2000; Garcia de Oliveira, 2008). Among different substances that can contaminate water, manganese (Mn) deserves special attention for being present in formation waters obtained from both oil and gas extraction in the Brazilian Amazon Basin, reaching concentrations up to 264 mg L^{-1} (Baldisserotto et al., 2012). Such contamination may represent risks for local populations, since the maximum permissible limit of Mn for Brazilian class I freshwaters is fixed at 0.1 mg L^{-1} , which may increase to 0.5 mg L^{-1} for class III waters (CONAMA, 2011). Therefore, Mn may be toxic for organisms when above these concentrations (Dolci et al., 2014).

Importantly, the gills play a complex role in the internal organization related to vital functions, including respiration, osmoregulation and excretion (Wendelaar-Bonga 1997; Cengiz 2006) in fish. Considering that they are the first structures exposed to waterborne contaminants, thus being highly representative in fish responses against toxic substances (Wendelaar Bonga and Lock, 1992; Bianchini et al., 2002), we investigated whether hypoxia acclimation could prevent Mn-induced oxidative status in the gills of silver catfish even after establishment of reoxygenation (restoration of oxygen levels to normoxia). Besides, the current study includes the assessment of possible morphological alterations in the gills during hypoxia acclimation, in search of a possible mechanism of action for the process of hypoxia adaptation in silver catfish.

2. Materials and Methods

2.1. Fish

One hundred (100) silver catfish (weight and length shown in Table 1) obtained from fishponds at Universidade Federal de Santa Maria, Rio Grande do Sul, Brazil, were acclimated in continuously aerated 250 L tanks with controlled temperature (20°C) for at least one week prior to experiments. During this period, they were fed once a day (42% crude protein) at 12 pm.

2.2. Reagents

Manganese sulfate hexahydrate [MnSO₄. 6H₂O] (Vetec®, Rio de Janeiro, RJ, Brazil) was used to maintain the waterborne Mn level. All chemicals and solvents used were of HPLC grade and purchased from Sigma Aldrich ® (Brazil).

2.3. Exposure protocol

Manganese level was selected according to previous studies by our laboratory (Dolci et al., 2014). After acclimation, 100 silver catfish were randomly transferred to 20 L tanks (5 fish per tank, five tanks/group, in duplicate), in a semi-static system, with 50% daily water exchange with dissolved oxygen levels previously adjusted through bubbling air (normoxia) and/or nitrogen (hypoxia) for 10 days (Fig. 1; Table 2A). On day 11, fish were euthanized and gills collected, and the remaining silver catfish (5 fish per tank, two tanks/group, in duplicate) were then exposed for additional 10 days to nominal waterborne Mn levels of 0 (control without Mn addition) or 10 mg L⁻¹ of Mn. This step yielded the following groups: normoxia, hypoxia, normoxia plus Mn, and hypoxia plus Mn (Fig. 1; Table 2A). Thus, on day 21, fish were euthanized and gills collected, and remaining silver catfish (5 fish per tank, one tank/group, in duplicate) were exposed to the reoxygenation protocol. In this step, groups previously acclimated to hypoxia were restored to normoxia values through pumping of bubbling air, according to the sequence: normoxia, reoxygenation, normoxia plus Mn, and reoxygenation plus Mn (Fig. 1; Table 2A). Finally, on day 31, fish were euthanized and gills collected. Waterborne manganese values were detected by inductively coupled plasma optical emission spectrometry (described in 2.6.2) and fixed in ~0.002 and ~8.1 mg L⁻¹ (Table 2A). Initial hypoxic environment was created from oxygen consumption due to normal fish metabolism and different dissolved oxygen (DO₂) levels were maintained through aeration with air pumps (air pump AC 2000; 0.0014 MPa pressure) or nitrogen gas when appropriate. Water reservoir tanks (250 L) containing waterborne manganese at appropriate concentration kept under normoxia (with air pumping) or under hypoxic conditions (with nitrogen gas bubbling) were used. This protocol was approved by the Ethics Committee of Animal Use (CEUA) (nº:104/2013), at Universidade Federal de Santa Maria (UFSM).

2.4. Tissues collection

Fish were sampled every 10 experimental days. They were anesthetized with MS 222, euthanized by beheading, and gills were then removed for further biochemical (5 fish were sampled from each duplicate, n=10) and histological analysis (3 fish per group). Gills were chosen since they reflect a model for environmental impact studies (Mallatt, 1985; Evans, 1987; Wendelaar Bonga and Lock, 1992).

2.5. Water parameters

Dissolved oxygen and temperature (YSI5512 oxygen meter; SI Inc., Yellow Springs, OH, USA), pH (DMPH-2 pH meter Digimed, São Paulo, SP, Brazil), and total ammonia nitrogen (TAN) levels (Eaton et al., 2005) were measured twice a day. Un-ionized ammonia (NH_3) levels were calculated according to Colt (2002). Once a day, water hardness (EDTA titrimetric method) and alkalinity (Boyd and Tucker, 1992) were determined. Water tanks collections (20L) were obtained from three points: surface, 8 cm deep, and bottom of the tank. In 250 L tanks, which served as a reservoir for replacement of water, water samples were obtained from three points: surface, 30 cm deep, and bottom of the tank. Tanks were cleaned late afternoon daily by siphoning, and replaced with pre-adjusted Mn levels ($\text{MnSO}_4 \cdot 6\text{H}_2\text{O}$). At the end of each exposure, Mn wastes were chemically treated and properly disposed.

2.6. Metal analyses

2.6.1. Gill samples

Digestion of gills was performed using a conventional heating block (Velp Scientifica, Model DK, Italy) with open glass vessels. The procedure was performed with concentrated nitric acid (14 Mol L⁻¹) (Merck, Darmstadt, Germany). H_2O_2 was added, and digests were heated up to 80°C for 1 h. After cooling, digests were diluted with purified water (Milli-Q system, Millipore Corp., Bedford, USA) for further analysis.

2.6.2. Sample Mn determination

Waterborne Mn level and Mn accumulation in the gills were determined using an inductively coupled plasma optical emission spectrometer (ICP OES, Model Spectro Ciros

CCD, Spectro Analytical Instruments, Kleve, Germany) equipped with an axial view configuration and a cross-flow nebulizer coupled to a Scott-type double pass nebulization spray chamber. Wavelengths for Mn determination were 257.611 nm. Radiofrequency power was 1400 W. Flow rates for plasma generation, auxiliary and nebulization gas were 12.0, 1.0 and 1.0 L min⁻¹, respectively. Argon (99.996%, White Martins–Praxair, São Paulo, SP, Brazil) was used for plasma generation for nebulization, and as an auxiliary gas. For accuracy assessment, certified reference material (CRM) of dogfish muscle tissue (DORM-2) from the National Research Council, Canada, was used.

2.7. Histological analysis

2.7.1 Sample preparation

The second left gill arch was prepared for light microscopy ($n = 3$). Following excision, it was rinsed in saline buffer, and immersed in Bouin's solution for 24 h. Then, its central portion was carefully excised, dehydrated in graded ethanol concentrations, and embedded in Technovit 7100 resin. A sagittal cut was obtained on a LEICA RM2245 microtome set to 1–2 μm , mounted on a glass slide, and stained with toluidine blue. Examination was performed at 400x using a Zeiss Axio VisionSystem with Remote Capture 4.7 Rel DC – Cannon Power shot G9. Slides were thoroughly examined in order to determine the presence of histological alterations. In addition, six high power fields were randomly selected on each slide to measure height of potentially functional lamella, epithelium epifilament thickness, lamella width, diffusion distance, lamella total area (modified from Hughes 1984). Chloride cells were counted in 1 mm of filament (Fig. 2).

2.8. Biochemical assays

2.8.1. Sample preparation

For all biochemical assays (except estimation of mitochondrial viability, in which a specific buffer preparation was used) gills were homogenized (1:5 w/v) in Tris-HCl buffer (10mM; pH 7.4), centrifuged at 3640 $\times g$ for 15 min, and supernatants were used for all determinations (except protein carbonyl, in which total homogenized was used).

2.9. Oxidative parameters

2.9.1. Reactive species (RS) determination

RS levels were measured using oxidant sensing fluorescent probe 2',7'-dichlorofluorescein diacetate (DCHF-DA) (Hempel et al., 1999). Oxidation (DCHF-DA) to fluorescent dichlorofluorescein (DCF) was determined at 488 nm for excitation, and 525 nm for emission. After homogenization and centrifugation of tissues, 3 mL of buffer (10 mM Tris-HCl, pH 7.4) was added. After 10 s, 10 µM (DCHF-DA) (prepared in ethanol) were added to the mixture, and fluorescence intensity from DCF was measured for 300 s and expressed as a percentage of the untreated control group. Protein content was normalized by quantification according to Lowry et al. (1951).

2.9.2. Protein carbonyl determination

Protein carbonyl (PC) was measured according to Yan et al. (1995), with some modifications. Soluble protein was mixed with 2,4-dinitro- phenylhydrazine (DNPH; 10 mM in 2 M HCl) or HCl (2 M) and incubated at room temperature for 1 h. Denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, with 3% sodium dodecyl sulfate), ethanol (99.8%) and hexane (99.5%) were added, mixed by shaking, and centrifuged. Protein isolated from the interface was washed twice with ethyl acetate/ethanol 1:1 (v/v) and suspended in denaturing buffer. Each DNPH sample was read at 370 nm in a spectrophotometer against the corresponding HCl sample (blank). Results were expressed as nmol carbonyl/g tissue⁻¹.

2.9.3. Estimation of mitochondrial viability

Mitochondria were isolated as previously described by Brustovetsky and Dubinsky (2000), with some alterations: gills were rapidly removed (within 1 min) and immersed in ice-cold “isolation buffer I” containing 225 mM mannitol, 75 mM sucrose, 1 mM K⁺-EGTA, 0.1% bovine serum albumin (BSA), and 10 mM K⁺- HEPES, pH 7.2. Tissue was minced using surgical scissors and extensively washed. Tissue was then homogenized in a power-driven, tight-fitting Potter-Elvehjem homogenizer with a Teflon pestle. The resulting suspension was centrifuged for 7 min at 2000 x g in a refrigerate centrifuge. After centrifugation, the supernatant was re-centrifuged for 10 min at 12 000 x g. The pellet was resuspended in

“isolation buffer II” containing 225 mM mannitol, 75 mM sucrose, 1 mM K⁺-EGTA, and 10 mM K⁺-HEPES pH 7.2, and re-centrifuged at 12 000 $\times g$ for 10 min. The supernatant was decanted and the final pellet gently washed and resuspended in 600 μ L of “isolation buffer III” containing 10 mM sucrose, 60 mM KCl, 10 mM K⁺-HEPES buffer (pH 7.2), 50 mM EGTA, 5 mM glutamate, and 5 mM succinate. Mitochondrial viability was developed by colorimetric reduction of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]- MTT whose color can be spectrophotometrically measured ($k = 570\text{-}630$ nm). This assay quantifies mitochondrial activity by measuring the formation of a dark violet formazan product formed by the reduction of a tetrazolium ring of MTT (yellow) (Mosmann, 1983). MTT reduction mainly occurs in mitochondria through the action of succinate dehydrogenase, therefore providing a measure of mitochondrial function (Slater et al., 1963). For MTT assay, mitochondria suspension was prior diluted to a protein concentration of 20 mg mL⁻¹ (Lowry et al., 1951). Data were expressed as a percentage of the control group (normoxia).

2.10. Enzymatic activities

2.10.1. Catalase activity assay

Catalase (CAT) activity was spectrophotometrically quantified by the method of Aebi (1984), which monitors the disappearance of H₂O₂ in presence of tissue at 240 nm. Enzymatic activity was expressed as μ mol H₂O₂ g tissue⁻¹ min⁻¹ (1 U decomposes 1 μ mol H₂O₂ min⁻¹ at pH 7 at 25°C).

2.10.2. Na⁺/K⁺-ATPase activity assay

Na⁺K⁺-ATPase activity was determined in gills according to Musbeck et al. (1977), with some modifications. Briefly, aliquots of tissue (20 μ L) were added to a reaction medium containing NaCl, MgCl₂, KCl, and Tris-HCl buffer (pH 7.4), with or without Na⁺K⁺-ATPase enzyme inhibitor ouabain. The method for ATPase activity measurement is based on the determination of inorganic phosphate (Pi) released to the reaction medium by hydrolysis of ATP, according to the method proposed by Atkinson et al. (1973). The reaction was initiated with the addition of ATP substrate to the reaction medium, and was finished by the addition of

color reagent (1 mL), containing ammonium molybdate (2%), triton X-100 and H₂SO₄ (10%) after 15 min of incubation at 37°C. Molybdate–Pi complexes formed were measured spectrophotometrically at 405 nm. Values were calculated in relation to a standard curve constructed with Pi at known concentrations and also corrected by protein content (Lowry et al., 1951).

2.11. Statistical analysis

Statistics were performed using Statistica (Statsoft Inc., Tulsa, USA, version 7). Homogeneity and normal distribution of data were initially tested by Levene's test. Data were analyzed by one-way ANOVA (dissolved oxygen levels: normoxia x hypoxia) or two-way ANOVA (Mn x dissolved oxygen levels), followed by Tukey's multiple range test, when appropriate. Pearson correlation was applied for data, expressed as mean ± S.E.M., and *p*<0.05 was regarded as statistically significant.

3. Results

3.1. Water parameters

All assessed parameters (pH; DO₂; hardness, alkalinity; total ammonia; NH₃; nitrite) were within the expected values, with no variations throughout the experiment (Table 2A, 2B).

3.2. Metal accumulation

Two-way ANOVA of Mn accumulation after both 20 and 30 experimental days revealed a significant main effect of oxygen level, Mn, and a significant oxygen level × Mn interaction [$F(1,36)_{10-20} = 8.04 p = 0.04$, $80.00 p = 0.0008$ and $7.82 p = 0.04$, respectively] and [$F(1,36)_{20-30} = 13.09 p = 0.02$, $58.14 p = 0.001$ and $12.05 p = 0.02$, respectively]. As expected, silver catfish exposed to Mn under normoxia for 10 days showed higher accumulation of metal in the gills, whose value was 1.89 fold greater than animals also exposed to Mn under hypoxia. Moreover, 10 additional days of Mn exposure under normoxia were able to increase metal levels in the gills (9.27%) in relation to previous exposure, while the reoxygenated group showed lower Mn accumulation (62.6%) in relation to normoxia (Table 3).

3.4. Histological analysis

Two-way ANOVA of functional height of lamella of gills revealed a significant main effect of oxygen level and Mn after 20 experimental days [$F(1,8)_{10-20} = 15.60 p = 0.0001$, $24.19 p = 0.0000$, respectively], and a significant main effect of Mn after 30 days [$F(1,8)_{20-30} = 32.5 p = 0.0000$]. Silver catfish acclimated to hypoxia for 10 (Fig. 2B and Fig. 3A) and 20 days (Fig. 2E and Fig. 3A) showed increased functional height of lamella in the gills (black arrows), what was not observed in the experimental group subsequently exposed to reoxygenation (Fig. 2I and Fig. 3A). Moreover, Mn caused a decrease in functional height of lamella after both 10 (Fig. 2D and Fig. 3A) and 20 (Fig. 2H and Fig. 3A) days of Mn exposure under normoxia. In contrast, hypoxia was able to prevent Mn-induced decrease in functional height of lamella in the gills of silver catfish (Fig. 2F and Fig. 3A). Regarding epithelium epifilament thickness, after both 20 and 30 experimental days, two-way ANOVA revealed a significant main effect of oxygen levels and Mn [$F(1,8)_{10-20} = 15.81$ and $17.14 p < 0.0000$, respectively; and $F(1,8)_{20-30} = 10.10 p = 0.002$ and $16.96 p = 0.0001$, respectively]. Epithelium epifilament thickness (black line) decreased *per se* after 10 (Fig. 2B and Fig. 3B) and 20 (Fig. 2E and Fig. 3B) days of hypoxia acclimation, persisting even after establishment of reoxygenation (Fig. 2I and Fig. 3B). On the other hand, after 10 (Fig. 2D, and Fig. 3B) and 20 (Fig. 2H and Fig. 3B) days of Mn exposure under normoxia, an increase in epithelium epifilament thickness was observed, which was prevented by hypoxia acclimation (Fig. 2F and Fig. 3B), but this effect was not maintained after reoxygenation (Fig. 2J and Fig. 3B).

After 30 experimental days, two-way ANOVA of lamella width revealed a significant main effect of Mn and a significant oxygen level \times Mn interaction [$F(1,8)_{20-30} = 8.98 p = 0.003$ and $5.37 p = 0.02$, respectively]. Lamella width (red line) showed increase only after 30 days of exposure to Mn under normoxia conditions (Fig. 2H and Fig. 3C). Regarding diffusion distance, two-way ANOVA revealed a significant main effect of Mn and a significant oxygen level \times Mn interaction [$F(1,8)_{10-20} = 69.90$ and $23.54 p < 0.0000$, respectively] after 20 experimental days. After 30 experimental days, two-way ANOVA of diffusion distance revealed a significant main effect of Mn [$F(1,8)_{20-30} = 34.45 p = 0.0000$]. Diffusion distance decreased *per se* after 10 (Fig. 2B and Fig. 3D) and 20 (Fig. 2E and Fig. 3D) days of hypoxia acclimation. Mn exposure was able to increase this morphological parameter in both normoxia (Fig. 2D and Fig. 3D) and hypoxia (Fig. 2F and Fig. 3D) acclimation, showing even higher

values for additional 10 days of Mn exposure under normoxia (Fig. 2H and Fig. 3D) and after reoxygenation (Fig. 2J and Fig. 3D).

Two-way ANOVA of lamella total area revealed a significant main effect of oxygen levels, Mn, and a significant oxygen level \times Mn interaction [$F(1,8)_{10-20} = 11.84 p = 0.0007$, $8.39 p = 0.04$ and $4.92 p = 0.02$, respectively], after 20 experimental days. After 30 experimental days, two-way ANOVA revealed a significant main effect of Mn [$F(1,8)_{20-30} = 10.03 p = 0.002$] on lamella total area. Interestingly, acclimation to hypoxia during both 10 (Fig. 2B and Fig. 3E) and 20 days (Fig. 2E and Fig. 3E) was able to increase *per se* lamella total area of gills of silver catfish. Likewise, presence of Mn allowed an increase in lamella total area in fish acclimated to normoxia for both 20 (Fig. 2D and Fig. 3E) and 30 days (Fig. 2H and Fig. 3E). Finally, after 20 experimental days, two-way ANOVA of chloride cells revealed a significant main effect of oxygen level and a significant oxygen level \times Mn interaction [$F(1,8)_{10-20} = 41.10 p = 0.0000$ and $11.352 p = 0.0008$, respectively]. After 30 experimental days, two-way ANOVA revealed a significant main effect of Mn [$F(1,8)_{20-30} = 34.45 p = 0.0000$]. Mn exposure under normoxia was able to increase the number of chloride cells in the interlamellar filament region in both 10 (Fig. 2D and Fig. 3F) and 20 (Fig. 2H and Fig. 3F) days of exposure to this metal. Such Mn-induced alteration was prevented by acclimation to hypoxia (Fig. 2F and Fig. 3F), but chloride cells number remained increased during establishment of reoxygenation (Fig. 2J and Fig. 3F).

3.5. Biochemical assays

3.5.1. Reactive species (RS) determination

Two-way ANOVA of reactive species (RS) after 20 experimental days revealed a significant main effect of oxygen level and Mn [$F(1,36)_{10-20} = 37.80 p = 0.0000$; $22.48 p = 0.0002$, respectively]. While silver catfish acclimated to hypoxia showed lower RS generation *per se* in the gills than animals acclimated to normoxia, Mn exposure was able to increase generation of RS at a bigger proportion in the normoxia group, since hypoxia prevented part of Mn-induced damage. Moreover, 10 additional days of Mn exposure was not related with increased RS generation, whose values were similar among all experimental groups, including the reoxygenated group (Fig. 4A).

3.5.2. Carbonyl protein determination

Two-way ANOVA of protein carbonyl (PC) after 20 experimental days revealed a significant main effect of oxygen level, Mn, and a significant oxygen level \times Mn interaction [$F(1,36)_{10-20} = 89.03 p = 0.0000$, $9.68 p = 0.008$ and $8.01 p = 0.01$, respectively]. After 30 experimental days, two-way ANOVA revealed a significant main effect of oxygen level and Mn [$F(1,36)_{20-30} = 210.31$, $333.24 p < 0.0000$, respectively]. While exposure of silver catfish to Mn under normoxia during both 10 and 20 days was related with increased levels of PC in the gills, notably, both hypoxia acclimation and reoxygenation were able to prevent and minimize, respectively, PC levels in the gills in presence of Mn or not (Fig. 4B).

3.5.3. Estimation of mitochondrial viability

Two-way ANOVA of mitochondrial viability after 20 experimental days revealed a significant main effect of oxygen level, Mn, and oxygen level \times Mn interaction [$F(1,36)_{10-20} = 17.34 p = 0.002$, $246.7 p = 0.0000$ and $85.80 p = 0.0000$, respectively]. After 30 experimental days, two-way ANOVA revealed a significant main effect of Mn and a significant oxygen level \times Mn interaction [$F(1,36)_{20-30} = 113.94 p = 0.0000$ and $11.53 p = 0.004$, respectively]. Silver catfish acclimated to hypoxia showed decreased mitochondrial viability *per se* in the gills when compared to normoxia, but this effect was reversed when oxygenation was reestablished. While 10 and 20 days of exposure to Mn under normoxia were able to decrease mitochondrial viability in the gills, silver catfish exposed to Mn under hypoxia and subsequently to reoxygenation showed minimized mitochondrial damage in comparison to normoxia group (Fig. 4C).

3.5.4. Catalase activity assay

Two-way ANOVA of catalase (CAT) activity after 20 experimental days revealed a significant main effect of oxygen level, Mn, and a significant oxygen level \times Mn interaction [$F(1,36)_{10-20} = 676.79 p = 0.02$, $7.75 p = 0.01$ and $6.12 p = 0.02$, respectively]. After 30 experimental days, two-way ANOVA revealed a significant main effect of oxygen level and a significant oxygen level \times Mn interaction [$F(1,36)_{20-30} = 9.49 p = 0.009$ and $6.13 p = 0.02$, respectively]. Acclimation of silver catfish to different oxygen levels (normoxia and hypoxia) as well as those subsequently reoxygenated caused no difference *per se* on CAT activity of the

gills. Mn exposure was able to increase CAT activity in both experimental hypoxia and reoxygenated groups, although CAT activity in the gills was not modified by Mn (Fig. 5A) under normoxia.

3.5.5. Na⁺/K⁺-ATPase activity assay

After 20 experimental days, two-way ANOVA of Na⁺/K⁺-ATPase activity revealed a significant main effect of oxygen level, and a significant oxygen level × Mn interaction [$F(1,36)_{10-20} = 22.77 p = 0.0007$, $66.03 p = 0.03$, respectively]. After 30 experimental days, two-way ANOVA revealed a significant main effect of oxygen level and Mn [$F(1,36)_{20-30} = 57.35 p = 0.0000$, $6.81 p = 0.02$, respectively]. Different levels of oxygen such as hypoxia and reoxygenation were not able to change Na⁺/K⁺-ATPase activity in the gills of silver catfish. However, animals exposed to Mn for 10 and 20 days under normoxia showed reduced activity of this enzyme in the gills. Notably, hypoxia acclimation was able to prevent the influence of Mn on Na⁺/K⁺-ATPase activity, but when the oxygen level was restored to normoxia, this activity was decreased to similar values as observed in animals exposed to Mn under normoxia (Fig. 5B).

4- Discussion

Recently it was showed that acclimation to moderate hypoxia was able to reduce both Mn accumulation and oxidative damage in different tissues of silver catfish (Dolci et al., 2013; 2014), indicating that hypoxia acclimation may favor cellular adaptation mechanisms or hormesis development in this species. In fact, additional studies have already demonstrated that moderate hypoxia may be beneficial to a number of aquatic organisms (Hamdoun and Epel, 2007; Nikinmaa and Rees, 2005). However, these findings gave rise to further questions: Could fish acclimated to hypoxia develop a greater ability to adapt to future adverse situations (such as water contamination) than those animals exposed to normal oxygen conditions? In an attempt to answer this question, the current experimental protocol was planned and performed.

In accordance with previous findings (Dolci et al., 2013), silver catfish exposed to Mn under normoxia showed higher metal accumulation in the gills than those exposed to Mn under hypoxia. However, it is important to highlight that this accumulation pattern is not followed by older silver catfish (~120 grams body weight), as recently described (Dolci et al., 2014), which

showed similar Mn accumulation in the gills, regardless of oxygen levels in water (normoxia and hypoxia). Moreover, an interesting outcome of this study was that hypoxia acclimation allowed a lower Mn accumulation in the gills, whose levels remained lower throughout reoxygenation. A hypothesis that could explain this pattern of Mn accumulation in the gills of silver catfish is that younger fish suffer greater modulation than older fish since their antioxidant system is in a developmental state.

In this context, gills are responsible for vital functions such as gas exchanges, ionic regulation, excretion of metabolic products (Evans et al., 2005), making this tissue susceptible to interferences from contaminants such as metals, whose accumulation depends on rates of absorption and elimination (Jezierska and Witeska, 2006). Moreover, gills represent 90% of total body area of fish, which is in constant interaction with the external aquatic environment, making this tissue a target to waterborne pollutants (Bianchini et al., 2002; Cengiz 2006). Thus, morphological changes in the gills may impair their physiological functions, including osmoregulation, ventilation and maintenance of acid-base balance (Wendelaar-Bonga 1997; Bianchini et al., 2002). In line with this, our current study included histopathological analysis of gills of silver catfish, which was able to reveal important effects of hypoxia acclimation, such as increased functional height and total area of lamella, besides decreased thickness of epithelium myofilament, which remained reduced even after reoxygenation. These findings suggest the development of an adaptive mechanism able to increase the area for gas exchange, since the available amount of oxygen is lower under hypoxia, thus favoring uptake of oxygen through the gills (Evans et al., 1999; Perry e cols., 2004).

Literature data have shown that hypoxia may activate nervous reflexes that stimulate catecholamine release (Baldisserotto, 2013), thus promoting a significant dilation of afferent arteries. Indeed, dilation of afferent arteries facilitates the blood supply to lamellae, causing an increase in blood pressure in gill lamellae. Due to this increase in the blood pressure, lamellae receive more blood, favoring an increase of area available for gas exchange, at the same time that lamella distension occurs (here confirmed by concomitant increase in lamella total area under hypoxia), reducing thickness of lamellar epithelium epifilament. This process facilitates gas exchange under hypoxia (Evans et al., 1999; Nilsson, 2007). Definitely, the current findings showed that exposure of silver catfish to Mn under normoxia was able to decrease the functional height of lamella and increase epithelium epifilament thickness in the gills, which were inversely affected under hypoxia acclimation. However, these morphological changes may be considered only temporary, since after reoxygenation, effects of Mn on the functional height of

lamellae and epithelium epifilament thickness in the gills was similar, regardless of oxygen levels. According to Mallat (1985), lifting and hyperplasia of lamellar epithelium could be interpreted as defense responses of fish rather than reflecting direct toxic action, as these alterations increase the distance across which waterborne pollutants such as Mn must diffuse to reach the bloodstream.

As expected, diffusion distance (the area available for gas exchanges) in the gills decreased at 10 and 20 days of hypoxia acclimation, thus favoring uptake of any amount of oxygen available (Nilsson, 2007). In contrast, diffusion distance in the gills was increased by Mn as well as in lead (Pb) contamination, as previously reported by Winkler et al. (2001), and Camargo and Martinez (2007). Our findings are in line with this, since animals exposed to Mn contamination under both oxygen levels presented increased diffusion distance in the gills with higher increase under hypoxia, which was maintained after reoxygenation. In fact, Mn is likely to generate an increase of diffusion space between blood and water, in an attempt to make the metal uptake more difficult through the gills. Taken together, the current findings are in accordance with Nilsson (2000), who suggested that chronic acclimation to hypoxia allows morphological remodeling of the gills. Our study revealed that such morphological changes may persist even after reestablishment of oxygen levels. So, 10 days of reoxygenation was not enough to recover diffusion distance shown by basal groups. We can infer that increase of diffusion space may be considered a protection mechanism against Mn-induced oxidative damage.

Notably, exposure of silver catfish to Mn for both 10 and 20 days increased the number of chloride cells in the interlamellar filament region of gills, which was prevented by hypoxia acclimation, possibly indicating adjustment of this structure to low oxygen levels. However, the presence of Mn together with reoxygenation increased the number of these cells. Chloride cells are the main cell type involved in uptake of minerals from water, and are located in basolateral membranes of the gill epithelium, where they are responsible for the ion transport mechanism in freshwater teleost fish (Sancho et al., 2003; Parvez et al., 2006). Thus, an increased density of chloride cells may indicate a compensatory response to Mn-induced ionoregulatory disturbances. In this context, a widely accepted model used to explain ion transport in freshwater fish is the coupling of Na^+ uptake to both NH_4^+ and H^+ excretion, besides uptake with Cl^- or to H^+ excretion (Dymowska et al., 2012), since this transport in chloride cells depends on Na^+K^+ -ATPase, a trans-membrane enzyme whose activity depends on the structural integrity of the membrane, which is also implicated in metabolic energy production (Doganli et al., 2013). The functionality of this enzyme can be inhibited by other

environmental pollutants such as pesticides (Bianchini et al., 2002; Vani et al., 2012), industrial waste (Parvez et al., 2006), and heavy metals (Rugimony et al., 2004). These pollutants may cause changes in the epithelium of gills, thus changing activity of Na^+/K^+ -ATPase, blocking normal ion fluxes, and consequently initiating physiological adjustments (Sancho et al., 2003).

Curiously, our findings showed that acclimation of silver catfish to hypoxia caused no significant changes *per se* in Na^+/K^+ -ATPase activity in the gills. However, chronic exposure to low levels of oxygen associated with Mn contamination was able to recover the activity of this enzyme, similarly to the beneficial effects of hypoxia observed in *Piaractus mesopotamicus* exposed to copper (Sampaio et al., 2008). Higher Na^+/K^+ -ATPase activity would also contribute to reduce ion loss, thus maintaining Na^+ ion homeostasis (Moyson et al., 2015) face to damage induced by Mn. In contrast, silver catfish exposed to Mn under normoxia showed decreased activity of the enzyme, which persisted even after 10 days of reoxygenation, indicating that Na^+/K^+ -ATPase is highly vulnerable to damage induced by Mn contamination. Such inhibition of Na^+/K^+ -ATPase activity in Mn-exposed fish under normoxia showed a negative correlation with number of chloride cells ($r^2 = -0,37$; $p = 0,02$). A similar correlation was found when *Oreochromis mossambicus* were exposed to cadmium (Pratap and Wendelaar Bonga 1993), indicating that the excess of ions in the aquatic environment increases chloride cell population to favor ionic exchanges, thus avoiding collapse of this enzyme.

Manganese is a recognized generator of reactive species (RS), since in a water environment it (Mn III) suffers reduction to Mn (III) under hypoxic/anoxic conditions through degradation of organic matter (Limburg et al., 2011; Itai et al., 2012). As a consequence, our findings showed that Mn exposure for 10 days under normoxia increased RS generation in the gills, whereas hypoxia acclimation decreased the production of this oxidative marker *per se*, minimizing Mn-induced RS generation. Interestingly, subsequent reoxygenation and/or Mn exposure for additional 10 days did not cause changes in RS generation, since basal levels of RS were maintained in all experimental groups. These outcomes allow us to propose that during this time, RS generation may have been balanced by the antioxidant defense system of this species. Indeed, as RS are characterized by having one or more unpaired electrons, it implies great instability and high reactivity with the extracellular environment (Halliwell and Gutteridge, 1999), damaging macromolecules, and triggering oxidation reactions on lipids, proteins, and DNA (Digiovanni, 1992; Marshall and Bangert, 1995).

In this sense, damage to proteins was assessed in this study, since it is an important marker of oxidative status of organisms in water contamination situations. This assessment

allows us to confirm the beneficial influence of hypoxia acclimation *per se*, which was observed by decreased PC levels after 20 days of acclimation and maintained after water reoxygenation. Interestingly, silver catfish exposed to Mn for 10 and 20 days under normoxia showed increased PC levels in the gills, while hypoxia acclimation was able to prevent this damage to proteins. In fact, this beneficial influence of hypoxia may be considered robust, since PC levels of gills were lower in the reoxygenated group in comparison to silver catfish exposed to Mn under normoxia. These protective effects of hypoxia on damage to Mn-induced proteins may be related to increased antioxidant defense system (Hermes-Lima, 2004), since organisms can neutralize reactive species (Halliwell and Gutteridge, 1999), avoiding impairments for vital organs and systems. Here, catalase (CAT) activity increased in Mn-exposed fish under hypoxia, thus confirming findings shown in previous studies of our (Dolci et. al., 2014) and other research group (Ransberry et al., 2016).

Moreover, increased activity of CAT observed in the hypoxia-acclimated group was maintained after reoxygenation, allowing to propose that hypoxia acclimation may have long-lasting effects, since in the current study lower PC levels and increased activity of CAT were maintained after reoxygenation. Thus, here we show for the first time that the beneficial influence of hypoxia acclimation can be long-lasting, contributing to maintaining the homeostatic balance of fish exposed to contamination of aquatic environment. Important to our findings, previous studies have shown that aquatic organisms can favorably adapt to this oxygen level, a fact that was reported by Hermes Lima et al. (1998) as "preparation for oxidative stress". Such phenomenon can be considered fundamental in the protection against post-hypoxia free-radical damage (Freire et al., 2011; Teixeira et al., 2013).

Subsequently, mitochondrial viability was used to assess cell viability (Liu et al., 1997) through MTT, which is an indirect indicator of cell metabolism when it is reduced to formazan by mitochondrial dehydrogenases in viable cells. Our results showed that silver catfish acclimated to hypoxia for 10 and 20 days showed major and minor reduction of mitochondrial viability in the gills, respectively, suggesting development of an adaptive process face to low water oxygen levels, and confirming previous findings regarding responsiveness of mitochondrial enzymes under hypoxia conditions (Dolci et. al, 2013). However, this effect was readily reversed when water oxygen levels were reestablished, indicating a fast recovery of mitochondrial enzymes activity under normoxia. The beneficial influence of hypoxia on mitochondrial viability was clearly observed, since: i) Silver catfish exposed to Mn under normoxia for 10 and 20 days showed a progressive reduction in mitochondrial viability,

whereas under hypoxia Mn toxicity was robustly reduced; ii) beneficial influence of hypoxia was preserved after water reoxygenation, even in a smaller proportion.

Additionally, interesting correlations were observed from 10 to 20 days of Mn exposure under hypoxia: i) negative correlations between Mn accumulation and RS generation, and mitochondrial viability ($r^2 = 0.76; p = 0.009$ and $r^2 = 0.42; p = 0.01$, respectively); ii) a negative correlation between mitochondrial viability and PC levels, and a positive correlation between RS generation and PC levels ($r^2 = 0.69; p = 0.000$ and $r^2 = 0.64; p = 0.0001$, respectively), indicating that mitochondrial enzymes functionality was impaired by Mn exposure due to the increased RS generation in the gills. After 20 days of Mn exposure and water reoxygenation it was possible to observe: iii) negative correlations between decreased activity of Na^+K^+ -ATPase and epithelium epifilament thickness, diffusion distance, and chloride cells ($r^2 = 0.47, p = 0.01$; $r^2 = 0.57, p = 0.01$; $r^2 = 0.37, p = 0.02$, respectively). This same water condition showed iv) a negative correlation between Na^+K^+ -ATPase activity and PC levels, and a positive correlation between Mn accumulation and PC levels in the gills ($r^2 = 0.76, p = 0.002$; $r^2 = 0.67, p = 0.04$, respectively), as well as a negative correlation between Mn accumulation and mitochondrial viability, and a positive correlation between Na^+K^+ -ATPase activity and mitochondrial viability ($r^2 = 0.73, p = 0.006$; $r^2 = 0.72, p = 0.0008$, respectively). Taken together, these correlations indicate that both Mn exposure and hypoxia acclimation produced histological and functional changes in the gills, exerting prolonged protective effects under hypoxia, which were preserved after water reoxygenation.

Considering Mn accumulation profile in the gills, as well as consequent morphological changes and oxidative status in silver catfish that were previously acclimated to hypoxia, it is possible to suggest that these changes are related to adaptation and/or hormesis process (as firstly described by Bengtsson, 1979 and Laughlin et al., 1981). This hypothesis admits that the presence of a small stimulus such as low oxygen levels may disrupt homeostasis, generating compensatory responses (Calabrese and Baldwin, 2002; Mattson, 2008; Calabrese, 2014) preparing organisms face to subsequent damage caused by stressors such as Mn exposure.

4. Conclusion

In conclusion, we are showing for the first time that a previous hypoxia acclimation was related to morphological rearrangements in gills of silver catfish, which was sufficient to minimize Mn accumulation and reduce Mn-induced oxidative damage. So far, no study showed the influence of hypoxia acclimation on Mn toxicity of aquatic species after water reoxygenation. From this study, we propose that hypoxia acclimation is capable to evoke protective response against hostile environments such as Mn contamination, reflecting the hormesis concept.

5. Limitation of the study

This study showed that acclimation to hypoxia favors morphological adaption in gills of silver catfish even after reoxygenation, thus reducing Mn-induced damage. Here, we explore possible molecular mechanisms such as analysis of hypoxia inducible factor (HIF-1 α) in the gills. We used HIF-1 α purified goat polyclonal antibody raised against a peptide mapping near the C-terminus of HIF- 1 α of human origin. This antibody is recommended for species such as mice, rats, humans, *Xenopus*, however, at the moment, there is no specific antibody HIF-1 α for *Rhamdia quelen*. Further studies should be performed in order to detect HIF-1 α immunoreactivity to this species.

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Table 1. Weight and length of the Silver catfish. Fish were acclimated for 10 days to normoxia (N) or hypoxia (H) and afterwards exposed or not to waterborne Mn [~ 8.1 mg L $^{-1}$], for additional 10 days (H+Mn; N+Mn)). After this period, fish previously hypoxia acclimated were submitted to reoxygenation (R; R+Mn) for 10 more days.

Exposure (Days)	Groups	Weight (g)	Length (cm)
0 – 10	N	28.66 ± 2.83	14.00 ± 0.63
	H	26.66 ± 4.80	13.33 ± 0.84
10 – 20	N	31.20 ± 3.81	16.00 ± 1.26
	H	29.00 ± 3.91	14.80 ± 1.49
	N + Mn	26.80 ± 2.08	15.20 ± 1.28
	H + Mn	26.80 ± 5.03	14.60 ± 1.50
20 – 30	N	27.60 ± 5.52	14.20 ± 1.20
	R	30.00 ± 4.13	15.40 ± 1.15
	N + Mn	29.80 ± 3.86	16.40 ± 1.21
	R + Mn	26.60 ± 1.43	15.40 ± 1.12

*Indicates significant difference between oxygen levels (N and H or N and R groups) in the same water condition (Mn or not);

[†]Indicates significant differences between Mn and control group in the same oxygen level ($p < 0.05$). Values are expressed as mean \pm SEM.

Table 2(A-B). Water parameters with Mn experimental values (obtained after inductively coupled plasma optical emission spectrometry (ICP-OES) analyses). Fish were acclimated for 10 days to normoxia (N) or hypoxia (H) and afterwards exposed or not to waterborne Mn [~ 8.1 mg L $^{-1}$], for additional 10 days (H+Mn; N+Mn). After this period, fish previously hypoxia acclimated were submitted to reoxygenation (R; R+Mn) for 10 more days.

2A

Exposure (Days)	Groups	Dissolved Oxygen	O ₂ Saturation (%)	[Mn] mg L $^{-1}$ ICP-OES	T (°C)	pH
0 – 10	N	7.36±0.06	81.89±0.86	0.002±0.00	21.30±0.18	5.85±0.17
	H	2.94±0.09*	40.19±1.13*	0.002±0.00	21.39±0.18	5.85 ± 0.14
10 – 20	N	7.43±0.10	82.00±1.00	0.002±0.00	22.02±0.14	6.00±0.23
	H	3.09±0.14*	41.54±1.14*	0.002±0.00	21.87±0.17	5.66±0.14
	N + Mn	7.57±0.22	82.79±2.84	8.13±0.21 ⁺	21.92±0.15	5.80±0.15
	H + Mn	3.32±0.10*	42.60±1.40*	8.30±0.31 ⁺	22.03±0.18	6.00±0.24
20 – 30	N	7.04±0.02	79.32±0.16	0.002±0.00	22.5±0.13	5.99±0.23
	R	7.15±0.05	79.94±0.26	0.002±0.00	22.5±0.10	5.83±0.21
	N + Mn	7.29±0.12	81.41±2.19	8.18±0.91 ⁺	22.5±0.13	5.95±0.20
	R + Mn	7.15±0.04	80.18±0.36	8.18±0.64 ⁺	22.5±0.11	5.67±0.15

2B

Exposure (Days)	Groups	Hardness (mg CaCO ₃ L $^{-1}$)	Alkalinity	Total ammonia	NH ₃	Nitrite
0 – 10	N	72.50 ± 2.19	47.60 ± 1.16	0.04 ± 0.002	0.0007 ± 0.00	0.17 ± 0.05
	H	78.20 ± 2.04	50.42 ± 1.34	0.04 ± 0.001	0.0008 ± 0.00	0.14 ± 0.01
10 – 20	N	77.20 ± 3.04	44.20 ± 2.37	0.03 ± 0.009	0.0008 ± 0.00	0.17 ± 0.03
	H	67.80 ± 2.50	48.50 ± 1.48	0.04 ± 0.003	0.0008 ± 0.00	0.14 ± 0.02
	N + Mn	76.00 ± 1.19	48.10 ± 1.51	0.03 ± 0.002	0.0008 ± 0.00	0.17 ± 0.05
	H + Mn	76.00 ± 3.21	45.40 ± 1.02	0.03 ± 0.003	0.0008 ± 0.00	0.17 ± 0.08
20 – 30	N	74.40 ± 1.40	46.15 ± 1.10	0.03 ± 0.003	0.0007 ± 0.00	0.13 ± 0.01
	R	67.80 ± 2.50	50.30 ± 1.67	0.04 ± 0.002	0.0007 ± 0.00	0.16 ± 0.03
	N + Mn	73.40 ± 1.40	46.10 ± 1.02	0.03 ± 0.002	0.0007 ± 0.00	0.15 ± 0.02
	R + Mn	75.40 ± 2.70	46.60 ± 0.53	0.04 ± 0.004	0.0007 ± 0.00	0.17 ± 0.05

*Indicates significant difference between oxygen levels (N and H or N and R groups) in the same water condition (Mn or not);

⁺Indicates significant differences between Mn and control group in the same oxygen level ($p < 0.05$). Values are expressed as mean ± SEM.

Table 3. Mn accumulation in silver catfish gill. Water parameters with Mn experimental values (obtained after inductively coupled plasma optical emission spectrometry (ICP-OES) analyses). Fish were acclimated for 10 days to normoxia (N) or hypoxia (H) and afterwards exposed or not to waterborne Mn [$\sim 8.1 \text{ mg L}^{-1}$], for additional 10 days (H+Mn; N+Mn). After this period, fish previously hypoxia acclimated were submitted to reoxygenation (R; R+Mn) for 10 more days.

Exposure (Days)	Groups	Mn accumulation in gills ($\mu\text{g g}^{-1} \text{ tissue}^{-1}$)
0 – 10	N	0.77 ± 0.20
	H	0.91 ± 0.18
10 – 20	N	0.83 ± 0.03
	H	0.71 ± 0.22
	N + Mn	$37.20 \pm 4.20 ^+$
	H + Mn	$19.75 \pm 4.25 ^{+*}$
20 – 30	N	0.82 ± 0.02
	R	0.29 ± 0.005
	N + Mn	$40.65 \pm 5.15 ^+$
	R + Mn	$15.20 \pm 5.0 ^{+*}$

*Indicates significant difference between oxygen levels (N and H or N and R groups) in the same water condition (Mn or not);

[†]Indicates significant differences between Mn and control group in the same oxygen level ($p < 0.05$). Values are expressed as mean \pm SEM.

Legend for figures:

Figure 1. Experimental design. Silver catfish were acclimated to normoxia (N) or hypoxia (H) for 10 days, and afterwards exposed or not to waterborne Mn [$\sim 8.1 \text{ mg L}^{-1}$], for additional 10 days, totaling 20 days (N+Mn; H+Mn). After this period, hypoxia-acclimated fish were exposed to water reoxygenation (R; R+Mn) for 10 more days, totaling 30 days.

Figure 2. - Gill histology qualitative parameters through light microscopy. Silver catfish were acclimated to normoxia (N) or hypoxia (H) for 10 days (2A, 2B), and afterwards exposed or not to Mn [$\sim 8.1 \text{ mg L}^{-1}$] for additional 10 days (2C, 2D, 2E, 2F). After this period, hypoxia-acclimated fish were submitted to water reoxygenation (R) for 10 more days (2G, 2H, 2I, 2J). Insert figure represents histological qualitative parameters in the gills assessed through light microscopy. Letters indicate: A = lamella total height; B = functional height lamella; C = epithelium epifilament thickness; D = lamella width; E = diffusion distance; F = lamella total area; White asterisk indicates chloride cell. Scale bar: 50 μm .

Figure 3. Analysis of histological quantitative parameters in the gills. Functional height lamella (A), epithelium epifilament thickness (B), lamella width (C), diffusion distance (D), lamella total area (E), and chloride cell (F). Silver catfish were acclimated to normoxia (N) or hypoxia (H) for 10 days, afterwards exposed or not to waterborne Mn [$\sim 8.1 \text{ mg L}^{-1}$], for additional 10 days (N+Mn; H+Mn). After this period, hypoxia-acclimated fish were submitted to water reoxygenation (R; R+Mn) for 10 more days.

*Indicates significant difference between oxygen levels (N and H or N and R groups) in the same water condition (Mn or not);

[†]Indicates significant differences between Mn and control group in the same oxygen level ($p < 0.05$). Values are expressed as mean \pm SEM.

Figure 4. Oxidative parameters. Reactive species generation (RS) (A), protein carbonyl (PC) (B), and MTT reduction levels (C) in the gills of silver catfish acclimated to normoxia (N) or hypoxia (H) for 10 days, and afterwards exposed or not to waterborne Mn [$\sim 8.1 \text{ mg L}^{-1}$] for additional 10 days (N+Mn; H+Mn). After this period, hypoxia-acclimated fish were submitted to water reoxygenation (R; R+Mn) for 10 more days.

*Indicates significant differences between oxygen levels (N and H or N and R groups) in the same water condition (Mn or not);

[†]Indicates significant differences between Mn and control group in the same oxygen level ($p < 0.05$). Values are expressed as mean \pm SEM.

Figure 5. Enzymatic activities. Catalase (CAT) (A) and Na^+/K^+ -ATPase (B) of silver catfish acclimated to normoxia (N) or hypoxia (H) for 10 days, and afterwards exposed or not to waterborne Mn [$\sim 8.1 \text{ mg L}^{-1}$] for additional 10 days (N+Mn; H+Mn). After this period, hypoxia-acclimated fish were submitted to water reoxygenation (R; R+Mn) for 10 more days.

*Indicates significant differences between oxygen levels (N and H or N and R groups) in the same water condition (Mn or not);

[†]Indicates significant differences between Mn and control group at the same oxygen level ($p < 0.05$). Values are expressed as mean \pm SEM.

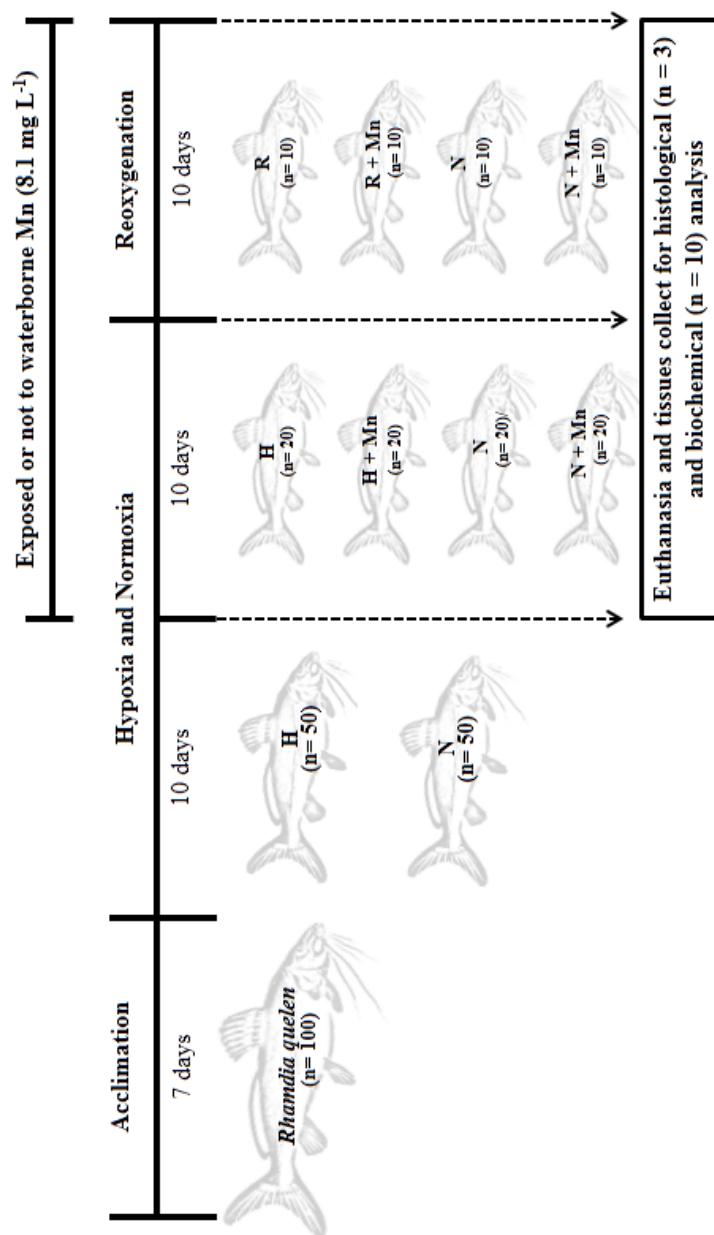
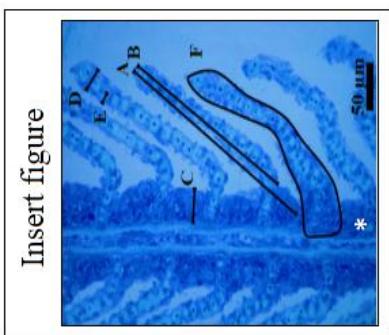
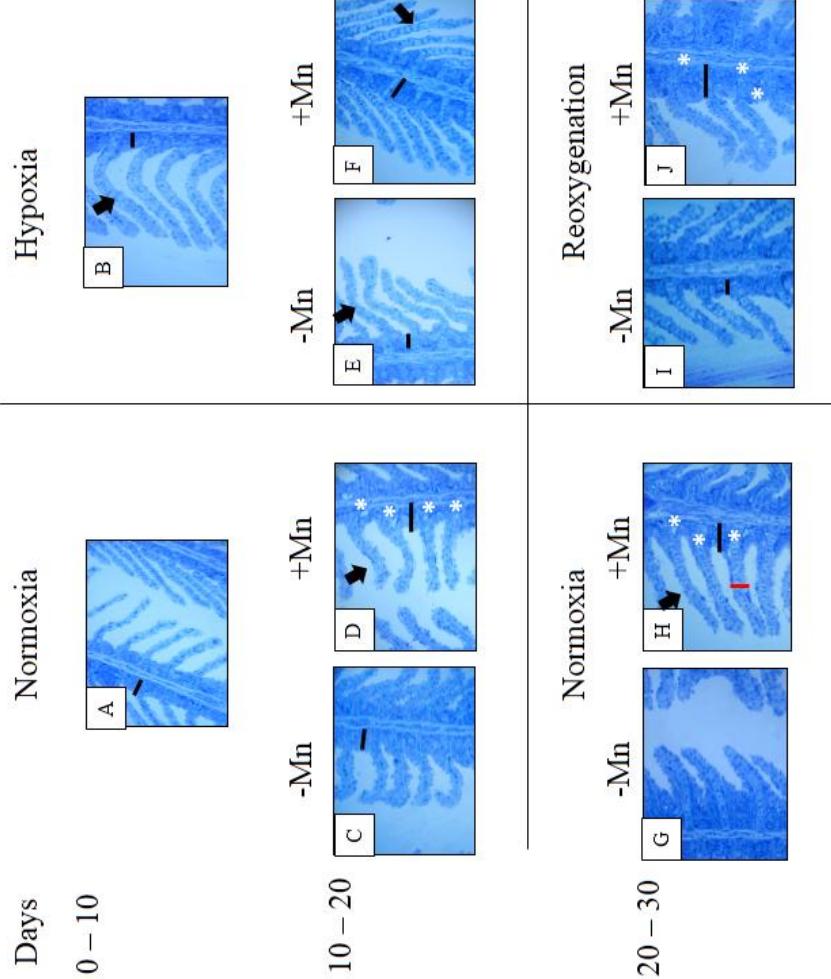


Figure 2



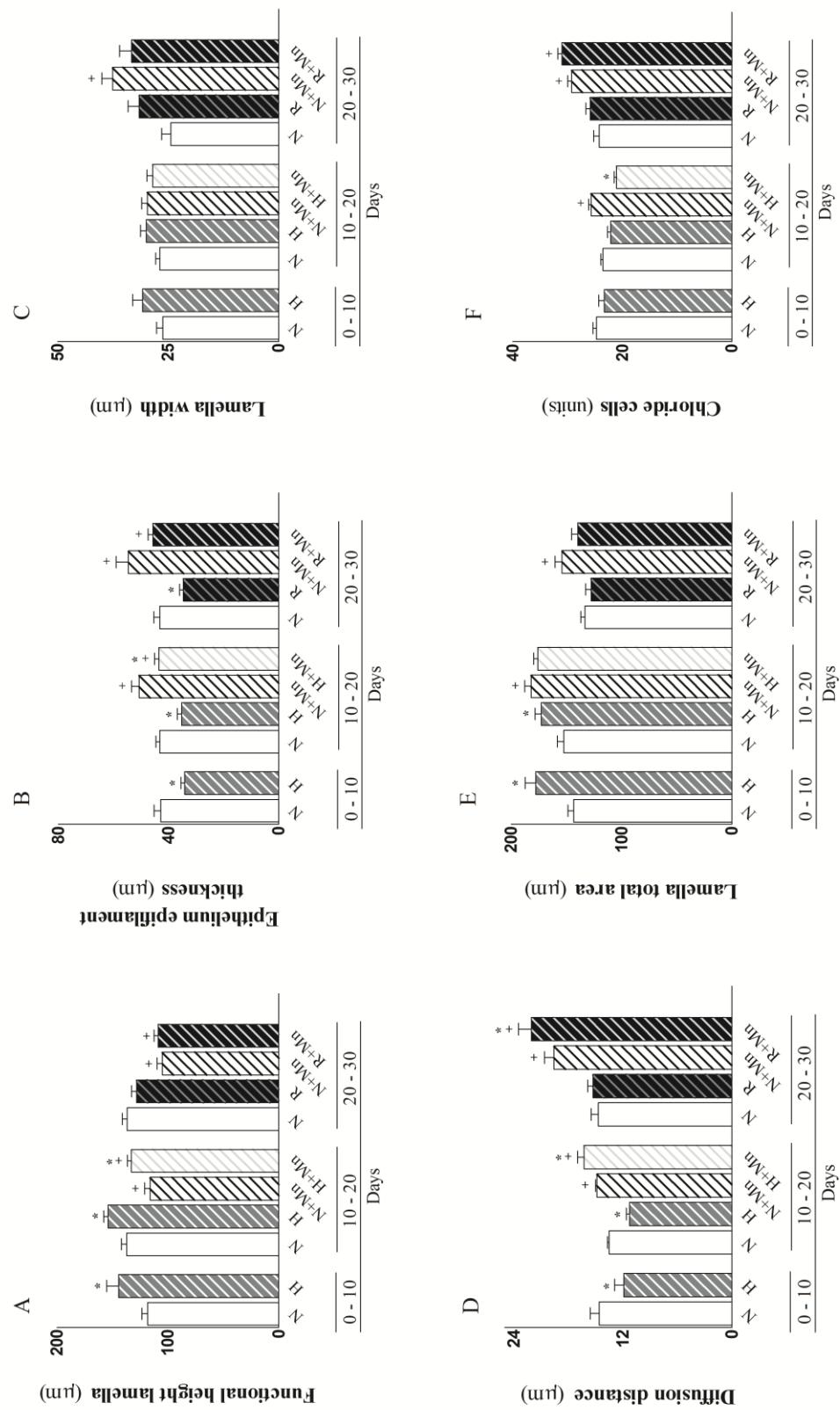


Figure 3

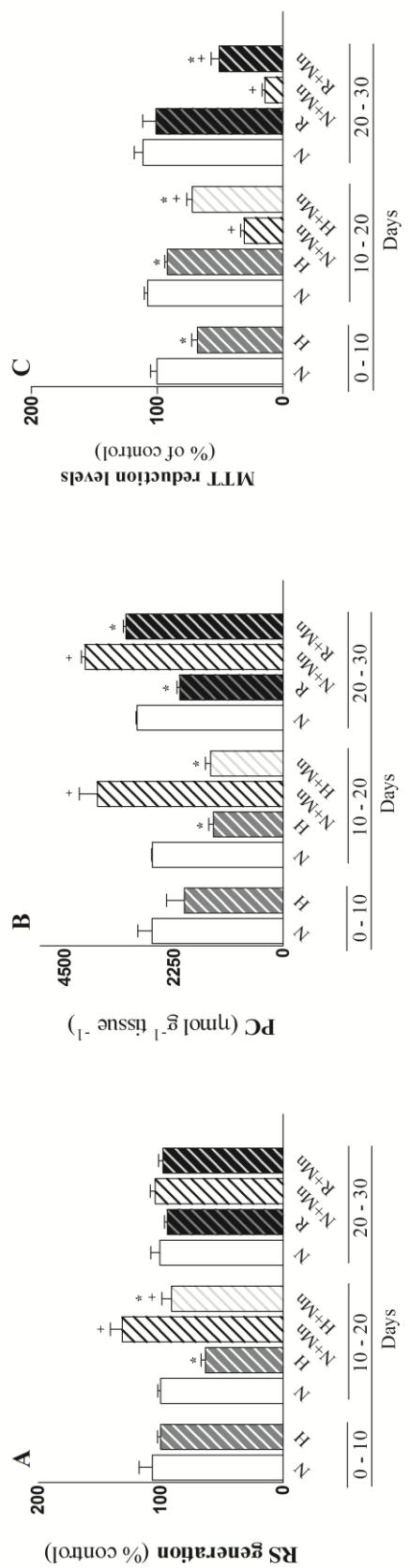


Figure 4

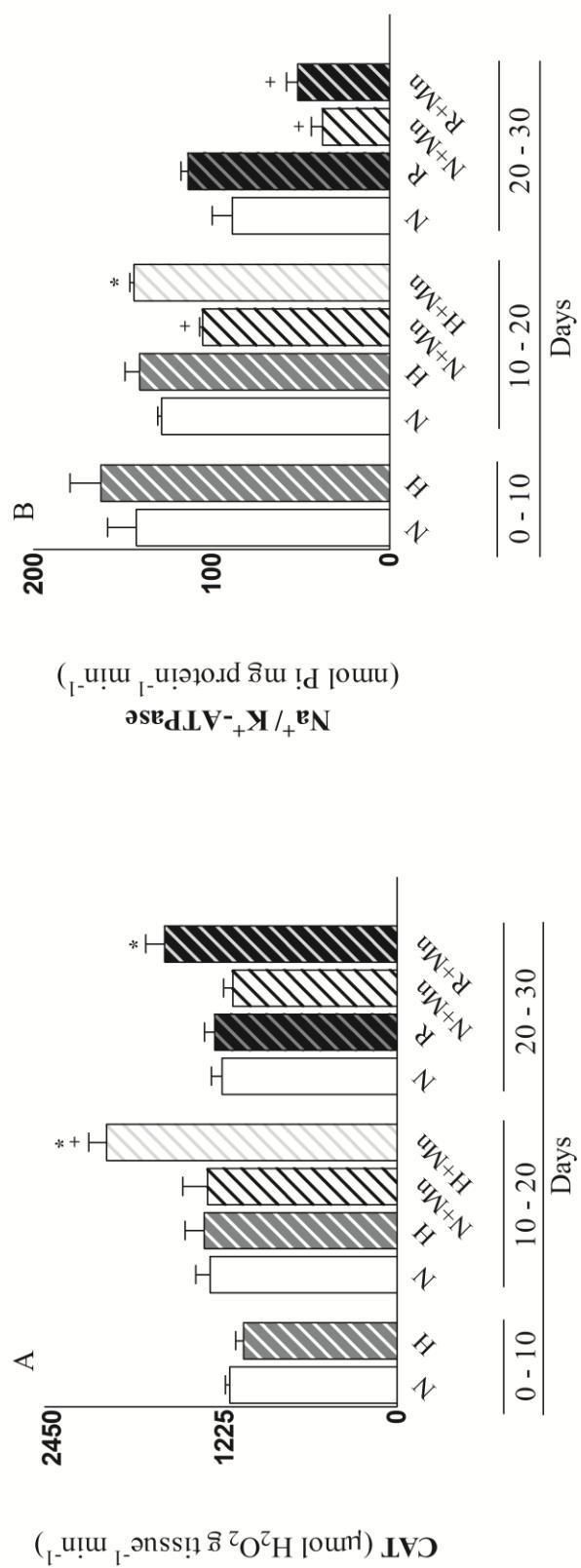


Figure 5

3.3 ARTIGO 3

ACLIMATAÇÃO À HIPÓXIA E SUBSEQUENTE REOXIGENAÇÃO REDUZEM OS DANOS INDUZIDOS POR Mn EM JUNDIÁS

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Hypoxia acclimation and subsequent reoxygenation reduces Mn-induced damage in silver catfish

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Abstract

This study was designed to investigate if hypoxia acclimation modifies the hematological and oxidative profiles in tissues of Mn-exposed silver catfish (*Rhamdia quelen*), and if such modifications persist upon subsequent reoxygenation. The findings showed that silver catfish acclimated to hypoxia (~3 mg L⁻¹, 41 % O₂ saturation) for 10 days and subsequently exposed to Mn (~ 8.1 mg L⁻¹) for additional 10 days exhibited lower Mn accumulation in plasma, liver and kidney, even after reoxygenation, as compared to normoxia-acclimated fish. Hypoxia acclimation increased *per se* red blood cells count and hematocrit, so favoring physiological adjustments in hemoglobin, which are indicative of adaptations to this oxygen level, while the reoxygenation process was also related to increased hematocrit and hemoglobin *per se*. Animals exposed to Mn under normoxia for 20 days showed decreased red blood cells count and hematocrit , while reoxygenation subsequent to hypoxia increased red blood cells count. Additionally, hypoxia acclimation was able to prevent Mn-induced oxidative damage, observed by increased reactive species (RS) generation and higher protein carbonyl (PC) levels in both liver and kidney under normoxia. Mn-exposed silver catfish under hypoxia and after reoxygenation showed decreased damage in liver mitochondria in relation to the normoxia group, with decreased plasma transaminases (glutamic oxaloacetic transaminase - GOT and glutamic pyruvic transaminase - GPT) and gamma glutamyl transferase (GGT). Moreover, acclimation of silver catfish to hypoxia was able to increase some antioxidant defenses, here represented by reduced glutathione (GSH) levels and catalase (CAT) activity in liver and kidney during Mn exposure, which remained increased even after reoxygenation. Similarly, Na⁺/K⁺-ATPase activity was increased in both tissues of Mn-exposed fish under hypoxia, remaining increased even after reoxygenation. These findings are showing for the first time that Mn-exposed silver catfish in normal oxygen conditions show impairments in both hematological and oxidative statuses, while their previous acclimation to moderate hypoxia is able to generate physiological adjustments, which generate coordinated responses that ameliorate the antioxidant status even after reoxygenation. Such responses represent a physiological regulation of this teleost fish against hostile conditions (oxygen deprivation and/or Mn toxicity) in order to preserve the stability of a particular tissue or system.

Keywords: *Rhamdia quelen*, moderate hypoxia, Mn toxicity, hematological profile, plasma transaminases, enantiostasis

1. Introduction

It is known that organisms contain different enzymatic and non-enzymatic antioxidant defenses that are able to maintain reactive species (RS) under control (Hermes-Lima, 2004). In this sense, RS comprise superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), among others (Halliwell and Gutteridge, 1999), which can be neutralized by recycling of glutathione (GSH), and also by antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Hermes-Lima, 2004). Consequently, environmental pollutants can modify the balance between pro-oxidant agents and the antioxidant defense system of the organisms (Winston and Di Giulio, 1991; Matthiessen and Law, 2002; Ransberry, 2016). So, evaluation of the oxidative status (protein and lipid peroxidation) together with antioxidant responses in aquatic species is usually applied as a non-specific biomarker of toxicity (Bainy et al., 1996; Geracitano et al., 2004a) in order to characterize areas of impact, where complex mixtures of pollutants can be present (Bainy et al., 1996; Amado et al., 2006a,b).

Manganese (Mn) is naturally present in freshwaters as a trace element ($<0.1\text{ mg L}^{-1}$) (CONAMA, 2011), being essential for biological functions, acting as enzymatic cofactor of mitochondrial superoxide dismutase (Mn-SOD) in very low concentrations (Li et al., 1995). However, contamination by Mn in freshwaters is connected with oil and gas exploration activities observed in formation waters in the Brazilian Amazon Basin (Baldisserotto et al., 2012; Gabriel et al., 2013), where Mn can reach high concentrations (266 mg L^{-1}). So, pollution in estuarine environments is considered a critical environmental concern due to the high variation in several abiotic factors that impose severe restrictions to organisms living in these areas (Amado et al., 2006a; Monserrat et al., 2007; Sany et al., 2014).

Among abiotic factors (temperature, salinity, etc.), oxygen levels deserve attention, since oxygen restriction (hypoxia) may exert detrimental influences on several aquatic species, as hypoxic zones range from mild hypoxia to complete anoxia (Welker et al., 2013). Natural hypoxic environments can be a result from excessive decomposition of organic matter, present in areas of dense vegetation, where there is proliferation of photosynthetic algae (Almeida-Val et al., 2000; Chippari-Gomes et al., 2005; Richards et al., 2007). On the other hand, environmental hypoxia can result from such anthropogenic action as urbanization and consequent pollution (Diaz, 2001; Ficke et al., 2007), which has contributed to the increased incidence of minimum oxygen in ocean zones, with crescent expansion to shallower waters (Keeling and Garcia 2002; Helly and Levin 2004).

Even though oxygen is essential for species survival, some organisms are able to adapt to limited amounts of oxygen, including hypoxia-tolerant species such as *Carassius auratus* and *Cyprinus carpio*, which can survive to extreme hypoxic situations, experiencing long periods of oxygen deprivation (Lushchak et al., 2001; Hattink et al., 2005). Furthermore, hypoxia-tolerant animals may resort to physiological adjustments by rising up antioxidant defenses against the deleterious influences of an overproduction of reactive species (RS) that occurs during reoxygenation (restoration of oxygen levels to normoxia) (Lushchak et al., 2001; 2005b).

In this regard, previous studies from our group (Dolci et al., 2014) showed increased catalase (CAT) as well as $\text{NA}^+ \text{K}^+$ -ATPase activity, an enzyme implicated in metabolic energy regulation and highly susceptible to oxidant agents (Chtourou et al., 2011), in liver and kidney of *Rhamdia quelen* after hypoxia acclimation, and such increases were maintained even after Mn exposure, thus indicating hypoxia tolerance. Likewise, Lushchak et al. (2001) observed increased CAT and glutathione reductase (GR) activities in liver of anoxia-exposed *Carassius auratus*, demonstrating activation of these enzymes under hypoxia, as already described by Hermes-Lima (1998) as a “preparation to oxidative stress”. The current study was performed to evaluate the influence of hypoxia acclimation and subsequent reoxygenation on Mn toxicity in plasma, liver and kidney of silver catfish, since these tissues are highly implicated in distribution (Sherwood, 2004), detoxification (Gluszak et al., 2000) and excretion (Xie et al., 2015) of xenobiotics in fish.

2. Materials and Methods

2.1. Fish

One hundred (100) silver catfish (27.02 g body weight; 14.45 cm length), obtained from fishponds at the Federal University of Santa Maria, Southern Brazil, were acclimated in continuously aerated 250 L tanks with controlled temperature (20°C) for at least one week prior to experiments. During this period, they were fed once a day (42% crude protein) at 12 p.m.

2.2. Reagents

Manganese sulfate hexahydrate [$\text{MnSO}_4 \cdot 6\text{H}_2\text{O}$] (Vetec®, Rio de Janeiro, RJ, Brazil) was used to maintain the waterborne Mn level. All chemicals and solvents used were of HPLC grade and purchased from Sigma Aldrich ® (Brazil).

2.3. Exposure protocol

The acclimation conditions, manganese levels and water quality parameters were the same as described by Dolci et al. (2016a) (*in preparation*). After seven days of laboratory acclimation, a hundred silver catfish were randomly transferred to 20 L tanks (5 fish per tank, five tanks/group, in duplicate), in a semi-static system, with 50% water exchange daily with dissolved oxygen levels previously adjusted through bubbling air (normoxia) and/or nitrogen (hypoxia) for 10 days (Fig. 1; Supplementary 1A). On day 11, blood, liver and kidney samples were collected for biochemical analyses. On day 10, remaining silver catfish (5 fish per tank, two tanks/group, in duplicate) were exposed for additional 10 days to waterborne Mn levels of 0.002 (control without Mn addition) and 8.1 mg L⁻¹, yielding the following groups: normoxia, hypoxia, normoxia plus Mn and hypoxia plus Mn (Fig. 1; Supplementary 1A). On day 21, fish were euthanized and plasma, liver and kidney samples were collected for biochemical analyses. On day 20, remaining silver catfish (5 fish per tank, one tank/group, in duplicate) were submitted to the reoxygenation protocol for 10 days, forming the following groups: normoxia, reoxygenation, normoxia plus Mn and reoxygenation plus Mn (Fig. 1; Supplementary 1A). Finally, on day 31, fish were euthanized and plasma, liver and kidney samples were collected for analyses. In this step, groups previously acclimated to hypoxia were restored to normoxia values through bubbling air. The initial hypoxic environment was created from oxygen consumption due to the normal fish metabolism, and different DO₂ levels were maintained through aeration with air pumps (air pump AC 2000; 0.0014 MPa pressure) or nitrogen gas when appropriate. Water reservoir tanks (250 L) containing waterborne manganese with pre-adjusted Mn levels (MnSO₄. 6H₂O) kept under normoxia (with air pumping) or under hypoxic conditions (with nitrogen gas bubbling) were used. The experimental tanks were cleaned daily in the late afternoon by siphoning and then replacing the water volume with reservoir tanks water. At the end of each exposure, Mn wastes were chemically treated and properly disposed of. This protocol was approved by the Ethics of Animal Use Committee (CEUA) (nº:104/2013), Federal University of Santa Maria.

2.4. Tissues collection

Fish were sampled every 10 experimental days (5 fish were sampled from each duplicate, n = 10). Silver catfish were anesthetized with MS222, euthanized by beheading and plasma, liver and kidney were then removed and stored at -80 °C for further analyses.

2.5. Metal analyses

2.5.1. Tissues samples

The digestion of plasma, liver and kidney was performed using a conventional heating block (Velp Scientifica, Model DK, Italy) with open glass vessels. The procedure was performed with concentrated nitric acid (14 Mol L^{-1}) (Merck, Darmstadt, Germany). H_2O_2 was added, and the digests were heated up to 80°C for 1 h. After cooling, the digests were diluted with purified water (Milli-Q system, Millipore Corp., Bedford, USA) for further analyses.

2.5.2. Sample Mn determination

The waterborne Mn level and Mn accumulation in plasma, liver and kidney were determined using an inductively coupled plasma optical emission spectrometer (ICP OES, Model Spectro Ciros CCD, Spectro Analytical Instruments, Kleve, Germany), which was equipped with an axial view configuration and a cross-flow nebulizer coupled to a Scott-type double pass nebulization spray chamber. The wavelength for Mn determination was 257.611 nm. The radio frequency power was 1400 W. The flow rates for plasma generation, auxiliary and nebulization gas were 12.0, 1.0 and 1.0 L min^{-1} , respectively. Argon (99.996%, White Martins–Praxair, São Paulo, SP, Brazil) was used for plasma generation for nebulization and as an auxiliary gas. For accuracy evaluation, certified reference material (CRM) of dogfish muscle tissue (DORM-2) from the National Research Council, Canada, was used.

2.6. Biochemical assays

2.6.1. Hematological profile

Blood samples were collected from caudal vertebrae vessels with heparinized syringes (3 ml) and needles (25x6) (5,000 IU/ml, Hemofol®, Cristália, Brazil). Subsequently, samples were stored in eppendorf tubes (2 ml) and immediately centrifuged ($1310 \times g$ for 15 min). The hematocrit percentage (Htc) was determined by the microhematocrit method as described by Goldenfarb et al. (1971). The total number of red blood cells per mm^{-3} was determined by counting in a Neubauer chamber, using rbc Thoma pipettes in a dilution of 1:200 with Natt-Herrick's solution. Hemoglobin (g dL^{-1}) was determined by the cyanmethemoglobin method after centrifugation, in order to remove RBCs free cores, and then spectrophotometrically read according to Thrall (2007). Total and differential leukocyte counts were performed in the blade, the former indirectly by random counting of 2000 blood cells according to Ranzani Paiva et al (2013b).

2.6.2. Specific markers of liver damage

2.6.2.1. Plasmatic transaminases (GOT, GPT, GGT and GOT/GPT ratio)

Plasma sample of silver catfish was obtained after blood centrifugation (1310 x g for 15 min) and plasmatic enzymes indicative of liver damage were assayed using Kit Labtest: glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and gamma glutamyl transferase (GGT). Additionally, the GOT/GPT ratio was calculated. Results of GOT/GPT ratio numerically equal or greater than two represent mitochondrial liver injury (Hafkenscheid and Dijt, 1979; Huang et al., 2006).

2.6.3. Oxidative status

2.6.3.1. Reactive species (RS) determination

Liver and kidney were homogenized (1:5 w/v) in Tris-HCl buffer (10 mM; pH 7.4), and centrifuged at 3640 x g for 15 min and supernatants were used for determination of RS levels using the oxidant sensing fluorescent probe 2',7'-dichlorofluorescein diacetate (DCHF-DA) (Hempel et al., 1999). The oxidation (DCHF-DA) to fluorescent dichlorofluorescein (DCF) was determined at 488 nm for excitation and 525 nm for emission. After homogenization and centrifugation of tissues, 3 mL of buffer (10 mM Tris-HCl, pH 7.4) was added. After 10 s, 10 µM (DCHF-DA) (prepared in ethanol) was added to the mixture, and the fluorescence intensity from DCF was measured for 300 s and expressed as a percentage of the untreated control group. The protein content was normalized by quantification according to Lowry et al. (1951).

2.6.3.2. Protein carbonyl determination

For protein carbonyl (PC) determination, liver and kidney were homogenized (1:5 w/v) in Tris-HCl buffer (10 mM; pH 7.4) and thus assayed according to Yan et al. (1995), with some modifications. Soluble protein was mixed with 2,4-dinitro- phenylhydrazine (DNPH; 10 mM in 2 M HCl) and incubated at room temperature for 1 h. Denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, with 3% sodium dodecyl sulfate), ethanol (99.8%) and hexane (99.5%) were added, mixed by shaking and centrifuged. The protein isolated from the interface was washed twice with ethyl acetate/ethanol 1:1 (v/v) and suspended in denaturing buffer. Each DNPH sample was read at 370 nm in a spectrophotometer against the corresponding HCl sample (blank). The results were expressed as the nmol carbonyl g⁻¹ tissue⁻¹.

2.6.3.3. GSH levels

GSH levels were determined fluorometrically as described by Hissin and Hilf (1976), using 0-phthalaldehyde (OPA) as fluorophore. Briefly, the samples were homogenized in perchloric acid (HClO_4) 0.1 M. Homogenates were centrifuged at $1310 \times g$ for 10 min and the low-speed supernatants were separated for measurement of GSH. The supernatant (100 μL) was incubated with 100 μL of OPA (0.1% in methanol) and 1.8 mL of 0.1 M phosphate buffer (pH 8.0) for 15 min at room temperature in the dark. Fluorescence was measured with a fluorescent spectrophotometer at excitation 350 nm wavelength and at emission 420 nm wavelength. GSH levels were calculated in relation to a standard curve constructed with GSH reduced at known concentrations and thus corrected by the protein content (Lowry et al., 1951) and expressed as $\text{GSH mg protein}^{-1}$.

2.6.3.4. Catalase activity assay

Catalase (CAT) activity was spectrophotometrically quantified by the method of Aebi (1984). Liver and kidney samples were homogenized (1:5 w/v) in Tris-HCl buffer (10 mM; pH 7.4), and centrifuged at $3640 \times g$ for 15 min and supernatants were used for analyses. The method used monitors the disappearance of H_2O_2 in the presence of tissue at 240 nm. The CAT enzymatic activity was expressed as $\mu\text{mol H}_2\text{O}_2 \text{ g tissue}^{-1} \text{ min}^{-1}$ (1 U decomposes 1 $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1}$ at pH 7 at 25°C).

2.6.3.5. Na^+/K^+ -ATPase activity assay

The Na^+/K^+ -ATPase activity was determined according to Musbeck et al. (1977), with some modifications. Briefly, the supernatant aliquots (20 μL) of liver and kidney previously homogenized (1:5 w/v) in Tris-HCl buffer (10 mM; pH 7.4) were added to a reaction medium containing NaCl, MgCl_2 , KCl and Tris-HCl buffer (pH 7.4), with or without the Na^+/K^+ -ATPase enzyme inhibitor ouabain. The method for ATPase activity measurement is based on the determination of the inorganic phosphate (Pi) released to the reaction medium by the hydrolysis of the ATP, according to the method proposed by Atkinson et al. (1973). The reaction was initiated with the addition of the substrate ATP to the reaction medium and was finished by the addition of the color reagent (1 mL), containing ammonium molybdate (2%), triton X-100 and H_2SO_4 (10%) after 15 min of incubation at 37°C . The formed molybdate-Pi complexes were measured spectrophotometrically at 405 nm. Values were calculated in relation to a standard curve constructed with Pi at known concentrations and corrected by the protein content (Lowry et al., 1951).

2.7. Statistical analysis

Statistics were performed using Statistica (Statsoft Inc., Tulsa, USA, version 7). Homogeneity of the data were initially tested by Levene's test. Data were analyzed by one-way ANOVA (dissolved oxygen levels: normoxia x hypoxia) or two-way ANOVA (Mn x dissolved oxygen levels), followed by Tukey's multiple range test, when appropriate. Pearson's correlation was applied. Data were expressed as the mean \pm S.E.M., and $p < 0.05$ was regarded as statistically significant.

3. Results

3.1. Water parameters

All of the assessed parameters (pH; DO₂; hardness, alkalinity; total ammonia; NH₃; nitrite) presented within the expected values, with no variations throughout the experiment (Supplementary 1A, 1B).

3.2. Metal accumulation

Two-way ANOVA of Mn accumulation (Table 1) revealed a significant main effect of oxygen level in plasma, liver and kidney at experimental day 20 [$F(1,36)_{10-20} = 16.49, p = 0.01$; 297.18, $p = 0.0000$; 15.10, $p = 0.01$], respectively. After 10 days of reoxygenation, Mn accumulation revealed a significant main effect of oxygen level in plasma, liver and kidney [$F(1,36)_{20-30} = 22.83, p = 0.008$; 167.36, $p = 0.0002$ and 140.51, $p = 0.0002$], respectively. Silver catfish exposed to Mn under hypoxia showed decreased Mn accumulation in plasma (27%), liver (50%) and kidney (41%) in relation to the normoxia group (Table 1). Likewise, after reoxygenation, animals previously acclimated to hypoxia showed decreased Mn accumulation in plasma (49%), liver (46%) and kidney (46%), in comparison to the normoxia group (Table 1).

3.3. Biochemical assays

3.3.1. Hematological profile

Two-way ANOVA of red blood cells, hematocrit and hemoglobin at experimental day 20, revealed a significant main effect of oxygen level [$F(1,36)_{10-20} = 15.57, p = 0.002$; 6.72, $p = 0.02$ and 12.26, $p = 0.005$], respectively. After 10 days of reoxygenation, two-way ANOVA of red blood cells, hematocrit and hemoglobin revealed a main effect of oxygen level and Mn [$F(1,36)_{20-30} = 6.59, p = 0.03$, 19.10, $p = 0.001$; 8.58, $p = 0.01$ 1, 7.77, $p = 0.002$ and 8.82, $p =$

0.01, 11.82, $p = 0.007$], respectively. There was an increase *per se* of red blood cells, hematocrit and hemoglobin in silver catfish acclimated to hypoxia from experimental day 0 to 10 and day 10 to 20, whereas animals exposed to reoxygenation for 10 days showed increased hematocrit and hemoglobin levels *per se* only. Notably, after 10 days of reoxygenation, silver catfish exposed to Mn under normoxia exhibited a reduction of red blood cells and hematocrit (Table 2A). After 10 days of acclimation, hypoxia decreased white blood cells, increased absolute and percentage of lymphocytes, and decreased neutrophils (Table 2B). These changes were not maintained beyond 10 days of hypoxia acclimation since Mn exposure and/or reoxygenation subsequent to hypoxia were related to no significant changes on white blood cells.

3.3.2. Plasmatic transaminases (GOT, GPT, GGT and GOT/GPT ratio)

Two-way ANOVA of GOT in plasma after 20 and 30 experimental days revealed a significant main effect of oxygen level, Mn and a significant oxygen level \times metal interaction [$F(1,36)_{10-20} = 88.57$; 417.29 and 79.71, $p = 0.0000$], respectively, and [$F(1,36)_{20-30} = 50.07$, 534.19 and 47.53, $p < 0.0000$], respectively. Hypoxia acclimation for 10 and 20 days as well as reoxygenation for additional 10 days did not affect plasma GOT levels in silver catfish (Fig. 2A). However, Mn exposure under normoxia for both 10 and 20 experimental days was able to increase *per se* plasma GOT levels (Fig. 2A). Notably, hypoxia acclimation and subsequent reoxygenation were able to partially prevent the increase of Mn-induced plasma GOT level (Fig. 2A).

Two-way ANOVA of GPT revealed a significant main effect of oxygen level and Mn after 20 [$F(1,36)_{10-20} = 6.65$, $p = 0.02$ and 683.25, $p = 0.0000$] and 30 [$F(1,36)_{20-30} = 36.73$ and 320.17 $p < 0.0000$] experimental days, respectively. Hypoxia acclimation for 10 and 20 days as well as reoxygenation for additional 10 days did not change plasma GPT levels in silver catfish (Fig. 2B). While Mn exposure for 10 and 20 days under normoxia increased plasma GPT level (Fig. 2B), hypoxia acclimation did not affect this plasma parameter. Contrarily, subsequent reoxygenation for additional 10 days was able to partially reduce the increased Mn-induced plasma GPT level (Fig. 2B).

Two-way ANOVA of GGT in plasma after 20 and 30 experimental days revealed a significant main effect of oxygen level, Mn and a significant oxygen level \times metal interaction [$F(1,36)_{10-20} = 25.16$, 164.12 $p < 0.0000$; 15.48 $p = 0.002$, respectively] and [$F(1,36)_{20-30} = 37.07$ $p = 0.0000$; 213.41 $p = 0.0000$; 25.29 $p = 0.0002$, respectively]. Plasma GGT level was not modified by 10 and 20 days of hypoxia acclimation and by 10 additional days of reoxygenation. Silver catfish exposed to Mn under normoxia for 10 and 20 days showed increased plasma GGT

level, but under hypoxia and subsequent reoxygenation, these plasma levels were partially reversed (Fig. 2C).

Two-way ANOVA of GOT/GPT ratio after 20 experimental days revealed a significant main effect of oxygen level, Mn and a significant oxygen level \times metal interaction [$F(1,36)_{10-20} = 58.50, 140.05$ and $50.73 p = 0.0000$], respectively. After 10 days of reoxygenation, two-way ANOVA of GOT revealed a significant main effect of Mn [$F(1,36)_{20-30} = 128.59 p = 0.0000$]. Hypoxia acclimation for 10 and 20 days as well as reoxygenation for additional 10 days did not change GOT/GPT ratio of silver catfish. Contrarily, Mn exposure under normoxia was able to increase this ratio, while hypoxia and the subsequent reoxygenation were able to prevent and minimize, respectively, the increase in Mn-induced GOT/GPT ratio (Fig. 2D).

3.4. Oxidative status

3.4.1. Reactive species (RS) determination

Two-way ANOVA of reactive species (RS) in liver at experimental days 20 and 30 revealed a significant main effect of oxygen level [$F(1,36)_{10-20} = 55.09$ and $98.59, p = 0.0000$], respectively, and Mn accumulation [$F(1,36)_{20-30} = 35.24, p = 0.0000$ and $6.47, p = 0.02$], respectively. Tukey's test showed that while hypoxia decreased RS generation *per se*, Mn exposure under normoxia was able to increase this oxidative parameter. In addition, fish acclimated to hypoxia and exposed to Mn showed minor increase of RS, in comparison to the normoxia group (Fig. 3A). After reoxygenation, only acclimation to hypoxia showed influence, decreasing RS generation independently of Mn exposure (Fig. 3A).

Two-way ANOVA of reactive species (RS) in kidney after 20 and 30 experimental days revealed a significant main effect of oxygen level [$F(1,36)_{10-20} = 18.18, p = 0.001$ and $15.60, p = 0.002$], respectively, and Mn accumulation $F(1,36)_{20-30}= 17.41, p = 0.001$ and $6.38, p = 0.02$], respectively. In kidney, hypoxia acclimation for 10 and 20 days, as well as the subsequent reoxygenation were related to decreased RS generation *per se* in relation to the normoxia group. Hypoxia was able to prevent the increased Mn-induced RS generation seen under normoxia (Fig. 3C). After reoxygenation, silver catfish not exposed to Mn showed decreased RS *per se*, while this oxidative marker was similarly generated in both experimental groups exposed to Mn under normoxia and reoxygenation (Fig. 3C).

3.4.2. Protein carbonyl (PC) determination

After 20 experimental days, two-way ANOVA of PC levels in liver of silver catfish revealed a significant main effect of oxygen level and Mn [$F(1,36)_{10-20} = 337.99$ and $4467.34 p < 0.0000$], respectively. After reoxygenation, two-way ANOVA of PC levels in liver revealed a main effect of oxygen, Mn and a significant oxygen level \times Mn interaction [$F(1,36)_{20-30} = 43.54, p = 0.0000$; $50.60, p = 0.0000$ and $4.99, p = 0.04$], respectively. Silver catfish acclimated to hypoxia for 10 and 20 experimental days, as well as fish subsequently reoxygenated for additional 10 days, exhibited a significant decrease *per se* in PC levels of liver in comparison to the normoxia group (Fig 3B). After 10 and 20 days of Mn exposure under normoxia, silver catfish showed increased PC level in the liver, while hypoxia acclimation, as well as subsequent reoxygenation were able to prevent the Mn-induced hepatic damage (Fig. 3B).

After 20 experimental days, two-way ANOVA of PC levels in kidney of silver catfish revealed a significant main effect of oxygen level and Mn [$F(1,36)_{10-20} = 8.08, p = 0.01$ and $22.30, p = 0.0002$], respectively. After reoxygenation, two-way ANOVA of PC levels revealed a significant main effect of Mn [$F(1,36)_{20-30} = 66.25, p = 0.0000$]. While Mn exposure was able to increase PC levels in kidney of normoxia-acclimated silver catfish, hypoxia acclimation was able to prevent this oxidative damage, which was not maintained after reoxygenation. In fact, after 30 experimental days, Mn exposure increased PC levels in kidney of silver catfish, independently of oxygen level (Fig. 3D).

3.4.3. GSH levels

Two-way ANOVA of GSH levels in liver for 20 and 30 days revealed a significant main effect of oxygen levels, Mn and a significant oxygen level \times Mn interaction [$F(1,36)_{10-20} = 66.77 p = 0.0001$; $66.72 p = 0.0000$; $25.21 p = 0.0001$], and [$F(1,36)_{20-30} = 42.28 p = 0.0000$; $18.03 p = 0.001$; $15.32 p = 0.002$], respectively. Two-way ANOVA of GSH levels in kidney after 20 and 30 experimental days revealed a significant main effect of oxygen level and Mn [$F(1,36)_{10-20} = 9.98 p = 0.006$ and $40.48 p = 0.0000$], respectively. After 30 experimental days, two-way ANOVA of GSH levels in kidney revealed a significant main effect of oxygen levels, Mn and a significant oxygen level \times Mn interaction [$F(1,36)_{20-30} = 51.15, 141.10$ and $50.54 p < 0.0000$], respectively. While hypoxia acclimation for 10 and 20 days, and also reoxygenation for additional 10 days, did not change GSH levels in liver and kidney (Figs. 4A and 4D, respectively), Mn exposure significantly decreased both hepatic and renal levels of this antioxidant compound. Hypoxia acclimation and subsequent reoxygenation were able to prevent this Mn effect (Figs. 4A and 4D).

3.4.4. Catalase activity

Two-way ANOVA of catalase (CAT) activity in liver after 20 experimental days revealed a significant main effect of oxygen level and Mn [$F(1,36)_{10-20} = 74.28$ and $228.34, p = 0.0000$], respectively. After 30 experimental days, a significant main effect of oxygen level, Mn and a significant oxygen level \times Mn interaction [$F(1,36)_{20-30} = 142.55, p = 0.0000, 20.58, p = 0.001$ and $62.27, p = 0.0000$] were observed, respectively. Two-way ANOVA of CAT activity in kidney after 20 experimental days revealed a significant main effect of oxygen level and Mn [$F(1,36)_{10-20} = 114.80$ and $49.29, p = 0.0000$], respectively. After 30 experimental days, two-way ANOVA of CAT activity in kidney revealed a significant main effect of oxygen level and a significant oxygen level \times Mn interaction [$F(1,36)_{20-30} = 106.56$ and $61.03, p = 0.0000$], respectively. Hypoxia acclimation for 10 days did not affect CAT activity in both liver and kidney (Figs. 4B and 4E, respectively). Additional 10 days of hypoxia acclimation increased CAT activity in liver, being maintained after reoxygenation (Fig. 4B), but this influence of hypoxia was not observed in kidney (Fig. 4E). In addition, Mn exposure under normoxia for 10 and 20 days was able to decrease CAT activity in liver (Fig. 4B) and kidney (Fig. 4E); however, when animals were exposed to Mn under hypoxia, this Mn-induced impairment was prevented, and the beneficial influence of hypoxia was maintained after reoxygenation in both liver and kidney (Figs. 4B and 4E).

3.4.5. Na^+/K^+ -ATPase activity

In liver, two-way ANOVA of Na^+/K^+ -ATPase activity revealed a significant main effect of oxygen level and Mn after 10 [$F(1,36)_{10-20} = 7.42$ and $7.38, p = 0.01$, respectively] and after 20 [$F(1,36)_{20-30} = 17.65$ and $32.55, p = 0.0001$, respectively] experimental days. In kidney, two-way ANOVA of Na^+/K^+ -ATPase activity revealed a significant main effect of oxygen level and a significant oxygen level \times Mn interaction [$F(1,36)_{10-20} = 9.82, p = 0.0005$ and $13.40, p = 0.002$, respectively] after 20 experimental days. After 30 days, two-way ANOVA revealed a significant main effect of oxygen level and Mn [$F(1,36)_{20-30} = 156.12$ and $65.60, p < 0.0000$, respectively]. Hypoxia acclimation for 10 and 20 days did not affect Na^+/K^+ -ATPase activity in liver and kidney (Fig. 4C and 4F, respectively), while subsequent reoxygenation for additional 10 days caused an increase *per se* in this enzyme's activity in both tissues (Fig. 4C and 4F). Under normoxia, Mn exposure for 10 and 20 days decreased this enzyme's activity in

both liver and kidney, but reoxygenation-exposed silver catfish presented a partial reversion of this Mn-induced effect (Figs. 4C and 4F).

4. Discussion

Several studies have reported that a large amount of RS generated during reoxygenation is the main factor responsible for damage to organisms during episodes of hypoxia/reoxygenation (Barry, 1994; Kelly et al., 1998; Lushchak et al., 2001). In previous studies, we showed that hypoxia-acclimated silver catfish presented increased antioxidant defenses, providing some protection to organs and systems when they were exposed to Mn (Dolci et al., 2014). Therefore, this study was conducted to explore the physiological mechanisms that may be involved in the lower toxicity of Mn, when silver catfish are acclimated to hypoxia, performing a detailed analysis of hematological parameters, oxidative profile and specific plasma markers of liver damage. The choice of the tissues was based on the specific functions of each tissue: plasma is related to homeostase functions, besides transport functions and delivery of substances to other tissues (Sherwood, 2004), while liver and kidney represent the primary organs that might be damaged by waterborne pollutants (Thophon et al., 2003), being involved in the xenobiotics detoxification and excretion.

Indeed, the current study showed that Mn exposure of silver catfish under normoxia was related to increased Mn accumulation in plasma, liver and kidney as compared to animals exposed to Mn under hypoxia. This pattern of Mn accumulation follows the description of Dolci et al. (2014) for silver catfish, excepting liver, which showed no influence of oxygen levels on the Mn accumulation pattern. The innovative finding in the current study was that acclimation of silver catfish to hypoxia allowed lower Mn accumulation in plasma, liver and kidney even after undergoing reoxygenation. This finding can be understood as enantiostasis, which is defined as a type of regulation that occurs when the effect of changes on chemical and/or physical properties (alterations in oxygen levels) experienced by the animal can be stabilized by opposite changes in other variables (such as activation of antioxidant defenses), preserving the stability of a particular physiological system.

In this context, Nilsson and Renshaw (2004) demonstrated that fish tolerant to hypoxic/anoxic conditions suffer physiological adaptations, including blood flow rearrangement, which follow hormonal control by erythropoietin (Epo) in the kidney, in order to maintain the homeostasis. Under hypoxic conditions, Epo production is increased in the kidney to collaborate in blood volume maintenance (Jelkmann, 2011). Here, a detailed analysis of the hematological profile showed that when this species was acclimated to hypoxia for 10

and 20 days, an increase of red blood cells, hematocrit and hemoglobin was observed, persisting even after reoxygenation, except for red blood cells. These data may be related to hypoxia-induced genetic control, which promotes availability of (α/β) hypoxia-inducible transcription factors (that are inactivated by hydroxylation in their α -subunits under normoxia), stimulating the Epo enhancer (Jelkmann, 2011). So, in hypoxic environments, fish can develop adaptive strategies such as alterations in cardiac output (Speers-roesch et al., 2010), increase of hemoglobin concentration and increase of affinity of Hb-O₂ binding (Wells, 2009), enabling O₂ extraction by the tissues.

Additionally, we found that there was no increase in leukocyte count throughout the experimental period, reinforcing that leukocytes are involved in nonspecific cellular defense, such as phagocytosis and phagocyte killing in fish, through oxidative and non-oxidative mechanisms (Secombes, 1997), being less responsive to pollutants like Mn. During phagocytosis, stimulated leukocytes produce reactive species (RS) intermediaries with potent cytotoxic activities (Neumann et al., 2001). The nonspecific immune activity can be also influenced by other factors, such as sex, age, stress and parasitism (Monserrat et al., 2007). Leukocytosis by absolute neutrophils and monocytes in fish are related to inflammatory processes, often derived from bacterial infection (Noro and Wittwer, 2012b; Ranzani Paiva, 2013b). Although Mn exposure did not affect leukocyte counts, hypoxia-acclimated fish had decreased white blood cells counts, as well as reduced percentage of neutrophils in comparison to normoxia-acclimated fish, indicating a better general condition under lower oxygen level. In fact, our study showed that Mn exposure did not affect white blood cells at both experimental days 20 and 30.

Subsequently, the potential Mn-induced oxidative damage was evaluated after hypoxia acclimation and reoxygenation. Hypoxia acclimation decreased *per se* RS generation in both liver and kidney of silver catfish. Under this condition, organisms seem not to undergo oxidative stress because the main source of RS, the electron transport chain, is blocked or is at low intensity. So, it is expected that hypoxia may decrease RS production (Monserrat et al., 2007). In contrast, Mn exposure under normoxia for 10 days increased RS generation in both liver and kidney confirming Mn toxicity, which was partially prevented in liver and entirely banned in kidney of hypoxia-acclimated silver catfish. Curiously, during 10 days of reoxygenation, Mn did not increase RS generation in liver and kidney, although reoxygenation of fish previously acclimated to hypoxia and exposed to Mn resulted in minor RS production in liver. In fact, some studies (Bols et al., 2001; Lushchak et al., 2001) have shown that during reoxygenation,

an overproduction of RS typically occurs, leading to an increase of oxygen consumption called “respiratory burst”. However, our study did not show such effect on RS generation in silver catfish exposed to reoxygenation. A plausible explanation for this would be that low iron concentrations present in almost any solutions are sufficient to catalyze hydroxyl radical ($\cdot\text{OH}$) generation from $\cdot\text{O}_2^-$ and H_2O_2 , generating RS. Consequently, RS reacts virtually with any cell component, the toxic effects being limited by its very short half-life (1ns) (Halliwell and Gutteridge, 1989).

Additionally, under normoxia, Mn increased the damage to proteins in both liver and kidney, as assessed by higher PC levels throughout 20 experimental days. This outcome was positively correlated with Mn accumulation in liver ($r_{10-20} = 0.79 p = 0.03$; $r_{20-30} = 0.72 p = 0.01$) and kidney ($r_{10-20} = 0.96, p = 0.0001$; $r_{20-30} = 0.85 p = 0.01$) at both experimental times. This finding can be due to proteins being more vulnerable to cell oxidative damage, as they are often catalysts rather than simply stoichiometric mediators (Dalle-Donne et al., 2003). Therefore, the higher amount of RS generated during 10 days of Mn exposure under normoxia was deleterious for both liver and kidney of silver catfish, since it caused concomitant protein carbonylation in these tissues. In addition, the shorter half-life of the RS after reoxygenation may have been due to its entire conversion in damage to proteins in such conditions. In contrast, hypoxia acclimation was able to reduce Mn-induced protein impairments, as assessed by lower PC levels in both tissues. However, after reoxygenation, only liver presented reduced PC levels.

In order to assess reliable indicators of Mn toxicity in silver catfish, our study included analysis of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT). These enzymes are found primarily in the liver and are released into the blood stream after liver injury, resulting in elevated enzyme levels (Lehninger, 2002), which are valuable in liver injury diagnosis (Huang et al., 2006). Curiously, our findings showed no influences of oxygen level on these plasmatic transaminases, which were assessed in silver catfish previously acclimated to normoxia and hypoxia and subsequently reoxygenated. On the other hand, animals exposed to Mn under normoxia for 10 and 20 days presented a significant increase in both GOT and GPT levels, showing positive correlations [liver Mn accumulation \times GOT ($r_{10-20} = 0.99, p = 0.0000$; $r_{20-30} = 0.99, p = 0.0000$) and liver Mn accumulation \times GPT ($r_{10-20} = 0.91, p = 0.001$; $r_{20-30} = 0.99, p = 0.0000$)], besides increased GOT/GPT ratio. This increased GOT/GPT ratio (≥ 2) is considered a reliable marker of hepatocellular damage (Kitamura et al., 1992; Huang et al., 2006), which confirms mitochondrial damage in liver of silver catfish exposed to Mn under normoxia. Inversely, Mn exposure under hypoxia resulted in lower

hepatic toxicity, since all plasmatic transaminases, including GOT/GPT ratio, were reduced, and remained so even after reoxygenation.

Another enzyme specifically used for the diagnosis of liver damage is the gamma-glutamyl transferase (GGT), whose plasma level is increased in all subtypes of liver damage (Thrall et al., 2007b). In this sense, besides biliary obstruction (Quaresma et al., 2007), exposure of organisms to heavy metals has also been related to high GGT plasma levels (Shalan et al., 2005). Our current findings are in agreement with this, since silver catfish exposed to Mn under normoxia for 10 and 20 days showed increased GGT plasma levels, presenting positive correlations [Mn accumulation liver \times GGT ($r_{10-20} = 0.98, p = 0.0000$; $r_{20-30} = 0.96, p = 0.0000$), which confirms Mn-induced hepatic damage. In contrast, hypoxia acclimation was able to reduce Mn-induced hepatic toxicity. Interestingly, silver catfish previously acclimated to hypoxia and subsequently reoxygenated showed lower levels of plasma GGT, indicating that hypoxia acclimation favored the activation of detoxification system, thus regulating glutathione levels in the liver, which persisted activated during reoxygenation.

In this context, it is known that antioxidants defenses (GSH, SOD, CAT, among others) (Hermes-Lima, 2004) are able to neutralize RS (O_2^- , H_2O_2 , $\cdot OH$) (Halliwell and Gutteridge, 1999) generated during its life, in order to avoid damage to vital organs and systems. Also, GSH (a tripeptide glutathione (L- γ -glutamyl-L-cysteinylglycine) is an antioxidant of low-molecular weight that has been involved in a number of crucial cellular functions, including RS scavenging, detoxification of electrophiles, maintenance of thiol-disulfide status and signal transduction (Dröge et al., 1994; Halliwell and Gutteridge, 1999), being present in high concentrations in the liver. Definitely, while acclimation of silver catfish to hypoxia did not modify GSH levels in both liver and kidney, hypoxia acclimation was able to partially restore the low Mn-induced GSH levels under normoxia in both tissues. Additionally, after reoxygenation, GSH levels were entirely restored in both tissues, confirming that activation of the antioxidant system is related to homeostasis preservation during Mn intoxication.

Interestingly, exposure of silver catfish to Mn under normoxia was related to a significant decrease in catalase (CAT) activity in both liver and kidney. In fact, CAT is an essential enzyme that is involved in H_2O_2 degradation, while the latter is a precursor of reactive species that can damage DNA (Halliwell and Gutteridge, 1999). Thus, the lack of CAT response in liver of silver catfish exposed to Mn under normoxia can be due to the inability of this enzyme to reduce higher amounts of hydroxyl radical promoters (Monserrat et al., 2007). In contrast, silver catfish acclimated to hypoxia for 20 days and subsequently reoxygenated showed an

increase *per se* in hepatic CAT activity. Similar findings were obtained in *Carassius auratus* acutely exposed to hypoxic conditions (Lushchak et al., 2001), confirming that the antioxidant defense system is more active under hypoxia (Lushchak et al., 2005a; Baker et al., 2007; Tripathi et al., 2013) in order to control pro-oxidants generated during reoxygenation. However, in kidney, hypoxia caused a more conventional response on CAT activity, which remained unchanged, because RS production is lower under hypoxia of, and there is no reason for enhancement of antioxidant defenses (Amado et al., 2006a). Additionally, hypoxia-acclimated silver catfish and thereafter submitted to reoxygenation showed no changes in renal CAT activity, leading us to propose that this organ is fundamentally involved in excretory function (Xie et al., 2015) and thus plays a reduced antioxidant role.

Notably, Mn exposure under normoxia for 10 and 20 days decreased the functionality of Na^+/K^+ -ATPase in both liver and kidney of silver catfish. Accordingly, literature data have shown that this enzyme's activity was also inhibited by heavy metals (Rugimony et al., 2004) besides other pollutants such as pesticides (Bianchini et al., 2002; Vani et al., 2012). In addition, our findings showed that hypoxia acclimation for 10 and 20 days was able to preserve Na^+/K^+ -ATPase activity unchanged in both tissues. This outcome indicates that there is a similar consumption of oxygen across hypoxia and normoxia, keeping the metabolic production unchanged (Doganli et al., 2013) in these organs. This idea was originally suggested by De Boeck et al. (2013), when the ionoregulation was maintained by higher branchial Na^+ uptake rates in *Astronotus ocellatus* after fasting and hypoxia acclimation for 14 days, confirming that this species is tolerant to hypoxia. Furthermore, our study also showed that under hypoxia, Mn-exposed silver catfish presented increased Na^+/K^+ -ATPase activity in both liver and kidney, as already previously shown (Dolci et al., 2014), and such effect also persisted after reoxygenation.

5. Conclusion:

Considering that hypoxia-acclimated silver catfish subsequently submitted to reoxygenation showed lower Mn accumulation in tissues ($\sim 3 \text{ mg L}^{-1}$), as well as better hematological and oxidative status, one can hypothesize that this species is able to activate the antioxidant defense system as observed by CAT and GSH activities during Mn exposure, which were maintained even after reoxygenation. Moreover, the initial disruption of homeostasis, which was observed after hypoxia acclimation, was able to produce compensatory adjustments (Monserrat et al., 2007; Mattson, 2008; Calabrese, 2014) in hematological and oxidative parameters, ameliorating physiological responses that control the damage generated by a toxicant agent like Mn. Such adjustments were initially defined by Magnum and Towle (1977) as “enantiostasis”, which is a physiological regulation that preserves the stability of a particular tissue or system, thus playing a key role in protection against post-hypoxia free radical damage (Freire et al., 2011; Teixeira et al., 2013).

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Supplementary 1(A-B). Water parameters with Mn experimental values (obtained after inductively coupled plasma optical emission spectrometry (ICP-OES) analyses). Fish were acclimated for 10 days to normoxia (N) or hypoxia (H) and afterwards exposed or not to waterborne Mn [$\sim 8.1 \text{ mg L}^{-1}$], for additional 10 days (H+Mn; N+Mn). After this period, fish previously hypoxia acclimated were submitted to reoxygenation (R; R+Mn) for 10 more days.

1A

Exposure (Days)	Groups	Dissolved Oxygen	O ₂ Saturation (%)	[Mn] mg L ⁻¹ ICP-OES	T (°C)	pH
0 – 10	N	7.36±0.06	81.89±0.86	0.002±0.00	21.30±0.18	5.85±0.17
	H	2.94±0.09*	40.19±1.13*	0.002±0.00	21.39±0.18	5.85 ± 0.14
10 – 20	N	7.43±0.10	82.00±1.00	0.002±0.00	22.02±0.14	6.00±0.23
	H	3.09±0.14*	41.54±1.14*	0.002±0.00	21.87±0.17	5.66±0.14
	N + Mn	7.57±0.22	82.79±2.84	8.13±0.21 ⁺	21.92±0.15	5.80±0.15
	H + Mn	3.32±0.10*	42.60±1.40*	8.30±0.31 ⁺	22.03±0.18	6.00±0.24
20 – 30	N	7.04±0.02	79.32±0.16	0.002±0.00	22.5±0.13	5.99±0.23
	R	7.15±0.05	79.94±0.26	0.002±0.00	22.5±0.10	5.83±0.21
	N + Mn	7.29±0.12	81.41±2.19	8.18±0.91 ⁺	22.5±0.13	5.95±0.20
	R + Mn	7.15±0.04	80.18±0.36	8.18±0.64 ⁺	22.5±0.11	5.67±0.15

1B

Exposure (Days)	Groups	Hardness (mg CaCO ₃ L ⁻¹)	Alkalinity	Total ammonia	NH ₃	Nitrite
0 – 10	N	72.50 ± 2.19	47.60 ± 1.16	0.04 ± 0.002	0.0007 ± 0.00	0.17 ± 0.05
	H	78.20 ± 2.04	50.42 ± 1.34	0.04 ± 0.001	0.0008 ± 0.00	0.14 ± 0.01
10 – 20	N	77.20 ± 3.04	44.20 ± 2.37	0.03 ± 0.009	0.0008 ± 0.00	0.17 ± 0.03
	H	67.80 ± 2.50	48.50 ± 1.48	0.04 ± 0.003	0.0008 ± 0.00	0.14 ± 0.02
	N + Mn	76.00 ± 1.19	48.10 ± 1.51	0.03 ± 0.002	0.0008 ± 0.00	0.17 ± 0.05
	H + Mn	76.00 ± 3.21	45.40 ± 1.02	0.03 ± 0.003	0.0008 ± 0.00	0.17 ± 0.08
20 – 30	N	74.40 ± 1.40	46.15 ± 1.10	0.03 ± 0.003	0.0007 ± 0.00	0.13 ± 0.01
	R	67.80 ± 2.50	50.30 ± 1.67	0.04 ± 0.002	0.0007 ± 0.00	0.16 ± 0.03
	N + Mn	73.40 ± 1.40	46.10 ± 1.02	0.03 ± 0.002	0.0007 ± 0.00	0.15 ± 0.02
	R + Mn	75.40 ± 2.70	46.60 ± 0.53	0.04 ± 0.004	0.0007 ± 0.00	0.17 ± 0.05

*Indicates significant difference between oxygen levels (N and H or N and R groups) in the same water condition (Mn or not);

[†]Indicates significant differences between Mn and control group in the same oxygen level ($p < 0.05$). Values are expressed as mean ± SEM.

Table 1. Mn accumulation in silver catfish tissues. Water parameters with Mn experimental values (obtained after inductively coupled plasma optical emission spectrometry (ICP-OES) analyses). Fish were acclimated for 10 days to normoxia (N) or hypoxia (H) and afterwards exposed or not to waterborne Mn [~ 8.1 mg L $^{-1}$], for additional 10 days (H+Mn; N+Mn). After this period, fish previously hypoxia acclimated were submitted to reoxygenation (R; R+Mn) for 10 more days.

Exposure (Days)	Groups	Mn accumulation ($\mu\text{g g}^{-1}\text{issue}^{-1}$)		
		Plasma	Liver	Kidney
0 – 10	N	0.06 ± 0.02	2.14 ± 0.07	0.84 ± 0.12
	H	0.07 ± 0.02	2.55 ± 0.49	0.92 ± 0.08
10 – 20	N	0.07 ± 0.01	1.54 ± 0.04	1.41 ± 0.00
	H	0.06 ± 0.01	1.52 ± 0.41	0.94 ± 0.28
	N + Mn	5.46 ± 0.24 ⁺	16.65 ± 0.15 ⁺	11.90 ± 0.20 ⁺
	H + Mn	3.98 ± 0.27 ^{++*}	8.29 ± 0.21 ^{++*}	7.06 ± 0.58 ^{++*}
20 – 30	N	0.07 ± 0.01	1.48 ± 0.03	0.73 ± 0.06
	R	0.07 ± 0.00	1.48 ± 0.24	0.65 ± 0.05
	N + Mn	3.39 ± 0.01 ⁺	18.92 ± 0.42 ⁺	14.76 ± 0.29 ⁺
	R + Mn	1.73 ± 0.34 ^{++*}	10.01 ± 0.48 ^{++*}	7.90 ± 0.50 ^{++*}

*Indicates significant difference between oxygen levels (N and H or N and R groups) in the same water condition (Mn or not);

⁺Indicates significant differences between Mn and control group in the same oxygen level ($p < 0.05$). Values are expressed as mean ± SEM.

Table 2. Hematological profile. Red blood cells (**A**) and white blood cells (**B**). Fish were acclimated for 10 days to normoxia (N) or hypoxia (H) and afterwards exposed or not to waterborne Mn [$\sim 8.1 \text{ mg L}^{-1}$], for additional 10 days (H+Mn; N+Mn). After this period, fish previously hypoxia acclimated were submitted to reoxygenation (R; R+Mn) for 10 more days.

Exposure (Days)		Groups	Red blood cells ($\times 10^6 \text{ mm}^{-3}$)	Hematocrit (% volume cell)	Hemoglobin (g dL^{-1})	MCHC (%)	VCM (μm^3)
0 – 10	N	1.63 ± 0.05	30.00 ± 1.00	5.88 ± 0.02	19.71 ± 0.65	185.21 ± 11.88	
	H	2.25 ± 0.04*	39.50 ± 3.17*	7.56 ± 0.05*	19.48 ± 1.42	176.74 ± 17.73	
10 – 20	N	1.40 ± 0.12	26.50 ± 3.77	5.87 ± 0.56	22.62 ± 1.19	186.36 ± 12.68	
	H	2.28 ± 0.20*	40.25 ± 3.54*	8.02 ± 0.49*	20.09 ± 0.83	176.54 ± 4.04	
N + Mn	N + Mn	1.89 ± 0.01	33.50 ± 0.28	7.14 ± 0.11	21.34 ± 0.51	177.33 ± 3.15	
	H + Mn	2.13 ± 0.02	35.66 ± 0.33	8.33 ± 0.39	23.35 ± 0.89	167.00 ± 3.42	
20 – 30	N	1.63 ± 0.15	29.00 ± 1.15	4.64 ± 0.32	15.94 ± 0.47	180.12 ± 9.79	
	R	1.94 ± 0.01	34.66 ± 0.66*	7.07 ± 0.38*	20.45 ± 1.41	178.07 ± 2.67	
N + Mn	N + Mn	0.91 ± 0.27†	18.00 ± 3.51†	3.46 ± 1.15	18.98 ± 4.93	208.96 ± 20.42	
	R + Mn	1.36 ± 0.05*	34.66 ± 0.66	4.37 ± 0.28†	17.75 ± 2.74	189.24 ± 12.54	

*Indicates significant difference between oxygen levels (N and H groups) in the same water condition (Mn or not);

†Indicates significant differences between Mn and control group in the same oxygen level ($p < 0.05$). Values are expressed as mean ± SEM.

Table 2. Hematological profile. Red blood cells (**A**) and white blood cells (**B**). Fish were acclimated for 10 days to normoxia (N) or hypoxia (H) and afterwards exposed or not to waterborne Mn [$\sim 8.1 \text{ mg L}^{-1}$], for additional 10 days (H+Mn; N+Mn). After this period, fish previously hypoxia acclimated were submitted to reoxygenation (R; R+Mn) for 10 more days.

Exposure (Days)		Groups	White blood cells ($\times 10^4 \text{ mm}^3$)	Absolute Lymphocyte ($\times 10^3 \text{ mm}^3$)	Lymphocyte (%)	Absolute Neutrophil (mm 3)	Neutrophil (%)
0 - 10	N		2.7 ± 0.43	1.4 ± 0.54	12.66 ± 5.07	1.03 ± 0.23	80.33 ± 3.65
	H		1.2 ± 0.26*	9.2 ± 0.31*	37.00 ± 6.98*	1.69 ± 0.43	58.50 ± 6.63*
10 - 20	N		2.4 ± 0.77	3.8 ± 1.57	27.75 ± 7.30	1.92 ± 0.72	76.00 ± 6.80
	H		1.0 ± 0.28	4.9 ± 1.87	40.75 ± 9.21	0.51 ± 0.08	54.00 ± 9.76
N + Mn			2.2 ± 0.10	7.2 ± 1.51	33.50 ± 8.37	1.23 ± 0.21	54.00 ± 6.98
	H + Mn		1.5 ± 0.04	5.84 ± 0.71	37.33 ± 5.33	0.88 ± 0.07	56.00 ± 4.16
20 - 30	N		0.9 ± 0.36	0.87 ± 0.04	15.00 ± 5.19	0.79 ± 0.32	77.00 ± 5.19
	R		1.0 ± 0.36	2.33 ± 1.13	20.66 ± 2.66	0.69 ± 0.27	66.00 ± 3.05
N + Mn			0.7 ± 0.46	0.92 ± 0.69	11.00 ± 1.73	0.56 ± 0.39	74.00 ± 6.11
	R + Mn		2.0 ± 0.74	1.23 ± 0.15	8.66 ± 2.05	1.77 ± 0.68	85.33 ± 1.69†

*Indicates significant difference between oxygen levels (N and H groups) in the same water condition (Mn or not);

†Indicates significant differences between Mn and control group in the same oxygen level ($p < 0.05$). Values are expressed as mean ± SEM.

Legend for figures:

Figure 1. Experimental design. Fish were acclimated for 10 days to normoxia (N) or hypoxia (H) and afterwards exposed or not to waterborne Mn [$\sim 8.1 \text{ mg L}^{-1}$], for additional 10 days, totaling 20 days (N+Mn; H+Mn). After this period, fish previously hypoxia acclimated were submitted to reoxygenation (R; R+Mn) for 10 more days, totaling 30 days.

Figure 2. Plasmatic transaminases. Plasma GOT (A), GPT (B), GGT (C) and GOT/GPT ratio (D) of the silver catfish acclimated for 10 days to normoxia (N) or hypoxia (H) and afterwards exposed or not to waterborne Mn [$\sim 8.1 \text{ mg L}^{-1}$], for additional 10 days (N+Mn; H+Mn). After this period, fish previously hypoxia acclimated were submitted to reoxygenation (R; R+Mn) for 10 more days.

*Indicates significant difference between oxygen levels (N and H or N and R groups) in the same water condition (Mn or not);

[†]Indicates significant differences between Mn and control group in the same oxygen level ($p < 0.05$). Values are expressed as mean \pm SEM.

Figure 3. Oxidative status. Reactive species generation (RS) (A, B) and protein carbonyl (PC) (C, D) in the liver and kidney of silver catfish, respectively. Fish were acclimated for 10 days to normoxia (N) or hypoxia (H) and afterwards exposed or not to waterborne Mn [$\sim 8.1 \text{ mg L}^{-1}$], for additional 10 days (N+Mn; H+Mn). After this period, fish previously hypoxia acclimated were submitted to reoxygenation (R; R+Mn) for additional 10 days.

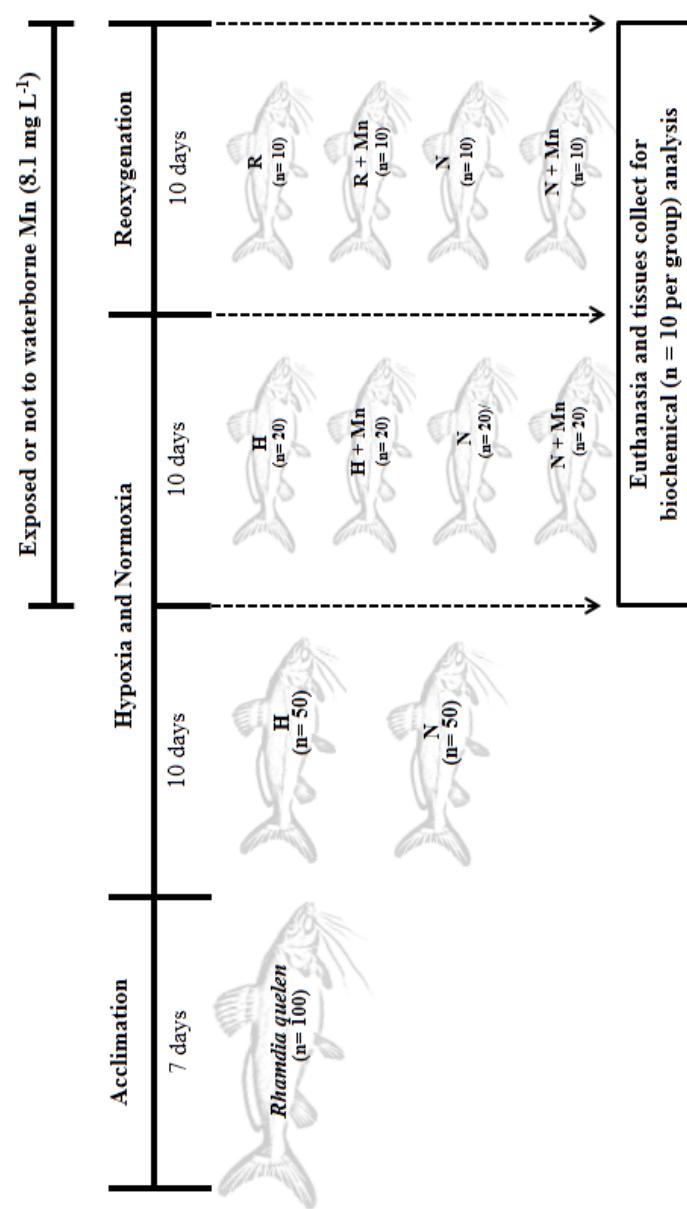
*Indicates significant difference between oxygen levels (N and H or N and R groups) in the same water condition (Mn or not);

[†]Indicates significant differences between Mn and control group in the same oxygen level ($p < 0.05$). Values are expressed as mean \pm SEM.

Figure 4. Oxidative status. Glutathione reduced (GSH) (A, D), Catalase (CAT) (B, E) and Na^+/K^+ -ATPase (C, F) in the liver and kidney of silver catfish, respectively. Fish were acclimated for 10 days to normoxia (N) or hypoxia (H) and afterwards exposed or not to waterborne Mn [$\sim 8.1 \text{ mg L}^{-1}$], for additional 10 days (N+Mn; H+Mn). After this period, fish previously hypoxia acclimated were submitted to reoxygenation (R; R+Mn) for 10 more days.

*Indicates significant difference between oxygen levels (N and H or N and R groups) in the same water condition (Mn or not);

[†]Indicates significant differences between Mn and control group in the same oxygen level ($p < 0.05$). Values are expressed as mean \pm SEM.



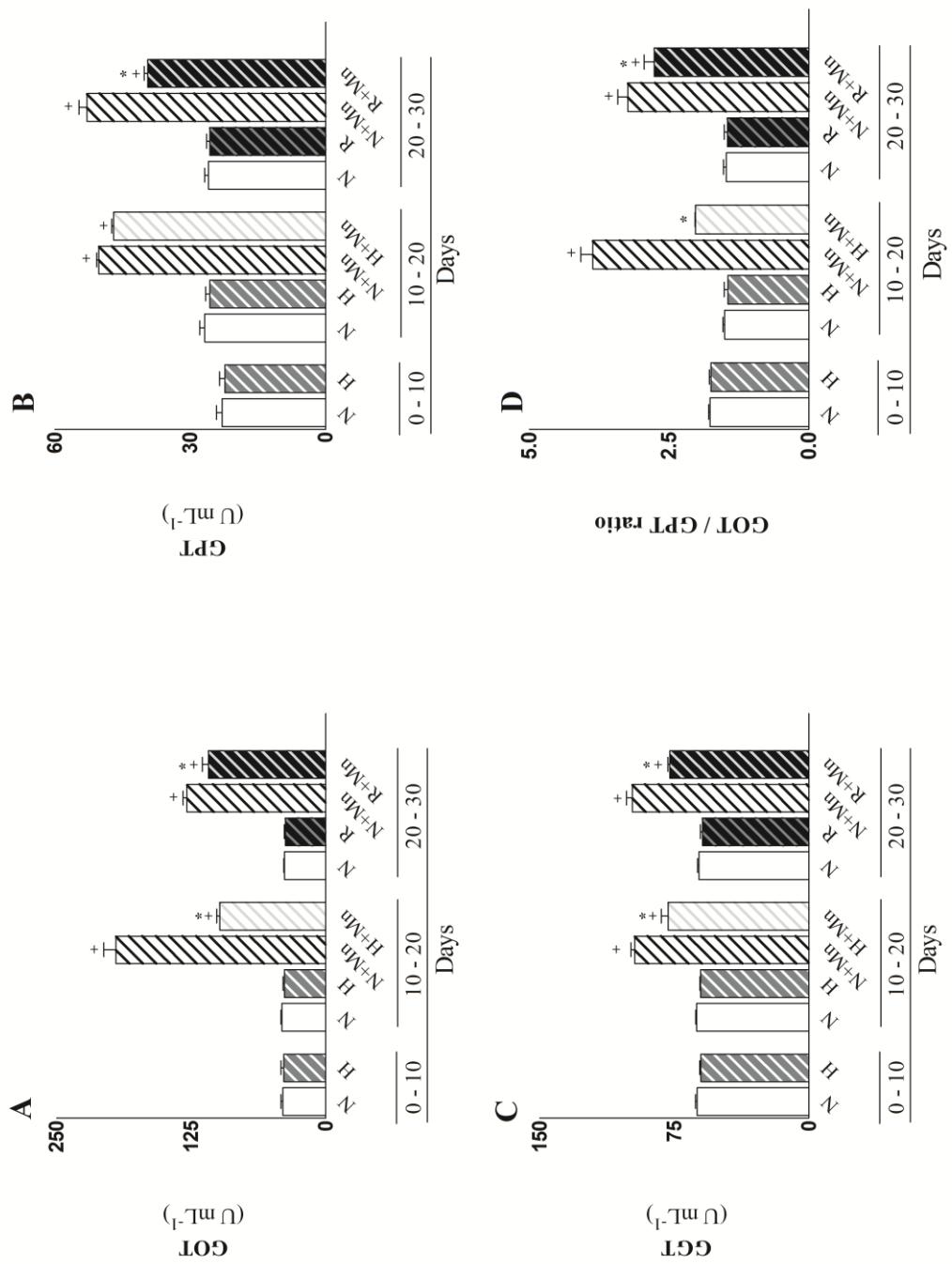


Figure 2

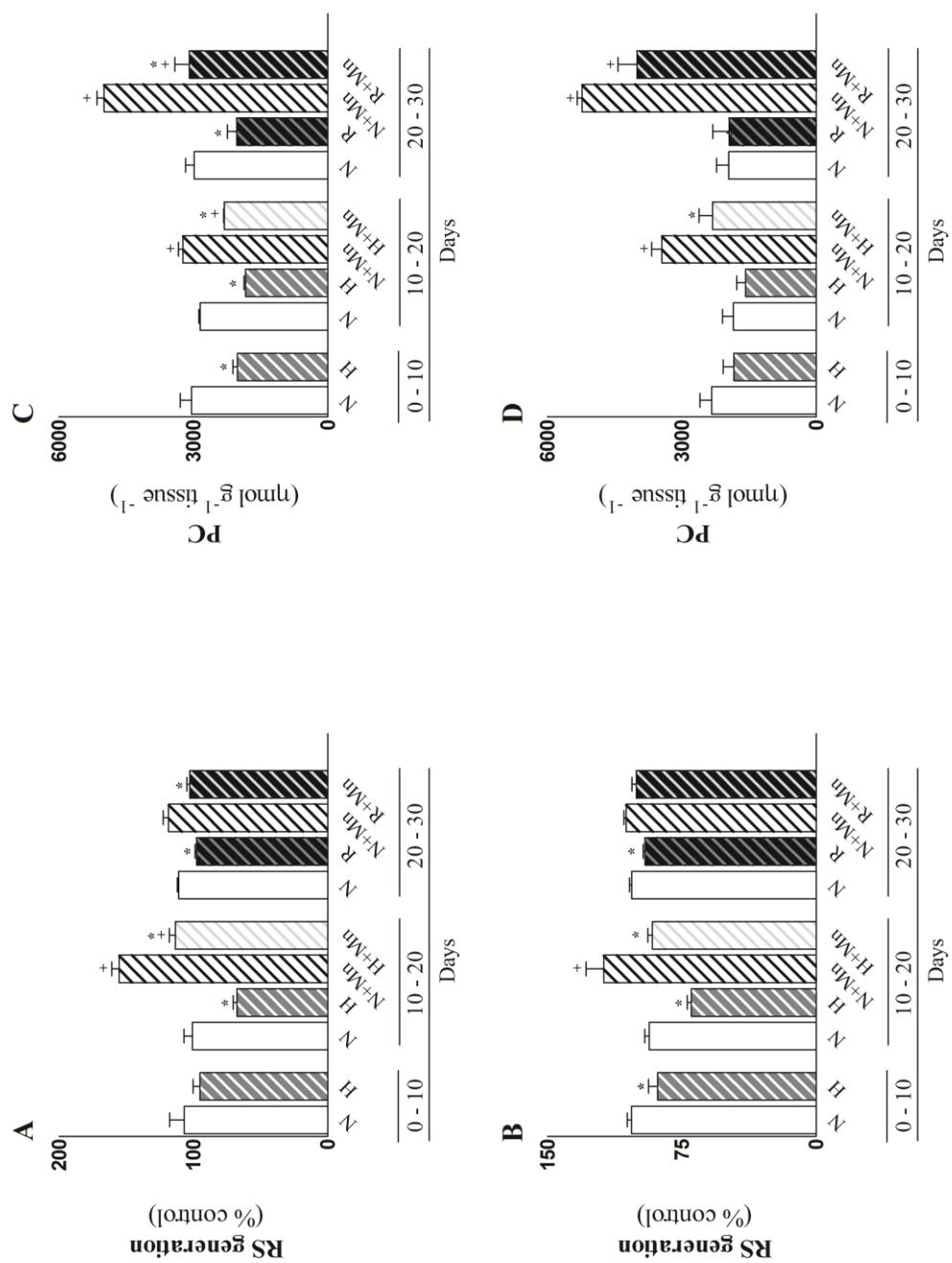


Figure 3

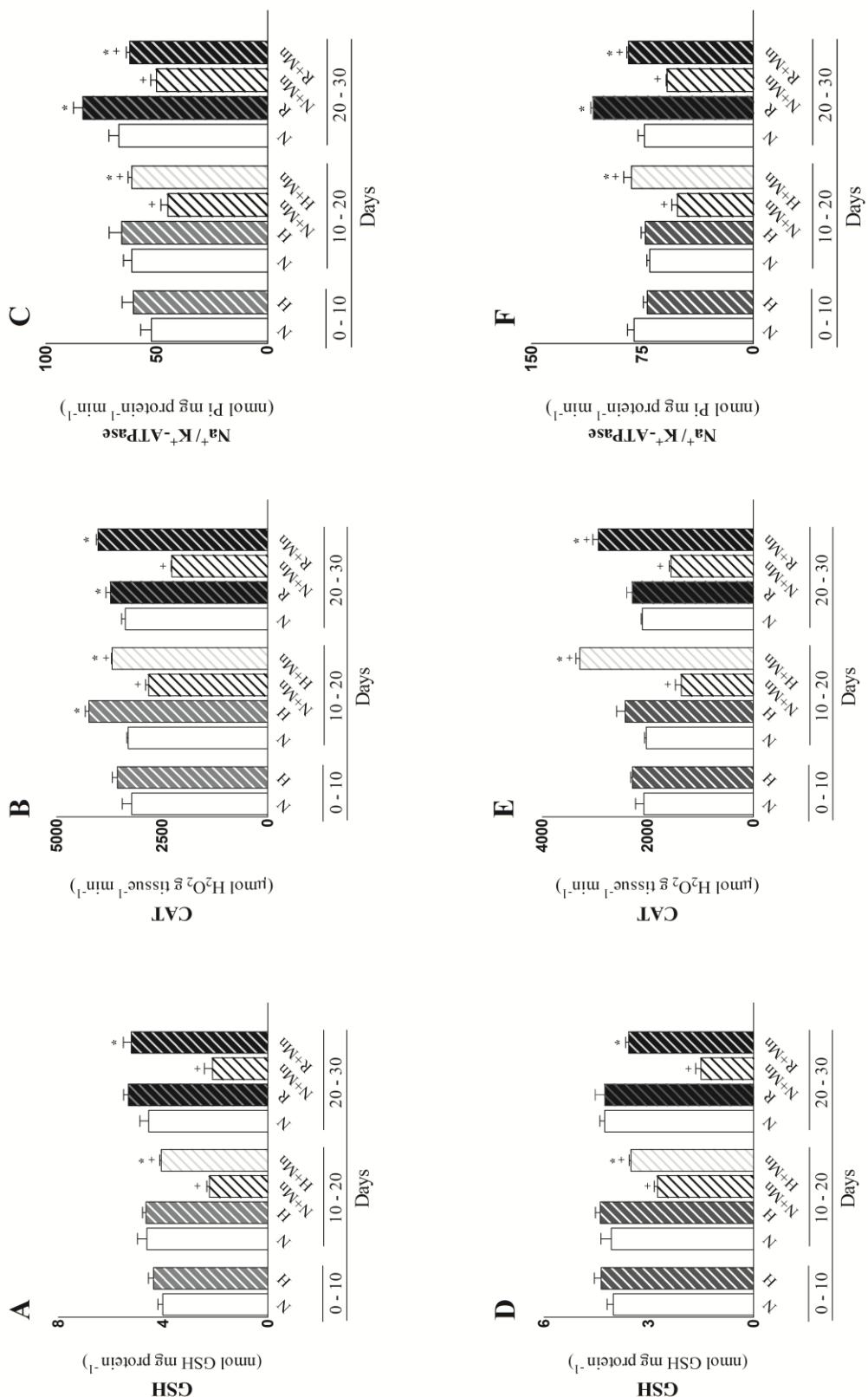


Figure 4

3.4 RESUMO EXPANDIDO

ACLIMATAÇÃO À HIPÓXIA E REOXIGENAÇÃO MODIFICAM PARÂMETROS COMPORTAMENTAIS, BIOQUÍMICOS E MOLECULARES EM JUNDIÁS EXPOSTOS AO Mn

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Hypoxia acclimation and reoxygenation modify behavioral, biochemical and molecular parameters in silver catfish exposed to Mn

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Oxygen is essential for the majority of organisms on Earth and its restriction exerts deleterious effects for several species. However, aquatic species hypoxia-tolerant are able to survive long periods of oxygen deprivation (Welker et al., 2013), as demonstrate in *Carassius auratus*, which maintain its antioxidant system more active when exposed to hypoxia (Lushchak et al. (2001, 2005b). Indeed, the reoxygenation (restoration of oxygen levels to normoxia), and not hypoxia (oxygen deprivation) cause oxidative impairments to the organisms, since the reoxygenation processes generates excessive amounts of reactive oxygen species (ROS) (Bols et al., 2001; Lushchak et al., 2001), fact described as “oxygen paradox” (Davies, 2000). The hypoxic aquatic ambient is generated mainly by eutrophication and organic pollution (Chippari-Gomes et al., 2005; Richards et al., 2007; Ficke et al, 2007) present in aquatic systems all over the world (Keeling and Garcia 2002; Helly and Levin 2004), can lead to endocrine disruption (Pait e Nelson, 2002) and behavioral changes (Poulsen et al., 2011) in fish species.

In this sense, the presence of environmental pollutants such as manganese (Mn) compounds (Gabriel et al., 2013; Dolci et al., 2013; Arndt et al., 2014; Dolci et al., 2014) can aggravate the fall in aquatic oxygen promoting deleterious effects to many aquatic organisms (Scott and Sloman, 2004). In this sense, the Mn is a constitutive element of a series of important enzymes and cofactors essentials for brain function, such as manganese superoxide dismutase (MnSOD) and glutamine synthetase (Yokel, 2009). In the presence of ammonia, glutamate (neurotransmitter related with learning and memory process) is metabolized to glutamine by the astrocyte-specific enzyme glutamine synthetase (GS) (Martinez-Hernandez et al., 1977), maintaining the glutamate balance in brain (Waniewski and Martin, 1986).

However, it is known that excessive Mn concentrations reduces significantly glutamate uptake in primary astrocyte cultures (Erikson and Aschner, 2002; Erikson et al., 2002) ensuring increase in extracellular glutamate concentrations being potentially excitotoxic to juxtaposed neurons (Erikson and Aschner, 2003). Moreover, excessive waterborne Mn, generated mainly from activities as oil and gas exploration in Amazon basin (Baldisserotto et al., 2012), implicate in oxidative stress in fish for increase the reactive species (RS) production (Gabriel et al., 2013; Dolci et al., 2014) which can inhibit the high affinity glutamate transporters (Trotti et al., 1998).

Consequently, environmental pollutants can lead to oxidative stress by modify the balance between pro-oxidants agents and the antioxidant defense system of the organisms (Hermes-Lima, 2004). The RS comprising superoxide anion ($\cdot\text{O}_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot\text{OH}^-$) among others (Halliwell and Gutteridge, 1999) while the

antioxidant system is compound by catalase (CAT), superoxide dismutase (SOD), and enzymes glutathione dependents (glutathione peroxidase, GPx, and glutathione reductase, GR) (Winston and Di Giulio, 1991; Matthiessen and Law, 2002; Ransberry, 2016). Therefore, since silver catfish (*Rhamdia quelen*) represents a Brazilian native species hypoxia-tolerant extensively cultivated due to its fillet quality and high carcass yield (Gomes et al., 2000), we investigate some physiological, biochemical and molecular mechanisms involved in its adaptation to low oxygen levels as well as the implications of an Mn exposure and of a subsequent reoxygenation in this fish.

For this, a hundred silver catfish were submitted to hypoxia or normoxia (5 fish per tank, five tanks/group, in duplicate), for 10 days (Fig. 1). Oxygen levels as well as the water parameters were monitored according data shown in Supplementary 1A, 1B. On day 10 behavioral tests were assessed and, subsequently, on day 11, brain and pituitary gland were collect for analyses (Fig 1). On day 10, the remaining silver catfish (5 fish per tank, two tanks/group, in duplicate) were then exposed for additional 10 days to waterborne Mn levels of 0.002 (control without Mn addition) and 8.1 mg L⁻¹, yielding the following groups: normoxia, hypoxia, normoxia plus Mn and hypoxia plus Mn (Fig. 1; Supplementary 1A). At the end of 20 days, behavioral tests were assessed, and on day 21, fish were euthanized and brain and pituitary gland were collected for analyses. On day 20, the remaining silver catfish (5 fish per tank, one tank/group, in duplicate) were submitted to reoxygenation protocol for additional 10 days. In the this step, groups previously hypoxia acclimated were restored to normoxia through of pumping bubbling air, providing following groups: normoxia, reoxygenation, normoxia plus Mn and reoxygenation plus Mn (Fig. 1; Supplementary 1A). Finally, on day 30, behavioral tests were assessed, and on day 31, fish were euthanized and brain and pituitary gland were collected for analyses. This protocol was approved by the Ethics of Animal Use Committee (CEUA) (nº:104/2013), Universidade Federal de Santa Maria.

The behavioral assessment was performed according Grassie et al. (2013), with some modifications. The fish were fasted for 24 hours prior to the behavior assessment. Learning ability (anxiety or social interaction memory) (n = 5 per group) was assessed using a maze trial (Figure 1, APÊNDICE E) that was placed inside the test tanks (250 L) which were maintained under the same conditions that original group. The test tank sides of the central arena were blue color, while the doorways at each of the false exits were transparent to help provide a potential social stimulus for fish, since in external side of the tanks existed fishes swimming. There were three ‘false’ exits and one ‘true’ exit leading out into the open area in the test tank. For the test, fish were placed in a transparent start cylinder (radius 8 cm) in the center of the maze. After 60

s the cylinder was manually removed and the fish was free to explore the maze. When the fish found way out of the maze, this way out was closed with manual guilhotine door. Trials ended after 5 min, or when the fish escaped successfully. Once fish that had leave the maze, it was immediately allocated in individual (4L) aquarium (APÊNDICE F) for posterior analysis of conspecifics. At the end of each experimental group, test tank was washed with clean water before the start of the next trial.

After ~30 minutes of the maze trial procedure, the test for evaluation of presence of the conspecifics (alarm substance) was started (adapted of Brown and Smith, 1998, Kochhann et al. 2009; Weber et al., 2012). In individual (4L) aquaria (APÊNDICE F), the behavior of each fish ($n= 5$ per group) was evaluated for 5 minutes (baseline) to basal ventilatory rate (opercular movements), which was estimated by counting the opercular beats for 15 s and multiplying by 4 to get opercular beats per minute (ob/min). Subsequently, was evaluated the defensive response (baseline) for additional 5 minutes, which is demonstrate through a fast swim in escape route in that the fish experiences a rapid and disorderly movement from one side to another side of the aquarium (dashes number) (Sanches et al., 2015). In the final step, was evaluate the fish feed habit, for additional 5 minutes (Foam et al., 2005), that consisted in provide feed and monitor its consumption by fish. All these parameters were assessed again after alarm substance administration (skin extract – SE) in aquarium test, totaling 30 minutes of observation for each fish. After all individuals of each group have been tested, the fish were then returned to their original tanks. The biochemical assays consisted of determination of brain oxidative status assessed through of reactive species (RS) (Hempel et al., 1999), protein carbonyl (PC) (Yan et al., 1995), besides enzymatic activities of catalase (CAT) (Aebi, 1984) and Na^+/K^+ -ATPase Musbeck et al., 1977; Atkinson et al., 1973). The molecular analysis consisted of gene expression of pituitary hormones such as proopiomelanocortin A and B (POMC-A; POMC-B), prolactin (PRL) and SL (somatolactin) The calculation of relative expression was performed as recommended by Pfaffl (2001). The sequences for design all the primers were prepared according Saccol et al. (*in preparation*) (Table 2).

Additionally, the fish color estimative was assessed ($n = 10$) after 10, 20 and 30 experimental days. The color estimative was adapted of Rezende et al. (2012). Briefly, the artificial lighting was positioned 80 cm above the fish for registration of photos and a camera (12.1 megapixels) was positioned 50 cm from the fish (90° angle in relation to the accommodation surface of the fish). Subsequently, the images obtained were scored by 10 observers blinded to treatments according to a color chart provided for evaluation. The color

range consists of four colors ranging from brown-coppery, purplish brown, brown and brown oil (APÊNDICE G), to which were assigned values of 1 to 4, where 1 should be assigned to the judgment of lighter color skin and 4 should be assigned to the darker color skin of the silver catfish.

The acclimation of silver catfish to hypoxia or normoxia for 20 did not change the Mn accumulation in brain for groups Mn exposed. However, in groups previously hypoxia acclimated and subsequently submitted to reoxygenation the Mn accumulation in brain decreased 73 % in comparison to fish exposed to Mn under normoxia (Table 1). Interestingly, when behavior was assessed, the acclimation to hypoxia or normoxia in fish exposed or not to Mn did not exert influence in the social memory interaction or anxiety, observed by similar entries number in all squares. However, when fish were exposed for the third time to the maze, fish they show a tendency to exhibit better memory in comparison to the previous exposures, regardless of the Mn presence (Fig. 2A, 2B, 2C, 2D, respectively).

Additionally, when aggressive responses (dashes number) against conspecifics (baseline and after skin extract – SE), (Fig. 3C, 3D, respectively) was assessed, silver catfish acclimated to hypoxia presented smaller aggressive responses than fish normoxia acclimated, regardless of Mn presence, which persisted lower even after the establishment of reoxygenation (Fig. 3C, 3D, respectively). In hypoxia, it is expected that fish decrease its aggressiveness, due a lower energy expenditure, since the oxidative phosphorylation is inhibited (Riepe et al., 1997) in animals hypoxia-tolerant. However, the decrease in aggressiveness against conspecifics exhibited by groups exposed to Mn under normoxia or after reoxygenation can be related to the toxicity of Mn to the brain, due increased RS generation in this organ. Moreover, it is possible that, similarly to the observed for rodents (Erikson and Aschner, 2003), a Mn excess causes impairments to the brain due both inhibition of affinity for glutamate transporters Mn-induced (Trotti et al., 1998), or by reduction of glutamate uptake in astrocytes (Erikson and Aschner, 2002; Erikson et al., 2002), triggering excitotoxicity to juxtaposed neurons. Additionally, it was observed an increase in the ventilatory rate (baseline and after SE) (Fig. 3A, 3B, respectively) in groups acclimated to hypoxia regardless of Mn presence throughout experiment when compared to groups normoxia acclimated. The different oxygen levels or Mn did not change the fish feed habit (baseline and after SE).

Hypoxia acclimation, for 20 days, and subsequent reoxygenation, for additional 10 days, decreased the oxidative damages (RS and PC) (Fig. 4A, 4B, respectively) to the brain and activates the antioxidant defense CAT (Fig. 4C), similarly to observed by Lushchak et al. (2005b) and Tripathi et al. (2013). However, Na^+/K^+ -ATPase (Fig. 4D) was reduced *per se* after

20 days of acclimation to hypoxia. While exposure to the Mn under normoxia produced a major decreased in this enzyme, fish acclimated to hypoxia and exposed to the Mn showed an increase on Na^+/K^+ -ATPase activity. Hypoxia acclimation, for 10 or 20 days, increases the POMC-A expression (Fig. 5A) while exposure to Mn under hypoxia decreased the expression of this pituitary hormone. In the hypothalamic – pituitary – adrenal (HPI) cascade, the synthesis of proopiomelanocortin (POMC) occurs from the stimulation of pituitary corticotrophic cells in the hypothalamus which are regulated by corticotropin-releasing hormone (CRH) which controls adrenocorticotropic hormone (ACTH) release, which, in turn, will stimulate the production and release of cortisol in interrenal cells in response to the stress (Mommsen et al., 1999). The ACTH also promotes release of α -MSH by adenohypophysis, which in teleost, induces the pigment dispersion (darkening of the skin) (Fugii, 2000; Takahashi and Kawauchi, 2006). Here, there was no changes in POMC-B (Fig. 5B), PRL (Fig. 5C) and SL expression (Fig. 5D) during total experimental period. Interestingly, acclimation to hypoxia for 10 to 20 days caused a darkening *per se* in the skin of the silver catfish, which was also observed after 10 days of reoxygenation (Fig. 6). Such outcome can be related to increased levels of POMC-A expression in response to the stress induced by hypoxia (Mommsen et al., 1999; Fugii, 2000; Takahashi and Kawauchi, 2006). Exposure to Mn under hypoxia produced a darkening of the skin of the silver catfish, but to a lesser degree than observed in hypoxia (Fig. 6). After subsequent reoxygenation, regardless of the presence of Mn, it was also observed darkening of the silver catfish skin.

Global findings indicate that while exposure of silver catfish to the Mn under normoxia was related to oxidative damages in brain, hypoxia acclimation was able to reduce waterborne Mn uptake in brain when reoxygenation was established. Importantly, the initial disruption of homeostasis induced by hypoxia was able to produce compensatory responses activating the antioxidant enzyme CAT and modifying POMC-A expression in pituitary, reinforcing hormesis hypothesis (Hermes-Lima, 2004, Mattson, 2008; Calabrese, 2014). Such changes were responsible for preserve the organism homeostasis in oxygen deprive conditions, in order to ensure the survival of this species against further hostile situations, as Mn contamination or subsequent reoxygenation, although, the long-term impacts caused by chronic hypoxia on silver catfish in theirs natural environment still remain unknown.

Keywords: *Rhamdia quelen*; Hypoxia-reoxygenation, social interaction memory, alarm substance, oxidative stress; pituitary hormones

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Supplementary 1(A-B). Water parameters with Mn experimental values (obtained after inductively coupled plasma optical emission spectrometry (ICP-OES) analyses). Fish were acclimated for 10 days to normoxia (N) or hypoxia (H) and afterwards exposed or not to waterborne Mn [$\sim 8.1 \text{ mg L}^{-1}$], for additional 10 days (H+Mn; N+Mn). After this period, fish previously hypoxia acclimated were submitted to reoxygenation (R; R+Mn) for 10 more days.

1A

Exposure (Days)	Groups	Dissolved Oxygen	O ₂ Saturation (%)	[Mn] mg L ⁻¹ ICP-OES	T (°C)	pH
0 – 10	N	7.36±0.06	81.89±0.86	0.002±0.00	21.30±0.18	5.85±0.17
	H	2.94±0.09*	40.19±1.13*	0.002±0.00	21.39±0.18	5.85 ± 0.14
10 – 20	N	7.43±0.10	82.00±1.00	0.002±0.00	22.02±0.14	6.00±0.23
	H	3.09±0.14*	41.54±1.14*	0.002±0.00	21.87±0.17	5.66±0.14
	N + Mn	7.57±0.22	82.79±2.84	8.13±0.21 ⁺	21.92±0.15	5.80±0.15
	H + Mn	3.32±0.10*	42.60±1.40*	8.30±0.31 ⁺	22.03±0.18	6.00±0.24
20 – 30	N	7.04±0.02	79.32±0.16	0.002±0.00	22.5±0.13	5.99±0.23
	R	7.15±0.05	79.94±0.26	0.002±0.00	22.5±0.10	5.83±0.21
	N + Mn	7.29±0.12	81.41±2.19	8.18±0.91 ⁺	22.5±0.13	5.95±0.20
	R + Mn	7.15±0.04	80.18±0.36	8.18±0.64 ⁺	22.5±0.11	5.67±0.15

1B

Exposure (Days)	Groups	Hardness (mg CaCO ₃ L ⁻¹)	Alkalinity	Total ammonia	NH ₃	Nitrite
0 – 10	N	72.50 ± 2.19	47.60 ± 1.16	0.04 ± 0.002	0.0007 ± 0.00	0.17 ± 0.05
	H	78.20 ± 2.04	50.42 ± 1.34	0.04 ± 0.001	0.0008 ± 0.00	0.14 ± 0.01
10 – 20	N	77.20 ± 3.04	44.20 ± 2.37	0.03 ± 0.009	0.0008 ± 0.00	0.17 ± 0.03
	H	67.80 ± 2.50	48.50 ± 1.48	0.04 ± 0.003	0.0008 ± 0.00	0.14 ± 0.02
	N + Mn	76.00 ± 1.19	48.10 ± 1.51	0.03 ± 0.002	0.0008 ± 0.00	0.17 ± 0.05
	H + Mn	76.00 ± 3.21	45.40 ± 1.02	0.03 ± 0.003	0.0008 ± 0.00	0.17 ± 0.08
20 – 30	N	74.40 ± 1.40	46.15 ± 1.10	0.03 ± 0.003	0.0007 ± 0.00	0.13 ± 0.01
	R	67.80 ± 2.50	50.30 ± 1.67	0.04 ± 0.002	0.0007 ± 0.00	0.16 ± 0.03
	N + Mn	73.40 ± 1.40	46.10 ± 1.02	0.03 ± 0.002	0.0007 ± 0.00	0.15 ± 0.02
	R + Mn	75.40 ± 2.70	46.60 ± 0.53	0.04 ± 0.004	0.0007 ± 0.00	0.17 ± 0.05

*Indicates significant difference between oxygen levels (N and H or N and R groups) in the same water condition (Mn or not);

⁺Indicates significant differences between Mn and control group in the same oxygen level ($p < 0.05$). Values are expressed as mean ± SEM.

Table 1. Mn accumulation in silver catfish brain. Water parameters with Mn experimental values (obtained after inductively coupled plasma optical emission spectrometry (ICP-OES) analyses). Fish were acclimated for 10 days to normoxia (N) or hypoxia (H) and afterwards exposed or not to waterborne Mn [~ 8.1 mg L $^{-1}$], for additional 10 days (H+Mn; N+Mn). After this period, fish previously hypoxia acclimated were submitted to reoxygenation (R; R+Mn) for 10 more days.

Exposure (Days)	Groups	Mn accumulation in brain ($\mu\text{g g}^{-1}$ tissue $^{-1}$)
0 - 10	N	0.02 ± 0.00
	H	0.02 ± 0.00
10 - 20	N	0.02 ± 0.00
	H	0.02 ± 0.00
	N + Mn	1.10 ± 0.11 *
	H + Mn	0.75 ± 0.07 *
20 - 30	N	0.02 ± 0.00
	R	0.02 ± 0.00
	N + Mn	1.80 ± 0.14 *
	R + Mn	0.48 ± 0.04 **

*Indicates significant difference between oxygen levels (N and H or N and R groups) in the same water condition (Mn or not);

†Indicates significant differences between Mn and control group in the same oxygen level ($p < 0.05$). Values are expressed as mean \pm SEM.

Table 2. Primers design for amplification of β-actin, PRL, SL, POMC-A and POMC-B genes based on the sequences of these genes described by Baldisserotto et al. (2014) and Saccol et al. (*in preparation*).

qPCR Primers	Nucleotide sequence
β-actin Fw	5' CGAATGCCAGGGTACATGGT 3'
β-actin Rv	5' CCACCTTCAACTCCATCATTGAA 3'
PRL Rw	5' ACCAGAGACAGGAGCTCGTTCT 3'
PRL Rv	5' AGCTCATGAGACCGTCCATGT 3'
SL Rw	5' CGAGGCCAGGACTTTGTTG 3'
SL Rv	5' GACGCGCACAAAGGTTGAT 3'
POMC-A Fw	5' ATGAAGCTCCAGAGTCCGTT 3'
POMC-A Rv	5' GATTCTCCTCCACTCCGTTG 3'
POMC-B Fw	5' AGTCCACACCACCTCTCCAT 3'
POMC-B Rv	5' TGCTCTGGCATCTGTGTTCT 3'

Legend for figures

Figure 1. Experimental design. Fish were acclimated for 10 days to normoxia (N) or hypoxia (H) and afterwards exposed or not to waterborne Mn [$\sim 8.1 \text{ mg L}^{-1}$], for additional 10 days, totaling 20 days (N+Mn; H+Mn). After this period, fish previously hypoxia acclimated were submitted to reoxygenation (R; R+Mn) for 10 more days, totaling 30 days. **Maze trial procedure.** Plan view (not drawn to scale) of the maze with test fish inside the isolation circular tube. The maze was raised up on a platform, creating a water depth of 15 cm inside the maze. Each false exit (arm) was enclosed, but they were formed by transparent walls. The true exit (arm) was open. Solid lines indicate blue walls that were used to minimize external cues; dashed lines are transparent walls.

Figure 2. Maze of anxiety or social interaction. Entries number in the first square (**A**), entries number in the second square (**B**), entries number in the third square (**C**) and, entries number in the fourth square – way out (**D**). Behavior presented for fish acclimated for 10 days to normoxia (N) or hypoxia (H) and afterwards exposed or not to waterborne Mn [$\sim 8.1 \text{ mg L}^{-1}$], for additional 10 days, totaling 20 days (N+Mn; H+Mn). After this period, fish previously hypoxia acclimated were submitted to reoxygenation (R; R+Mn) for 10 more days, totaling 30 days.

*Indicates significant difference between oxygen levels (N and H or N and R groups) in the same water condition (Mn or not);

⁺Indicates significant differences between Mn and control group in the same oxygen level ($p < 0.05$). Values are expressed as mean \pm SEM.

Figure 3. Evaluation of conspecifics (alarm substance). Ventilatory rate (baseline) (**A**) and ventilatory rate after exposure to skin extract (alarm substance) (**B**), dashes number (baseline) (**D**) and dashes number after exposure to skin extract (alarm substance) (**E**). Parameters analysed in silver catfish acclimated for 10 days to normoxia (N) or hypoxia (H) and afterwards exposed or not to waterborne Mn [$\sim 8.1 \text{ mg L}^{-1}$], for additional 10 days (N+Mn; H+Mn). After this period, fish previously hypoxia acclimated were submitted to reoxygenation (R; R+Mn) for 10 more days.

*Indicates significant difference between oxygen levels (N and H or N and R groups) in the same water condition (Mn or not);

⁺Indicates significant differences between Mn and control group in the same oxygen level ($p < 0.05$). Values are expressed as mean \pm SEM.

Figure 4. Oxidative parameters and enzymatic activities. Reactive species generation (RS) (**A**), protein carbonyl (PC) (**B**), and enzymatic activities of catalase (CAT) (**C**) and Na^+/K^+ -ATPase (**D**) in the brain of silver catfish acclimated for 10 days to normoxia (N) or hypoxia (H) and afterwards exposed or not to waterborne Mn [$\sim 8.1 \text{ mg L}^{-1}$], for additional 10 days (N+Mn; H+Mn). After this period, fish previously hypoxia acclimated were submitted to reoxygenation (R; R+Mn) for 10 more days.

*Indicates significant difference between oxygen levels (N and H or N and R groups) in the same water condition (Mn or not);

⁺Indicates significant differences between Mn and control group in the same oxygen level ($p < 0.05$). Values are expressed as mean \pm SEM.

Figure 5. Pituitary hormones. Proopiomelanocortin A (POMC-A) (**A**), proopiomelanocortin B (POMC-B) (**B**), prolactin (PRL) (**C**), somatotropin (SL) (**D**) expression in the pituitary of silver catfish acclimated for 10 days to normoxia (N) or hypoxia (H) and afterwards exposed or not to waterborne Mn [$\sim 8.1 \text{ mg L}^{-1}$], for additional 10 days (N+Mn; H+Mn). After this period, fish previously hypoxia acclimated were submitted to reoxygenation (R; R+Mn) for 10 more days.

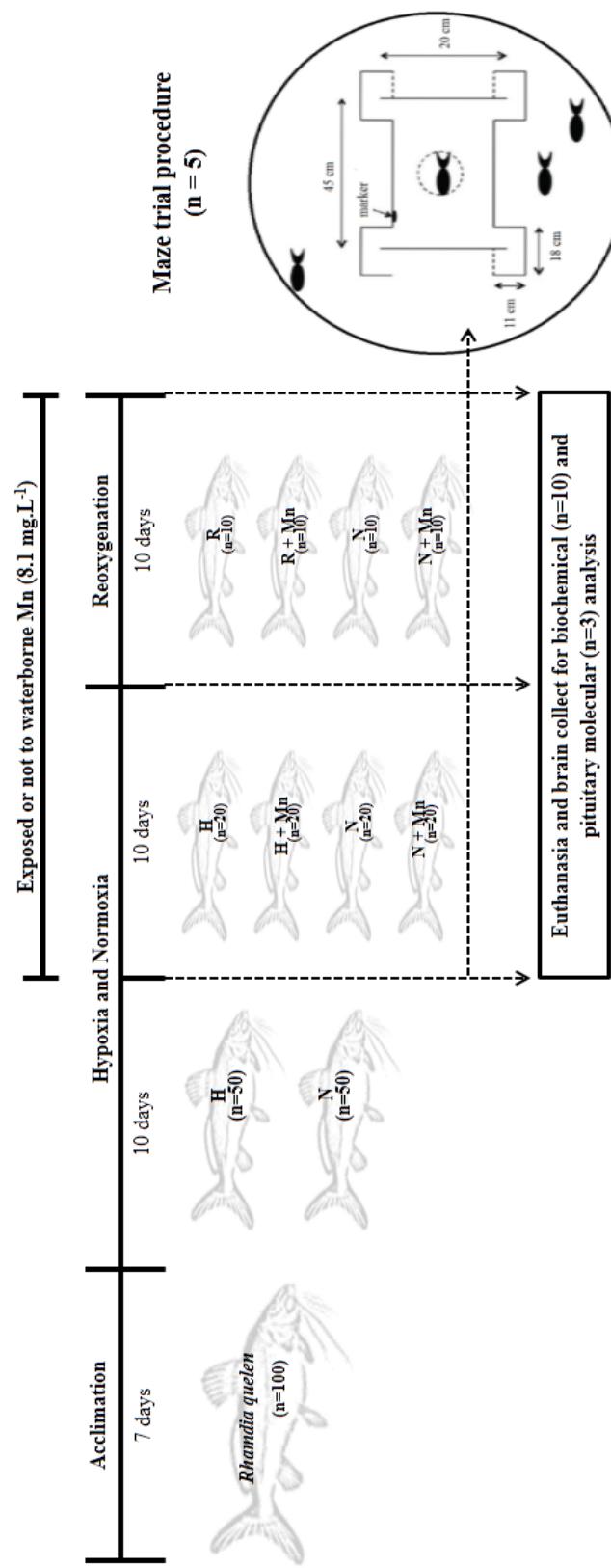
*Indicates significant difference between oxygen levels (N and H or N and R groups) in the same water condition (Mn or not);

[†]Indicates significant differences between Mn and control group in the same oxygen level ($p < 0.05$). Values are expressed as mean \pm SEM.

Figure 6. Color estimative. Silver catfish color analysis after 10, 20 or 30 experimental days. Silver catfish were acclimated for 10 days to normoxia (N) or hypoxia (H) and afterwards exposed or not to waterborne Mn [$\sim 8.1 \text{ mg L}^{-1}$], for additional 10 days (N+Mn; H+Mn). After this period, fish previously hypoxia acclimated were submitted to reoxygenation (R; R+Mn) for 10 more days.

*Indicates significant difference between oxygen levels (N and H or N and R groups) in the same water condition (Mn or not);

[†]Indicates significant differences between Mn and control group in the same oxygen level ($p < 0.05$). Values are expressed as mean \pm SEM.



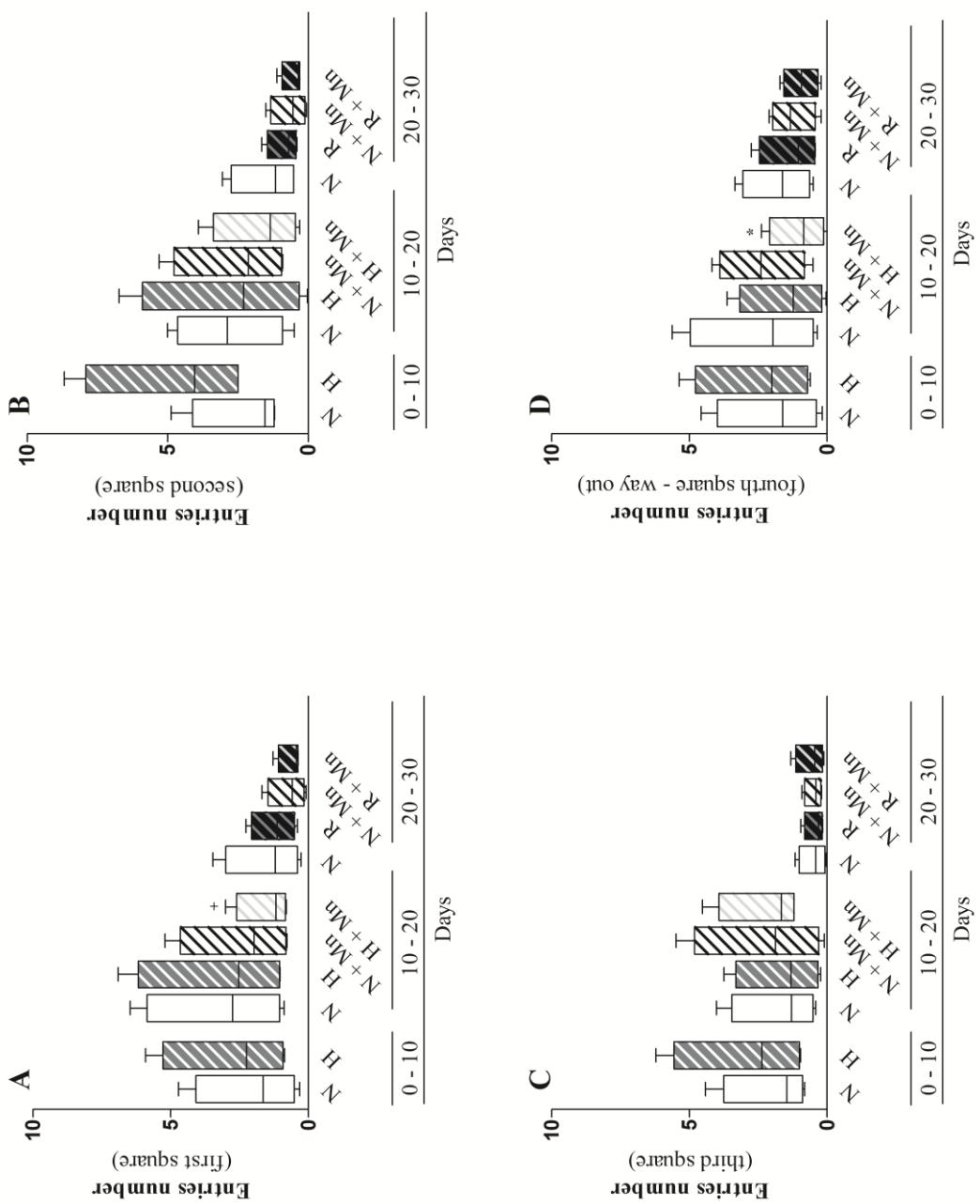


Figure 2

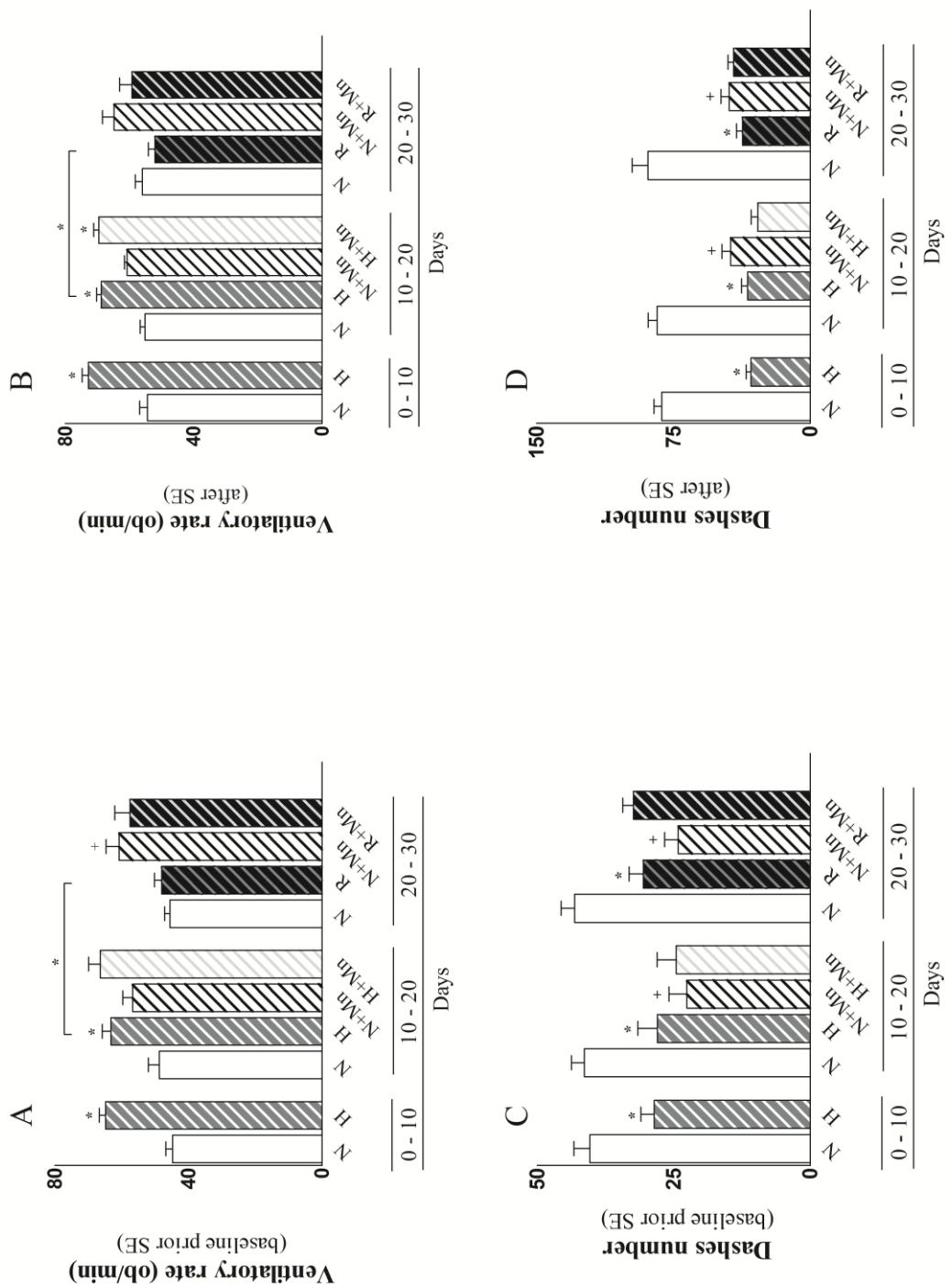


Figure 3

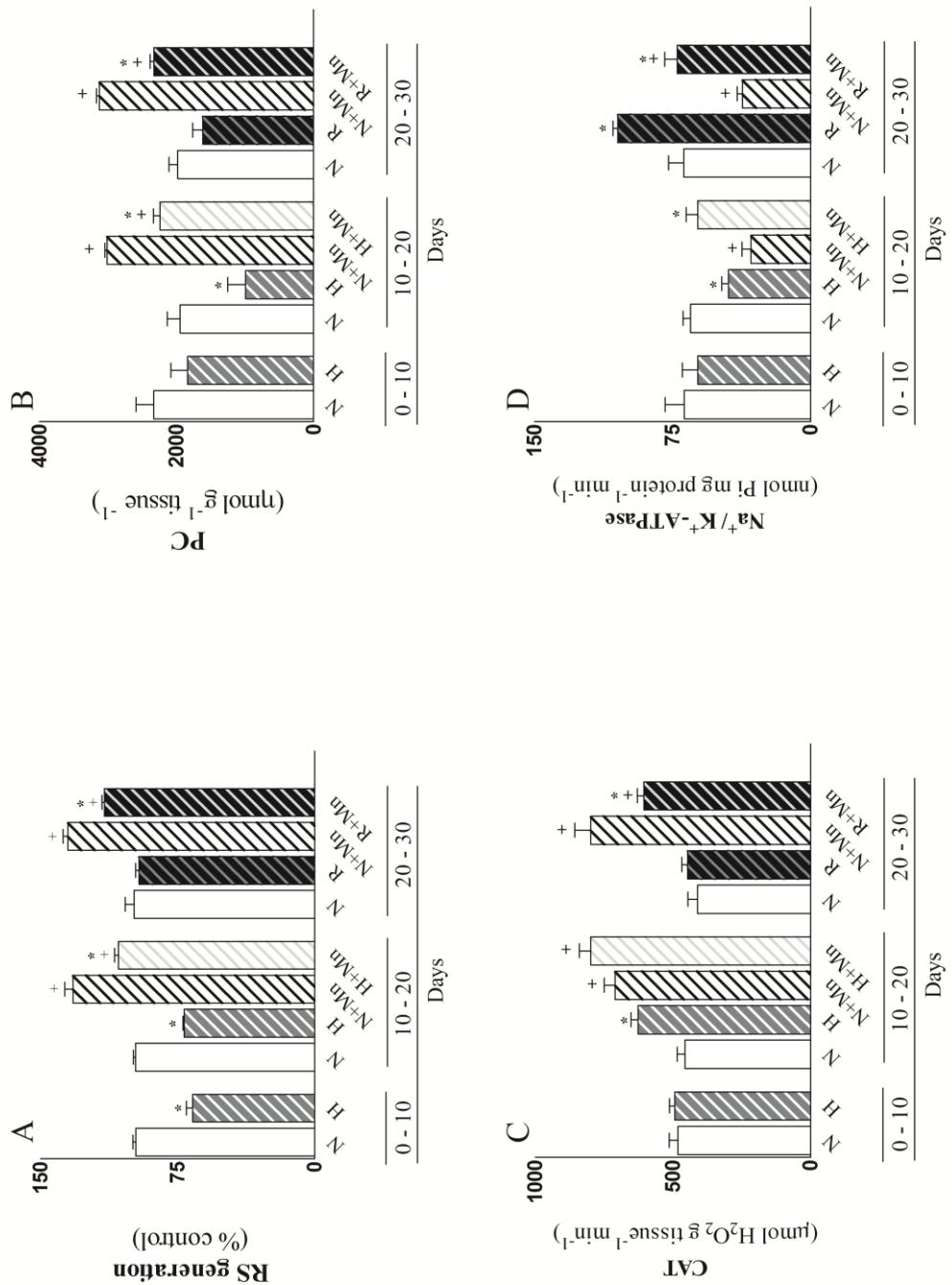


Figure 4

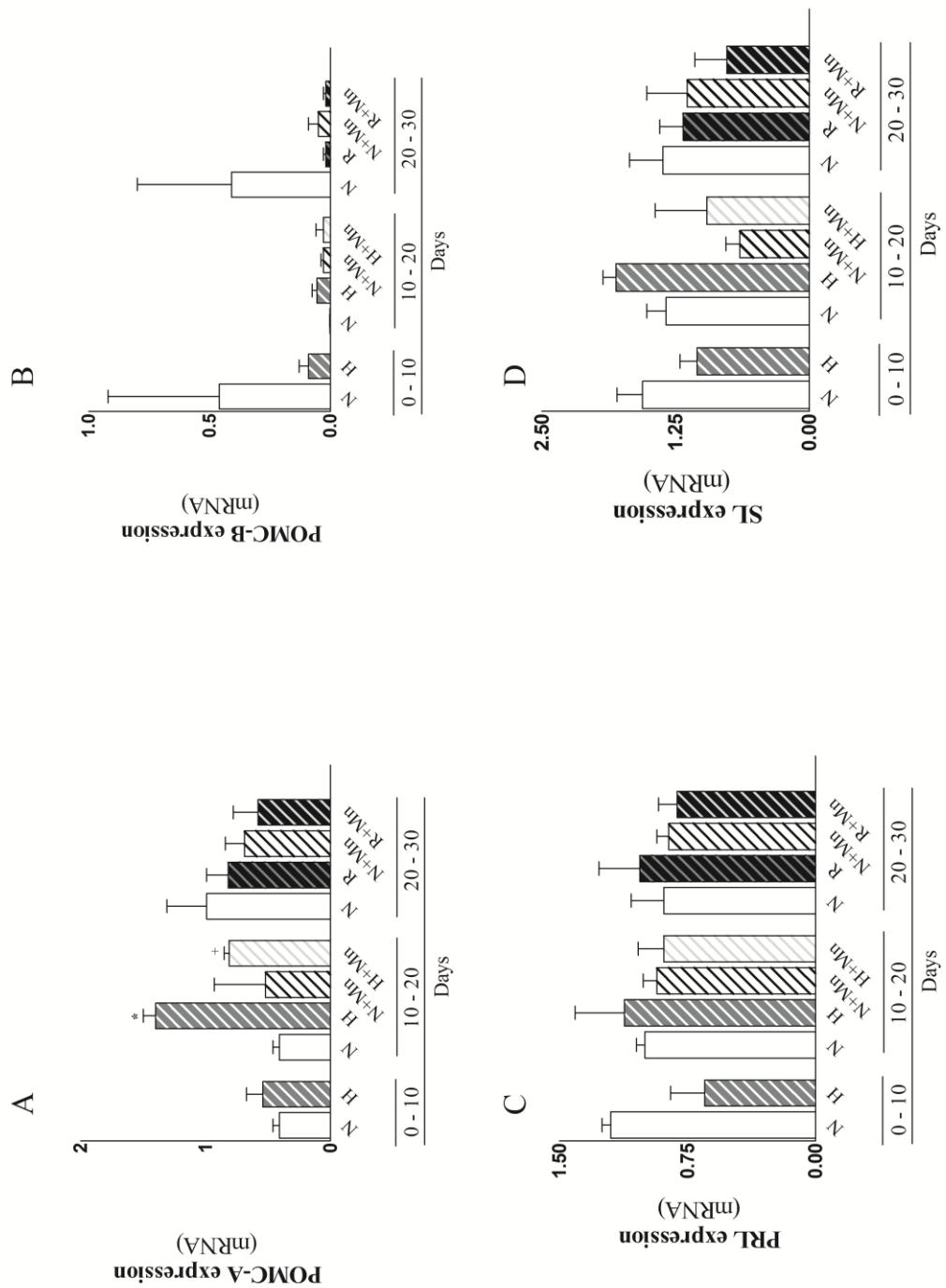


Figure 5

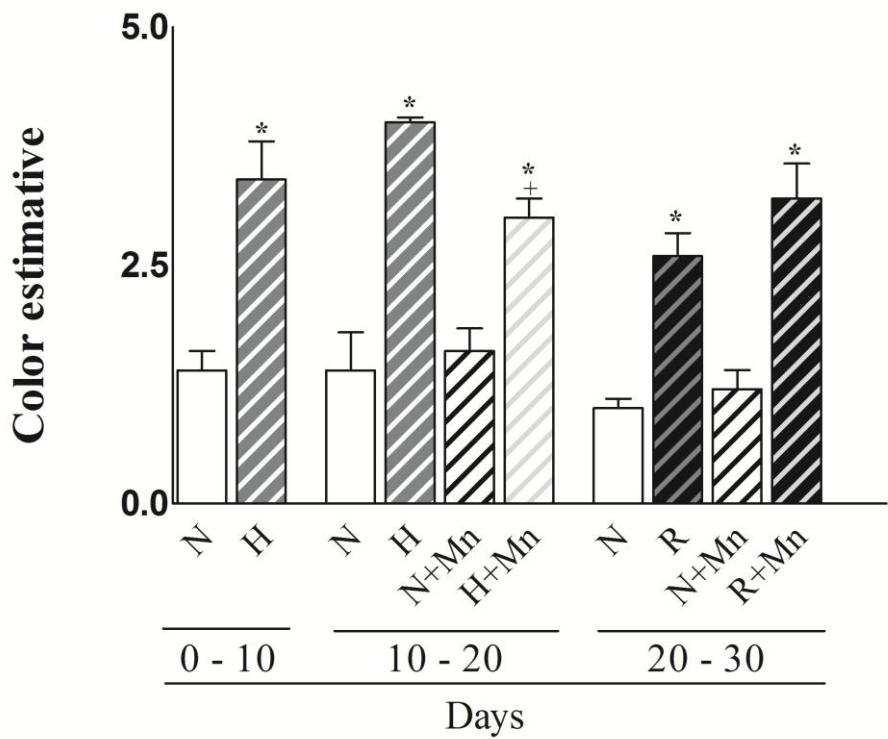


Figure 6

CAPITULO III

4 DISCUSSÃO

A constante interferência do homem no ambiente destaca-se como um dos fatores responsáveis pela ocorrência de alterações nas condições oceanográficas mundiais e, neste sentido, a redução da emissão de poluentes ao meio ambiente tem sido motivo de preocupação de líderes de países desenvolvidos e em desenvolvimento, a exemplo da conferência para mudanças climáticas (COP21) realizada em Paris em 2015. Como resultado dessas interferências observa-se o aquecimento global que afeta negativamente o teor de oxigênio dissolvido dos oceanos, aumentando a incidência de zonas aquáticas com deficiência em oxigênio (KEELING e GARCIA 2002; HELLY E LEVIN 2004). Conectado a esses fatos, o ambiente aquático representa um dos últimos receptáculos de poluentes uma vez que os vertebrados, devido à bioconcentração e biomagnificação, se encontram muitas vezes expostos a alta carga de poluentes (WESTER e VOS, 1994). Adicionalmente, a carga de poluentes sedimentados nessas águas contribui ainda mais para exacerbação da hipóxia aquática exigindo complexos processos de adaptação pelas espécies que habitam essas áreas.

Além das mudanças globais representarem um fator de risco para o aumento da incidência de hipóxia aquática, outros fatores podem atuar agravando a sobrevivência das espécies nessas condições como, por exemplo, a constante emissão de agentes químicos ao meio ambiente, responsáveis por contaminações tanto de reservatórios de águas doces, quanto de águas oceânicas (OCDE, 2012; ATSDR, 2000). Portanto, estudos com substâncias potencialmente tóxicas são necessários para a melhor compreensão dos diferentes ajustes fisiológicos experimentados pelas espécies durante episódios de contaminação.

O estudo focou sobre dois aspectos que comumente podem ocorrer isoladamente ou simultaneamente na natureza, ou seja, uniu a situação de hipóxia ambiental, mimetizando uma área com baixos níveis de oxigênio dissolvido (naturalmente causada por diversos fatores, como aumento da temperatura global, eutrofização, etc.), com a situação de contaminação por metais. Optou-se por utilizar o manganês (Mn), uma vez que este metal está geralmente presente em altas concentrações em águas estuarinas onde ocorre a intensa prospecção de combustíveis fósseis como o petróleo (BALDISSEROTTO e cols., 2012; GABRIEL e cols., 2013). Para a realização do estudo, em todas as suas etapas, utilizou-se como modelo experimental o jundiá (*Rhamdia quelen*), por ser um peixe de crescente consumo na região sul do Brasil, com alto potencial de comercialização e de grande aceitação pela população pela qualidade da carne.

Este estudo apresentou alguns mecanismos pelos quais a hipóxia causa tolerância na espécie *Rhamdia quelen*, tais como aumento das enzimas antioxidantes frente ao estresse

oxidativo, aumento de alguns parâmetros hematológicos, alterações morfológicas branquiais, assim como redução do consumo de energia nas brânquias demonstrada pela inibição de desidrogenases mitocondriais (redução do MTT) e também pela reduzida atividade da Na⁺/K⁺-ATPase no encéfalo. Associadamente, esses fatores contribuem para uma melhor adaptação a situações adversas, porém as bases moleculares responsáveis por desencadear tais respostas nessa espécie necessitam ser melhor esclarecidas. Neste contexto, é conhecido que muitos peixes podem expressar proteínas específicas chave no processo de adaptação a hipóxia, dentre as quais, o fator induzível por hipóxia – HIF-1 α – “*Hypoxia inducible factor*”, responsável pela regulação de outros genes alvo, como por exemplo eritropoietina (Epo) (NAGAO e cols., 2008; ROBERTSON e cols., 2015).

O HIF-1 constitui-se em um heterodímero composto de uma subunidade HIF-1 α regulada por oxigênio e uma subunidade HIF-1 β expressa constitutivamente (WANG e cols., 1995; SEMENZA, 2007). A expressão desse fator, que já foi demonstrada em diferentes organismos ao longo da evolução (WU, 2002; NIKINMAA e REES, 2005), confere as espécies que expressam esse fator em seu código genético uma proteção natural a ambientes hipóxicos. Como o jundiá constitui-se em uma espécie euritolerante com capacidade de adaptação a diferentes condições de temperatura, salinidade e oxigênio (CHIPPARI-GOMES, GOMES e BALDISSEROTTO, 1999; MARCHIORO, 1997; BRAUN e cols. 2008), especula-se a também a expressão do HIF na espécie, observada sua alta capacidade de adaptação à hipóxia. De acordo com Bhatia e cols. (2013) quando os animais são expostos pela primeira vez à hipóxia a sua transcrição de HIF-1 α não é afetada, porém quando esses organismos experimentam episódios crônicos de hipóxia, a sua transcrição é estimulada. Então, em indivíduos que se encontram regularmente expostos a hipóxia em seu habitat, a expressão de HIF-1 α pode ser regulada tanto transcrecionalmente quanto pós-transdacionalmente.

Neste sentido, sabe-se também que a regulação pós-transdacional de HIF-1 α é realizada por prolil hidroxilases e asparginil hidroxilases (KAELIN e RATCLIFFE, 2008; RYTKÖNEN, e cols., 2012) por hidroxilação de resíduos de prolina específicos. Portanto, na presença dessas hidroxilases ocorre a rápida degradação proteossômica do HIF-1 α no citosol (JAAKKOLA, e cols., 2001), o que resulta na inibição da interação do HIF-1 α com os seus coativadores transcrecionais. Porém, quando ocorre um aumento do HIF-1 α citosólico, devido a uma hipóxia sustentada, por exemplo, essas hidroxilases são inibidas e, consequentemente, ocorre uma estimulação dos coativadores transcrecionais do HIF-1 α , que resultam na ativação do ARE “*antioxidant response element*” a nível de DNA. Em nosso estudo, a expressão do HIF-1 α não foi investigada a nível nuclear, portanto, estudos futuros devem ser conduzidos a fim de

investigar se a hipóxia crônica produzida em jundiás poderia de alguma forma estar ativando o ARE à nível de DNA.

De fato, a aclimatação á hipóxia (~ 40 % de saturação de O₂, ou seja, abaixo do valor crítico de saturação de oxigênio para o crescimento de teleósteos) reduziu os danos gerados pelo Mn na maioria dos tecidos analisados (DOLCI e cols., 2014) revelando que, aclimatação a níveis moderadamente baixos de oxigênio, permite ajustes fisiológicos em jundiás, garantindo uma maior proteção aos órgãos frente a agentes estressores, como Mn ou até os perigos potenciais de uma subsequente reoxigenação. Adicionalmente, a ordem de captação para o Mn seguiu um padrão relativamente homogêneo entre os estudos desenvolvidos nesta tese, sendo encontrado um acúmulo de Mn em maior grau para fígado e brânquias, seguido pelo rim e, finalmente, similares quantidades de Mn captadas pelo plasma e encéfalo.

A análise morfológica de brânquias de jundiás aclimatados a hipóxia, permitiu observar que, sob hipóxia, ocorreram ajustes como aumento da área de superfície branquial e da área funcional lamelar (SOLLID e cols., 2006; SOLLID e NILSSON, 2006; DOLCI e cols., 2016a – *em preparação*) que, somados ao aumento do número de eritrócitos, aumentada concentração de hemoglobina e hematócrito, contribuíram para uma maior perfusão branquial (RUSSELL, DOMBKOWSKI e OLSON, 2008) e assim maior afinidade de ligação da hemoglobina ao oxigênio (Hb-O₂) que segundo Baldisserotto (2013) é provocada pela diminuição dos níveis de GTP e ATP intraeritrocitários. Embora essa maior afinidade de ligação da hemoglobina ao O₂ dificulte a distribuição uniforme de oxigênio aos tecidos durante hipóxia, essa resposta representa a capacidade dessa espécie em poupar energia frente a situações de privação de oxigênio garantindo a sobrevivência dos organismos.

Em relação aos danos provocados pelo Mn sob normóxia houve uma maior geração de espécies reativas (ERO), maior peroxidação lipídica (PL), maior dano às proteínas (PC), perda da viabilidade mitocondrial, dano direto aos hepatócitos, diminuição da atividade da bomba Na⁺/K⁺-ATPase, além da inibição de importantes enzimas antioxidantes (DOLCI e cols., 2014; CHTOUROU e cols., 2011; ATLI e cols., 2006; ZHANG e cols., 2004). De fato, os tecidos analisados apresentaram maior acúmulo do metal sob normóxia, indicando uma relação causal entre acúmulo de Mn e desenvolvimento de estresse oxidativo nesses tecidos, comprovando assim prejuízos fisiológicos produzidos pelo Mn nas concentrações compreendidas entre 9,1 e 8,1 mg L⁻¹.

Adicionalmente, no primeiro estudo descrito nesta tese, jundiás aclimatados a hipóxia não apresentaram alterações na expressão de hormônios hipofisários, enquanto que a

aclimatação de jundiás à normoxia e exposição posterior ao Mn resultou em aumentada expressão gênica da prolactina (PRL) semelhante ao observado para roedores expostos ao Mn ([MARREILHA DOS SANTOS e cols., 2011](#)). Concomitantemente, houve uma reduzida expressão gênica da somatolactina (SL), a qual desempenha funções sobre a regulação do metabolismo lipídico ([FUKAMACHI e MEYER, 2007](#)) assim como sobre a maturação sexual ([RAND-WEAVER e SWANSON, 1993; BHANDARI e cols., 2003; ONUMA e cols., 2003](#)), sugerindo que o Mn possa ter causado prejuízos reprodutivos ao jundiá, embora a avaliação de parâmetros reprodutivos não tenham sido alvo do estudo. Outro fato interessante é que durante a realização do segundo experimento, o qual foi investigada a influência do estabelecimento da reoxigenação em jundiás após um período prévio de hipoxia, não foram observadas alterações na expressão dos hormônios PRL e SL. É possível que tal fato esteja relacionado à idade dos animais, uma vez que para o primeiro estudo foram utilizados animais mais velhos, enquanto que, no estudo subsiguiente, peixes mais jovens foram utilizados.

Na verdade, o monitoramento da super ou sub expressão dos hormônios hipofisários, tais como PRL, SL, hormônio do crescimento (GH) e proopiomelanocortina (POMC) constituem excelentes alvos para investigar os prejuízos endócrinos gerados pela exposição dos organismos a xenobióticos como o Mn, pois fornecem informações sobre possíveis alterações do eixo endócrino, o qual está intimamente relacionado à regulação dos níveis de cortisol e desenvolvimento de estresse em peixes ([LIMA e cols., 2006; JIAO e cols., 2006](#)). De fato, neste estudo, a expressão da isoforma A da POMC (POMC-A) foi aumentada em jundiás aclimatados à hipoxia por 20 dias, demonstrando uma possível relação entre a expressão aumentada desse hormônio com o estresse gerado pela aclimatação à hipoxia ([MOMMSEN, VIJAYAN e MOON, 1999; FUGII, 2000; TAKAHASHI e KAWAUCHI, 2006](#)) enquanto a exposição ao Mn sob hipoxia por 20 dias reduziu a expressão da POMC-A. Entretanto, não foram observadas alterações na expressão gênica da isoforma POMC-B para nenhum grupo durante todo o período experimental.

Curiosamente, foi observado um escurecimento *per se* na cor da pele de jundiás aclimatados à hipoxia por 20 dias experimentais, enquanto que a exposição ao Mn sob hipoxia, nesse mesmo período, levou a um clareamento da cor da pele dos jundiás quando comparados ao grupo aclimatado à hipoxia. Neste sentido, é possível que os sistemas da POMC possam estar relacionados à regulação da liberação do α-MSH em jundiás, estimulando a dispersão dos pigmentos, uma vez que a POMC é precursor do α-MSH ([TAKAHASHI e KAWAUCHI, 2006](#)). Contudo, não foram observadas alterações na expressão da POMC-A durante os 10 dias do estabelecimento da reoxigenação, embora tenha ocorrido um escurecimento em jundiás

submetidos à reoxigenação expostos ou não ao Mn nesse período. Essa aparente divergência de respostas pode ser explicada de duas maneiras distintas; tanto pelo fato de que o aumento na expressão de um gene nem sempre reflete-se em um aumento de proteínas transcritas nos seus órgãos alvo (SMITH e ADKISON, 2010), assim como pelo fato de que na pele, o controle da pigmentação pelo α -MSH é duplo, ou seja, endócrino pela hipófise, além do controle parácrino pela própria pele, tornando as funções do α -MSH na pigmentação muito específicas, uma vez que uma simples acetilação na extremidade N-terminal de α -MSH inibe a sua atividade dispersante de pigmento (TAKAHASHI e cols., 2009).

De importância para o estudo, a análise da resposta coordenada do aumento da expressão da POMC-A com a alteração de cor em jundiás permitiu demonstrar um dos prováveis mecanismos envolvidos no processo de adaptação de jundiás à hipóxia, revelando uma relação entre o aumento da expressão da POMC-A com aumentada liberação de α -MSH pela adenohipófise. É possível que a resposta antioxidante em jundiás possa estar interligada a alterações controladas pelo eixo endócrino (através de vias ainda não definidas), uma vez que, sob hipóxia, os jundiás experimentam uma situação de estresse e, em resposta à esse estresse, aumentam suas defesas antioxidantes (DOLCI e cols. 2014).

Em relação aos parâmetros comportamentais analisados, tanto a aclimatação de jundiás à hipóxia ou normoxia, quanto a exposição ou não ao Mn, não exerceram influência sobre a memória de interação social ou ansiedade. Entretanto, quando jundiás foram submetidos à reoxigenação, sendo expostos pela terceira vez ao labirinto de interação social ou ansiedade, estes revelaram tendência a apresentar melhor memória em relação aos grupos previamente expostos ao labirinto. Adicionalmente, houve uma tendência dos grupos aclimatados à hipóxia por 10 dias apresentarem maior ansiedade em relação aos aclimatados à normoxia, observada pelo maior número de visitas aos três primeiros quadrantes sob níveis reduzidos de oxigênio. É possível que essa maior ansiedade seja produzida pela busca do animal por outro ambiente com probabilidade de maior disponibilidade de oxigênio, tal como ocorre em ambientes naturais (BALDISSEROTTO, 2013). Adicionalmente, jundiás aclimatados à hipóxia sofreram aumento da taxa ventilatória nas brânquias, o qual foi acentuado após exposição à coespecíficos nesse mesmo nível de oxigênio. Contudo, o estabelecimento da reoxigenação provocou redução na taxa ventilatória das brânquias de jundiás antes e após a exposição à coespecíficos.

A partir do teste de coespecíficos, outro dado importante foi observado, uma vez que jundiás aclimatados à hipóxia, assim como aqueles expostos ao Mn sob normoxia, mostraram menor responsividade, tanto antes quanto após o desafio frente à coespecíficos. Na verdade, é

esperado que a hipóxia cause uma diminuição na atividade dos peixes com afinalidade de reduzir dessa forma o gasto energético, uma vez que sob hipóxia a fosforilação oxidativa está comprometida (RIEPE e cols., 1997). O interessante é que essa menor responsividade frente à coespecíficos foi observada também durante a exposição ao Mn independente do nível de oxigênio (hipóxia ou normoxia) e persistiu mesmo após o estabelecimento da reoxigenação. É possível que o Mn reduza essa responsividade de jundiás frente a coespecíficos por causar prejuízos em importantes centros cerebrais como telencéfalo (*medial pallium*) (PORTAVELLA, e cols., 2002, 2004) diencéfalo (hipotálamo) (BAATRUP e DOVING, 1990) e metencéfalo (cerebelo), estruturas responsáveis pelos processos de memória, olfato e coordenação muscular em peixes (YOSHIDA, OKAMURA e UEMATSU, 2004; SACCHETTI, SCELFO e STRATA, 2005; ITO, 2006), similar aos danos cerebrais induzidos pelo Mn sobre roedores (ERIKSON e ASCHNER, 2003, MARREILHA DOS SANTOS e cols., 2011).

5 CONCLUSÕES FINAIS

5.1 ARTIGO 1

✚ Aclimatação de jundiás à hipóxia proporcionou um menor acúmulo de Mn em fígado, rim, encéfalo e plasma, exceto nas brânquias, cujo acúmulo foi igual para ambos níveis de oxigênio (hipóxia ou normoxia).

✚ A aclimatação de jundiás à hipóxia preveniu a redução do hematócrito observada em jundiás expostos ao Mn sob normoxia. A exposição de jundiás ao Mn não alterou os valores de hemoglobina em ambos níveis de oxigênio testados.

✚ A aclimatação à hipóxia reduziu prejuízos oxidativos induzidos pelo Mn sob normoxia em brânquias, rim e encéfalo, com exceção do fígado, enquanto a CAT foi ativada em brânquias e rim pela aclimatação de jundiás à hipóxia e posterior exposição ao Mn. Contudo, no rim de jundiás aclimatados à normoxia o Mn reduziu a atividade dessa enzima;

✚ A aclimatação de jundiás à hipóxia com subsequente exposição ao Mn revelou um aumento na atividade da Na^+/K^+ -ATPase em brânquias, fígado e encéfalo. A atividade dessa enzima foi reduzida pela exposição ao Mn sob normoxia em brânquias, rim e encéfalo de jundiás;

✚ A aclimatação à hipóxia não modificou a expressão de hormônios hipofisários GH, PRL e SL. Entretanto, a exposição ao Mn sob normoxia promoveu o aumento da expressão gênica da PRL e, contrariamente, diminuição da SL.

5.2 ARTIGO 2

✚ Aclimatação de jundiás à hipóxia proporcionou um menor acúmulo de Mn em brânquias, permanecendo mais baixo mesmo após subsequente reoxigenação;

✚ A aclimatação à hipóxia produziu alterações morfológicas em brânquias que foram importantes para contrabalancear aquelas provocadas pela exposição ao Mn sob normoxia. Para a maioria dos parâmetros morfológicos, sob reoxigenação tais modificações induzidas pela hipóxia não foram mantidas, demonstrando que as brânquias podem ser facilmente remodeladas em resposta a mudanças nos níveis de oxigênio da água.

 Aclimatação de jundiás à hipóxia reduziu prejuízos oxidativos (demonstrados por aumentada geração ER, maior dano às proteínas, menor viabilidade mitocondrial) induzidos pelo Mn sob normoxia através da ativação da enzima CAT e aumentada atividade da Na^+/K^+ -ATPase em brânquias. Durante a reoxigenação, a atividade da CAT continuou elevada, enquanto para a Na^+/K^+ -ATPase o mesmo efeito não tenha sido observado.

5.3 ARTIGO 3

 A aclimatação de jundiás a hipóxia proporcionou um menor acúmulo de Mn em plasma, fígado e rim, permanecendo mais baixo mesmo após subsequente reoxigenação;

 A aclimatação de jundiás à hipóxia por 10 e 20 dias produziu um aumento *per se* no número de eritrócitos, hematócrito e hemoglobina, que foram observados mesmo após a reoxigenação, exceto para os eritrócitos). Adicionalmente, a aclimatação à hipóxia por 10 dias reduziu *per se* o número de leucócitos e a percentagem de neutrófilos em comparação com jundiás aclimatados à normoxia. Em 20 dias de exposição ao Mn ocorreu redução dos eritrócitos e hematócrito, que foi revertida apenas em eritrócitos, pelo estabelecimento da reoxigenação.

 Aclimatação de jundiás à hipóxia reduziu os danos hepáticos (avaliados por TGO, TGP, GGT e razão TGO/TGP plasmáticos) induzidos pela exposição ao Mn sob normoxia. Esses danos foram menores também em jundiás expostos ao Mn sob reoxigenação.

 A aclimatação à hipóxia diminuiu os danos oxidativos induzidos pelo Mn (representados pela aumentada geração ER e maior dano às proteínas) sob normoxia. A aclimatação à hipóxia ativou defesas antioxidantes, como GSH e CAT, além de aumentar a atividade da Na^+/K^+ -ATPase em fígado e rim de jundiás expostos ao Mn. Essas enzimas permaneceram ativadas mesmo após exposição ao Mn durante a reoxigenação.

5.4 RESUMO EXPANDIDO

 Aclimatação de jundiás à hipóxia não exerceu influência no acúmulo de manganês em encéfalo de jundiás, porém, durante a reoxigenação houve uma redução do acúmulo de Mn em encéfalo, em comparação aos jundiás aclimatados sob normoxia;

 Aclimatação à hipóxia ou normoxia, assim como a exposição ao Mn, não exerceram influência sobre o labirinto de interação social ou ansiedade em jundiás. Entretanto, quando os peixes foram expostos pela Terceira vez ao labirinto, durante o estabelecimento da

reoxigenação, jundiás mostraram uma tendência a exibir uma melhor memória em comparação com as exposições prévias, independente da presença de Mn.

✚ Aclimatação de jundiás à hipóxia, por 10 e 20 dias, aumentou *per se* a taxa ventilatória, a qual também sofreu aumento proporcional, porém em maior grau, após a exposição a coespecíficos sob hipóxia. O estabelecimento da reoxigenação causou redução *per se* na taxa ventilatória de jundiás aclimatados à hipóxia, que foi reduzida mesmo após a exposição à coespecíficos. Em relação ao teste de agressividade, jundiás aclimatados à hipóxia, assim como aqueles expostos ao Mn sob normóxia, mostraram menor responsividade, tanto antes do desafio com coespecíficos (por apresentarem menor mobilidade) quanto após o desafio frente à coespecíficos (demonstrando maior agressividade) em relação aos animais aclimatados à hipóxia. Essa menor responsividade observada durante hipóxia, permaneceu quando os níveis de oxigênio foram restaurados (reoxigenação). A aclimatação aos diferentes níveis de oxigênio, assim como posterior reoxigenação, não exerceu influência sobre a alimentação de jundiás, avaliada no teste de coespecíficos, independentemente da presença de Mn;

✚ A aclimatação à hipóxia reduziu prejuízos oxidativos induzidos pelo Mn sob normóxia em encéfalo de jundiás (representados pela aumentada geração ER e maior dano às proteínas). Após reoxigenação, essa prevenção continuou sendo observada no encéfalo de jundiás;

✚ A aclimatação à hipóxia, assim como a exposição ao Mn, aumentaram a atividade da CAT em encéfalo de jundiás. A atividade da CAT permaneceu aumentada após a subsequente reoxigenação;

✚ A aclimatação à hipóxia por 20 dias reduziu *per se* a atividade da Na⁺/K⁺-ATPase. Similarmente, a exposição ao Mn sob normóxia causou redução, porém em maior grau, da atividade dessa enzima, que foi aumentada novamente quando jundiás foram expostos ao Mn sob hipóxia. Sob reoxigenação houve aumento *per se* da atividade da Na⁺/K⁺-ATPase mantida inclusive durante a exposição ao Mn;

✚ A aclimatação a hipóxia, de 10 à 20 dias, aumentou a expressão da POMC-A enquanto a expressão desse hormônio hipofisário foi diminuída durante a exposição ao Mn sob hipóxia. A POMC-B, assim como PRL e SL permaneceram inalteradas durante todo o período experimental.

✚ A aclimatação à hipóxia por 10 e 20 dias causou um escurecimento *per se* na pele de jundiás, o qual foi observado também após 10 dias de reoxigenação. A exposição ao Mn sob hipóxia produziu um escurecimento na pele dos jundiás, porém em menor grau que

aquele observado em hipóxia. Após subsequente reoxigenação, independente da presença de Mn, também foi observado o escurecimento na pele de jundiás.

6 CONCLUSÃO GERAL E PERSPECTIVAS

Este estudo demonstrou os benefícios que uma aclimatação de jundiás (*Rhamdia quelen*) a níveis moderadamente baixos de oxigênio (hipóxia moderada) exerce frente a contaminação por manganês (Mn) sobre diferentes tecidos dessa espécie e, adicionalmente demonstrou, pela primeira vez, que a maioria dos ajustes fisiológicos e bioquímicos observados durante a hipóxia persistem mesmo após a reoxigenação (restauração dos níveis normais de oxigênio) revelando que o jundiá apresenta uma relativa tolerância à hipóxia moderada, possivelmente pelo fechamento dos canais de Na⁺, uma vez que a atividade da bomba Na⁺/K⁺ ATPase foi reduzida frente à hipóxia. Por isso, análises sobre outros marcadores devem ser conduzidas no futuro, como o próprio HIF-1 α , responsável pela regulação de outros genes alvo, como a eritropoietina (Epo), ou como o fator nuclear k β (NFk β), o qual responde a uma variedade de fatores como estresse oxidativo, xenobióticos e patógenos e que, quando ativado, promove a transcrição de numerosos genes inflamatórios que poderiam elucidar a complexidade de mecanismos pelos quais a hipóxia exerce seus efeitos benéficos em jundiás expostos ao Mn, os quais persistem, na sua maioria, mesmo após a reoxigenação.

Embora esse estudo tenha contribuído para aumentar a compreensão sobre alguns dos sistemas envolvidos nos processos de adaptação de jundiás frente a baixos níveis de oxigênio, ainda existem muitos outros pontos a serem elucidados. Estudos adicionais também devem ser realizados sobre os efeitos da hipóxia sobre a expressão de genes envolvidos nos processos de aprendizagem e memória, como por exemplo o fator de diferenciação neurogênico (NeuroD), que regula e controla a diferenciação neuronal, uma vez que a neurogênese inclui a proliferação, migração, diferenciação e sobrevivência de neurônios, sendo essencial na modulação da aprendizagem e memória sob estresse. Até o momento não se investigou a expressão de genes ligados à neurogênese em jundiás, contudo, é possível que as alterações comportamentais apresentadas por esta espécie possam estar relacionadas com a expressão modificada desses fatores de transcrição e, portanto, representar a chave para o conhecimento dos mecanismos responsáveis pelas diferentes respostas comportamentais apresentadas pelos jundiás frente a níveis distintos de oxigênio (normóxia ou hipóxia), assim como frente à contaminação por Mn.

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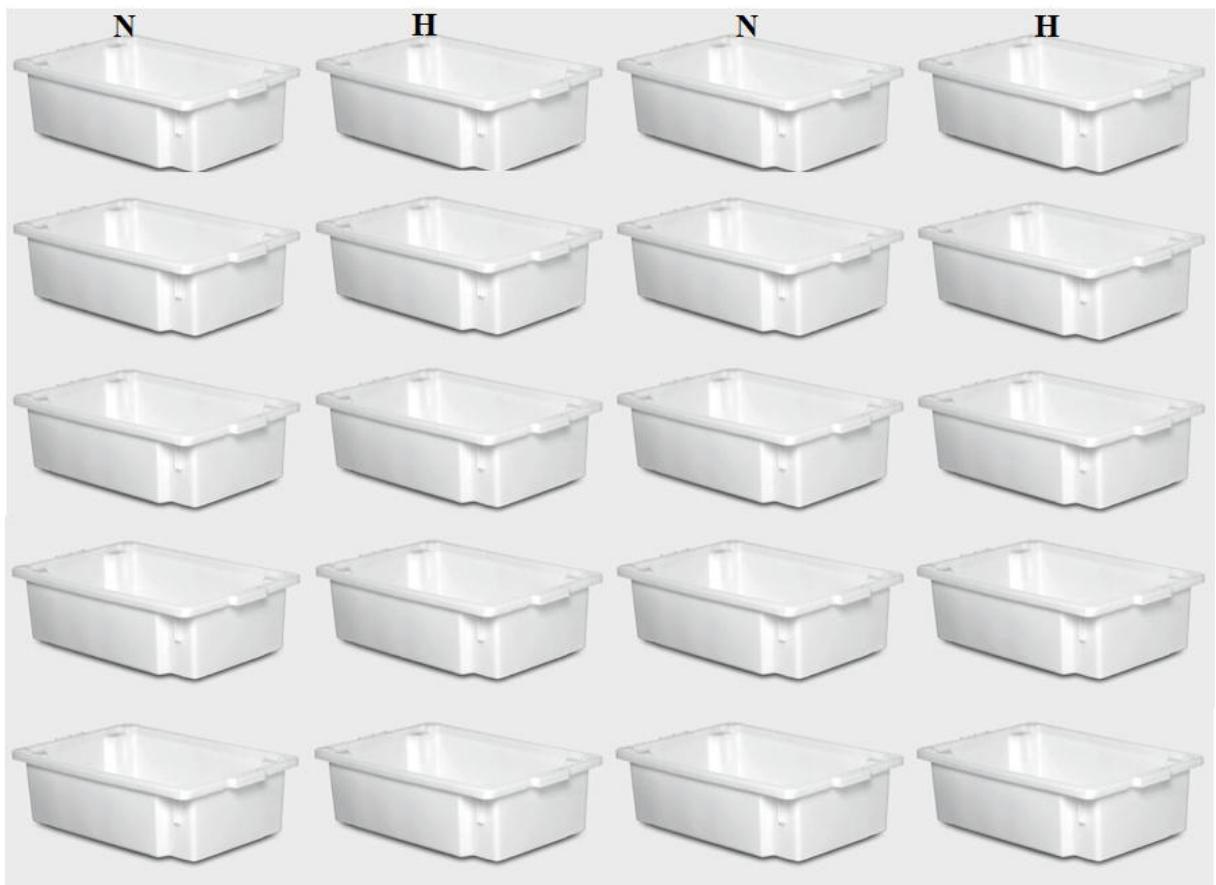
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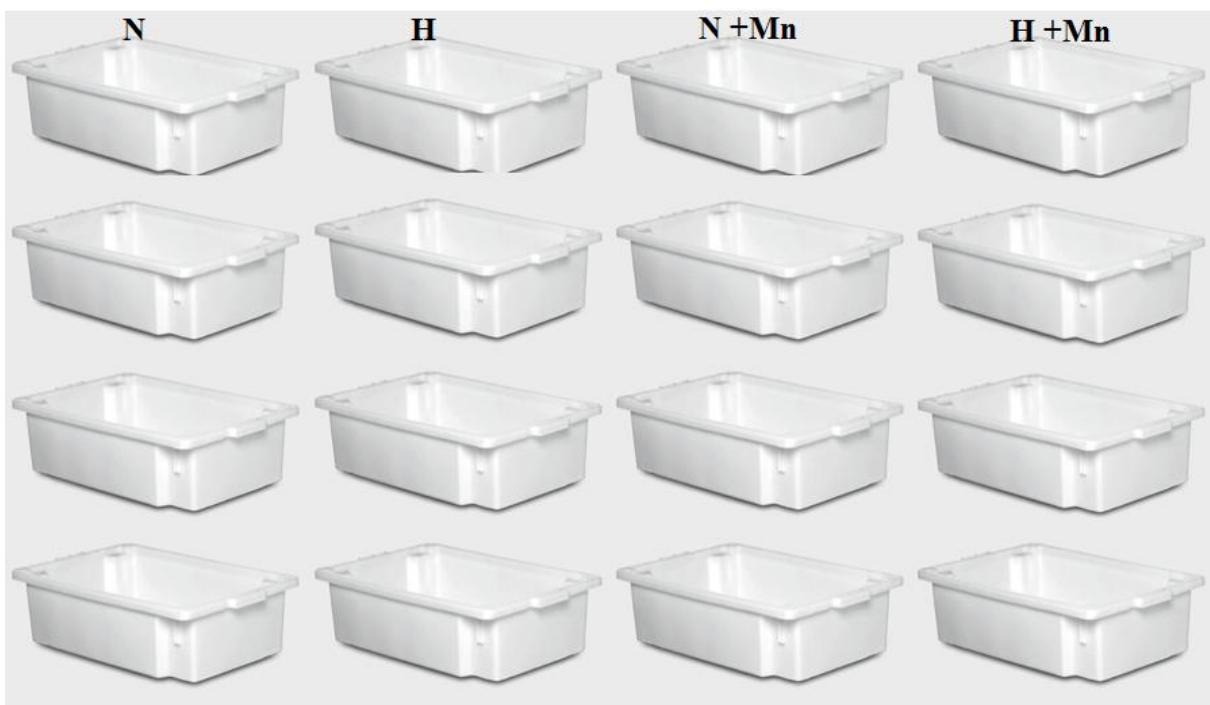
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8 APÊNDICES

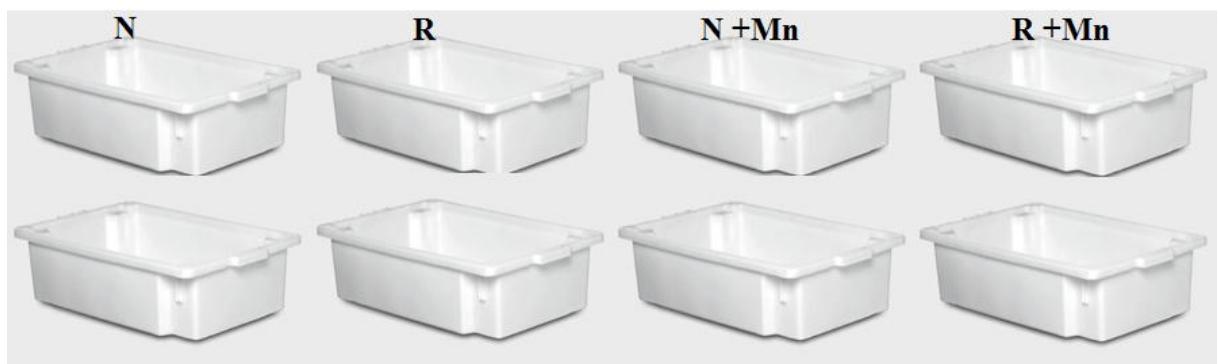
APÊNDICE A – REPRESENTAÇÃO ESQUEMÁTICA DA DISPOSIÇÃO DOS TANQUES AOS PRIMEIROS 10 DIAS EXPERIMENTAIS. GRUPOS: NORMÓXIA (N) E HIPÓXIA (H). CADA TANQUE CONTINHA 5 PEIXES (N TOTAL = 100).



APÊNDICE B – REPRESENTAÇÃO ESQUEMÁTICA DA DISPOSIÇÃO DOS TANQUES DO 10º DIA ATÉ O 20º DIA EXPERIMENTAL. GRUPOS: NORMÓXIA (N) E HIPÓXIA (H), MANGANÊS SOB NORMÓXIA (N + MN) E MANGANÊS SOB HIPÓXIA (H + MN) CADA TANQUE CONTINHA 5 PEIXES (N TOTAL = 80).



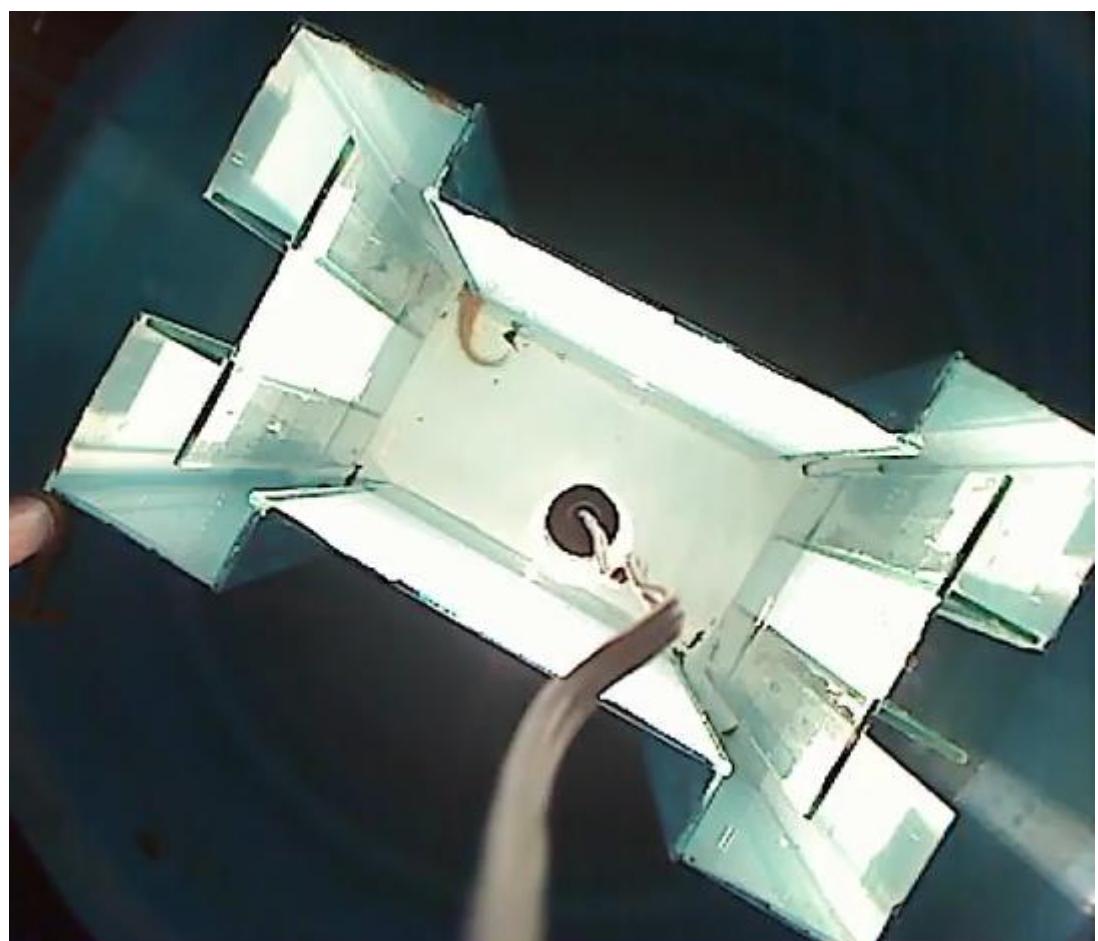
APÊNDICE C – REPRESENTAÇÃO ESQUEMÁTICA DA DISPOSIÇÃO DOS TANQUES DO 20º DIA ATÉ O 30º DIA EXPERIMENTAL. GRUPOS: NORMÓXIA (N) E REOXIGENAÇÃO (R), MANGANÊS SOB NORMÓXIA (N + MN) E MANGANÊS SOB REOXIGENAÇÃO (R + MN) CADA TANQUE CONTINHA 5 PEIXES (N TOTAL = 40).



APÊNDICE D – TANQUES RESERVATÓRIOS PARA REPOSIÇÃO DE ÁGUA NOS TANQUES EXPERIMENTAIS. À ESQUERDA (A), TANQUES EM HIPÓXIA COM OU SEM ADIÇÃO DE MANGANÊS E À DIREITA (B) TANQUES EM NORMÓXIA COM OU SEM ADIÇÃO DE MANGANÊS.



APÊNDICE E – APARATO UTILIZADO PARA AVALIAÇÃO DA MEMÓRIA DE INTERAÇÃO SOCIAL / ANSIEDADE. OS TESTES FORAM REALIZADOS AOS 10, 20 E 30 DIAS EXPERIMENTAIS.



APÊNDICE F – AQUÁRIOS UTILIZADOS PARA OS EXPERIMENTOS DE AVALIAÇÃO DA RESPONSIVIDADE FRENTE A COESPECÍFICOS (SUBSTÂNCIA DE ALARME).



APÊNDICE G – ESCALA DE CORES UTILIZADA PARA AVALIAÇÃO DA COLORAÇÃO DE JUNDIÁS. DA ESQUERDA PARA A DIREITA: JUNDIÁ MENOS ESTRESSADO (EXTREMIDADE ESQUERDA DA PÁGINA) E MAIS ESTRESSADO (EXTREMIDADE DIREITA DA PÁGINA).

